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EXPERIMENTAL STUDIES

Biodegradation Kinetics of Poly(3-hydroxybutyrate)-Based Biopolymer Systems

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Abstract—The aim of this study was to evaluate and to compare the long-term kinetics curves of biodegradation of poly(3-hydroxybutyrate) (PHB), its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), and a PHB/polylactic acid composite. The total weight loss and the change of average viscosity molecular weight were used as the parameters reflecting the biodegradation degree. The rate of biodegradation was analyzed in vitro in the presence of lipase and in vivo after film implantation in animal tissues. The morphology of the PHB film surface was studied by the atomic force microscopy technique. It was shown that PHB biodegradation involves both polymer hydrolysis and its enzymatic biodegradation. The results obtained in this study can be used for the development of various PHB-based medical devices.

Key words: polyhydroxyalkanoates (PHA), poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), polylactic acid (PLA), biodegradation, biopolymer systems.

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INTRODUCTION

Bacterial polyhydroxyalkanoates (PHA) and its most prominent member, poly(3-hydroxybutyrate) (PHB), represent a competitive alternative to traditional polymers such as propylene, polyethylene, polyesters, etc. Due to convenient combination of compatibility and biodegradability they are widely used in various medical devices produced for long-term implantation and contact with blood and tissues of animals. These polymers are non-toxic and substrate resources for their production are renewable and independent of hydrocarbon extraction; it is also important that products of their degradation (carbon dioxide, water and (for PHB) 3-hydroxybutyrate) do not exhibit negative effects on the organism [1–5].

In contrast to other biodegradable polyesters (polylactic and polyglycolic acids or their copolymers) degradation of PHB and its copolymers in the animal tissues occurs at a lower rate and therefore potential risk of intoxication by biodegradation products accumulated near implants is significantly lower for this class of polymers [6].

Use of PHB copolymers (most frequently copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate) improves characteristics of highly crystalline PHB (fragility and rigidity) and decrease processing temperature. In addition, these PHB copolymers with 3-hydroxyvalerate [7], 3-hydroxyhexanoate [6], 3-hydroxyoctanoate [8] exhibit improved thermal and mechanical properties and, thus, extend the spectrum of medical materials/devices.

Prediction of behavior of PHB and its derivatives in the animal tissues requires investigation of kinetics and mechanism of their hydrolytic and enzymatic biodegradation. Since the history of such studies is not very long (less than 25 years) problems related to biodegradation of amorphous and crystalline regions of polymers still require detailed investigation. Besides vary rare classic studies on PHB biodegradation [9], several detailed publications on the mechanism of degradation of PHB and some its copolymers have been recently appeared [6, 7, 9–11]. However, kinetic studies of biodegradation especially during long-term exposure (incubation) still appear very rarely [6]. Thus, the aim of this study was to evaluate and to compare the long-term kinetics of biodegradation of PHB, its copolymer with 3-hydroxyvalerate, and a PHB composite with polylactic acid (PLA). For comparison, the same kinetic curves were studies for pure PLA; this allowed us to compare the biodegradation rates for the most widely used medical biopolymers. Special attention has been paid to the effect of initial molecular mass (MM) on the rate of polymer degradation.

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MATERIALS AND METHODS

The following reagents were used in this study: polylactic acid with molecular weight (Mw) of 42.4 and 388.7 kDa from Fluka (Switzerland); NaH_2PO_4 (Mw = 121) from Khimmed (Russia); Tris-HCl ($C_4H_{11}NO_3$, MM = 121.1) from Serva (Germany), sodium azide (NaN_3) from Sigma-Aldrich (USA), porcine pancreatic lipase (MM = 50 kDa, activity of 20 U/mg) from KMF Laborchemie Handels Gmbh (Germany), chloroform (trichloromethane, $CHCl_3$) from JSC EKOS-1 (Russia), hydrochloric acid (specially pure grade 20-4) and sodium hydroxide (NaOH; chemically pure) from Khimmed, sodium thiopental ($C_{11}H_{17}N_2O_2SNa$) from Biochemi Gmbh, Kundle (Austria). In surgical operations the surgical thread (silk braided thread 4/0) from Volot' (Russia) was used.

Biochemical synthesis of PHB was performed using a highly productive strain of PHB, *Azotobacter chroococcum* 7B, which can synthesize PHB up to 80% of dry weight of cells. The collection strains of *Azotobacter* were maintained on the Ashby medium. High productivity of the cell culture was achieved by cultivation on the Burk medium under conditions of excessive content of a carbon source in the medium [12, 13]. Isolation and purification of the polymer from *Azotobacter chroococcum* have been described earlier [13, 14]. Determination of PHB content in cells was performed by the method of Zevenhuisen [15].

Initial molecular weights of polymers and their changes were determined by the viscosimetry method. Measurements of viscosity of PHB in chloroform were carried out at 30°C. Molecular weight was calculated by the Mark-Houwink-Coon equation using the equation with the following coefficients [16].

$$[\eta] = 7.7 \times 10^{-5} \times M^{0.82},$$

where [η] and M are viscosity of PHB solution and average viscous molecular weight of a biopolymer, respectively.

The type of crystal lattice and degree of polymer crystallinity of the polymer samples were characterized earlier [17].

Microphotographs of the surface of PHB films were obtained be means of atomic force microscopy (AFM). The PHB films were studied using an atomic force microscope Solver PRO-M (Zelenograd, Russia). A piece of the film (~2 × 2 mm^2) was fixed on a holder by double-sided adhesive tape. Scanning was performed using a semi-contact mode and NSG01 cantilevers (with typical spring constant of 5.1 N/m), scanning frequency of 1–3 Hz, scanning areas from 3 × 3 to 20 × 20 μm^2.

The study of hydrolysis and biodegradation in vitro and in vivo was performed using a series of PHB films 40 μm thick, 30 mm in diameter and various Mw: 159 kDa (defined as 150), 329 (defined as 300), 463 kDa (defined as 450), 987 kDa (defined as 1000), 1482 kDa (defined as 1500) and also copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV) with Mw of 1056 kDa. In addition we have investigated *in vitro* degradation of polylactic acid films (PLA) 40 μm with various Mw (40 and 400 kDa and prepared a composite of PHB and PLA. High molecular weight PHB (Mw = 1000 kDa) and low molecular weight PLA (Mw = 40 kDa) were dissolved in chloroform at the ratio 1 : 1 and the composite films were then obtained. The composite and individual polymer films were prepared by solvent evaporation (chloroform) on a glass scaffold. The film samples weighed 50–70 mg each. The loss of polymer weight due to destruction was determined gravimetrically using a AL-64 balance (Max = 60 g, d = 0.1 mg; Acculab, USA).

Measurement of hydrolytic destruction of the PHB, PLA, PHBV films and the PHB-PLA composite was performed as follows. Films were incubated in 100 mM Tris buffer, pH 7.7, at 37°C in a ES 1/80 thermostat (SPU, Russia) for 83 days; pH was controlled using an Orion 420+ pH-meter (Thermo Electron Corporation, USA). For polymer weight measurements films were taken from the buffer solution every three day, dried, placed into a thermostat for 1 h at 70°C and then weighed on a balance (measurement error d = 0.1 mg). For investigation of *in vitro* biodegradation the PHB and PHBV films were incubated in 100 mM Tris buffer, pH 7.7, containing additions of porcine pancreatic lipase (10 mg/ml, A = 20 U/mg, Mw = 50 kDa) for 3 months at 37°C. The buffer solution also contained additions of NaN_3 (2 g/l) for inhibition of microbial growth and prevention of possible contribution of microorganisms in biodegradation. Every three (in the case of phosphate buffer) and two days (in the experiments with lipase) the buffer was replaced by a fresh one [10]. Before weighing, films were treated with 0.1% SDS in distilled water for 2 h, washed with water (to remove detergent) and dried.

For evaluation of in vivo biodegradation of PHB and PHBV films 40 μm thick and 15 × 15 mm^2 in size were implanted subcutaneously onto peritoneum of male Wister rats for 180 days [7]. Before implantation films were autoclaved and additionally treated with alcohol and distilled water. Rats were anesthetized with intraperitoneal injection of sodium thiopental (5 mg per 100 g of body weight). Implantation of films was performed after achievement of the surgical stage of anesthesia under strictly aseptic conditions. Rat abdominal wool was removed by trimming. Skin was treated twice with 70% ethanol and cut in the longitudinal direction up to 1 cm. At the cut, skin and weakly developed subcutaneous cut were separated to get access to peritoneum. An implant (one per one rat) was placed onto the prepared area. The implanted films were not fixed to the adjacent tissues. After the implantation skin cut was ligated by the Volot surgical thread 4/0 and the suture area was treated by 70% ethanol and iodine. On day 7, 14, 30, 90, and 180 after implantation animals were examined. Removed films were treated with 0.1% SDS in distilled water for 2 h,

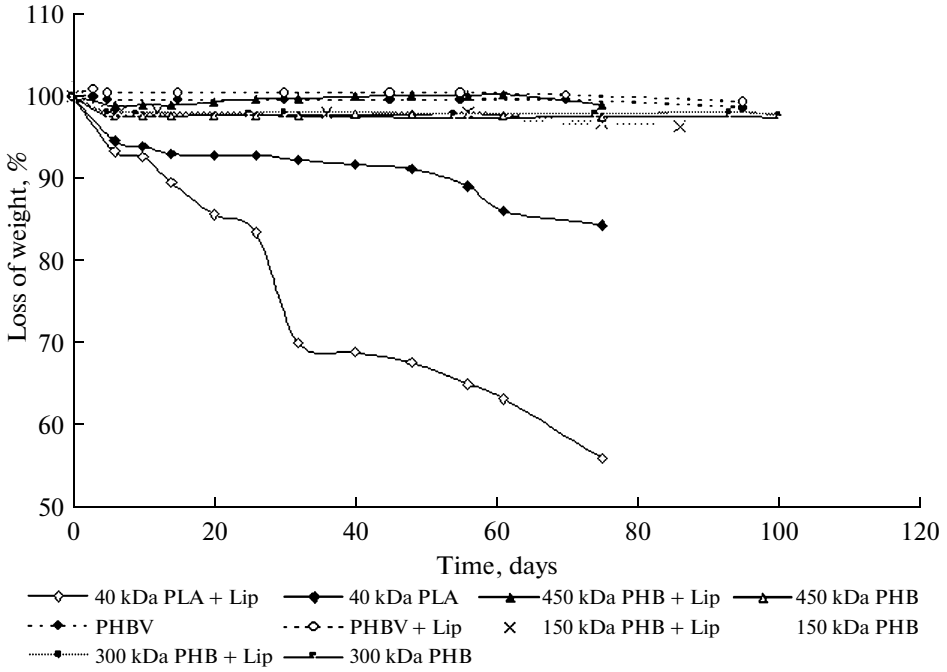

Fig. 1. Incubation of PHB, PLA and PHBV films in the absence and in the presence (+Lip) of lipase in Tris buffer, pH 7.7, at 37°C.

washed with distilled water (to remove detergent) and their masses and molecular mass of polymer changed during biodegradation were measured. 150 animals were used in these experiments. Postoperative observation did not find transplant rejection. Examination of inner organs did not reveal any differences between control and experimental groups.

RESULTS AND DISCUSSION

Kinetics of degradation of PHB and its derivatives in vitro

Study of kinetics of enzymatic PHB degradation in vitro is important for understanding of mechanisms responsible for PHB degradation in animal tissues and environment. There are reports in the literature on PHB degradation by bacterial enzyme PHB depolymerase. For example, Sudesh et al. [18] reviewed results on enzymatic degradation of PHB by polyhydroxybutyrate depolymerase. It should be noted that PHB depolymerase is rather specific enzyme and its effect on PHB is rather rare and untypical situation. In reality, enzymatic degradation of PHB in animal tissues (and even in environment) involves nonspecific esterases [18, 19]. We have performed here two series of in vitro (films incubated in Tris buffer containing lipase) and in vivo (films implanted subcutaneously into rats) experiments. In the in vitro experiment we have investigated 40 kDa PLA, PHB of 150, 300, and 450 kDa and also PHBV (Fig. 1). During incubation for 95 days we did not find any difference in the weight loss of PHB and PHBV films incubated with (experimental group) and without lipase (control group). In the case of PLA incubation for 75 days the presence of lipase resulted in the loss of 54% of initial weight of this polymer, whereas in the control group (Tris buffer without lipase) the loss of PLA weight was just 15%.

It is known that enzymatic degradation of the PHB films is a heterogeneous process that occurs in two steps, including enzyme adsorption followed by its catalytic action on the functional (ester) groups of the polymer [20]. The first step, enzyme adsorption on the PHB surface, involves its specific domains and then an active site of the adsorbed enzyme performs hydrolysis of polyester chains. In addition, hydrolytic destruction may also contribute to cleavage of polymeric chains and therefore we have investigated hydrolytic destruction of the polymers in tris buffer.

Implantation of PHB into animal organisms is accompanied by inflammatory-reparative changes, which result in formation of a connective tissue capsule (in our case see below, Fig. 2). Consequently, polymer (PHB) biodegradation in vivo occurs inside the capsule. It is known that degradation of an encapsulated polymer mainly involves macrophages and foreign body giant cells (FBGC). In the in vivo experiment we observed almost total biodegradation of the polymer on month 4 after implantation of 450 kDa PHB (Fig. 3). During this period initial film weight of 1000 and 1500 kDa PHB decreased by 93 and 83%, respectively. These experiments have demonstrated that PHD degradation in the animal body occurs faster than in vitro and this may be attributed to the presence of various nonspecific esterases capable to

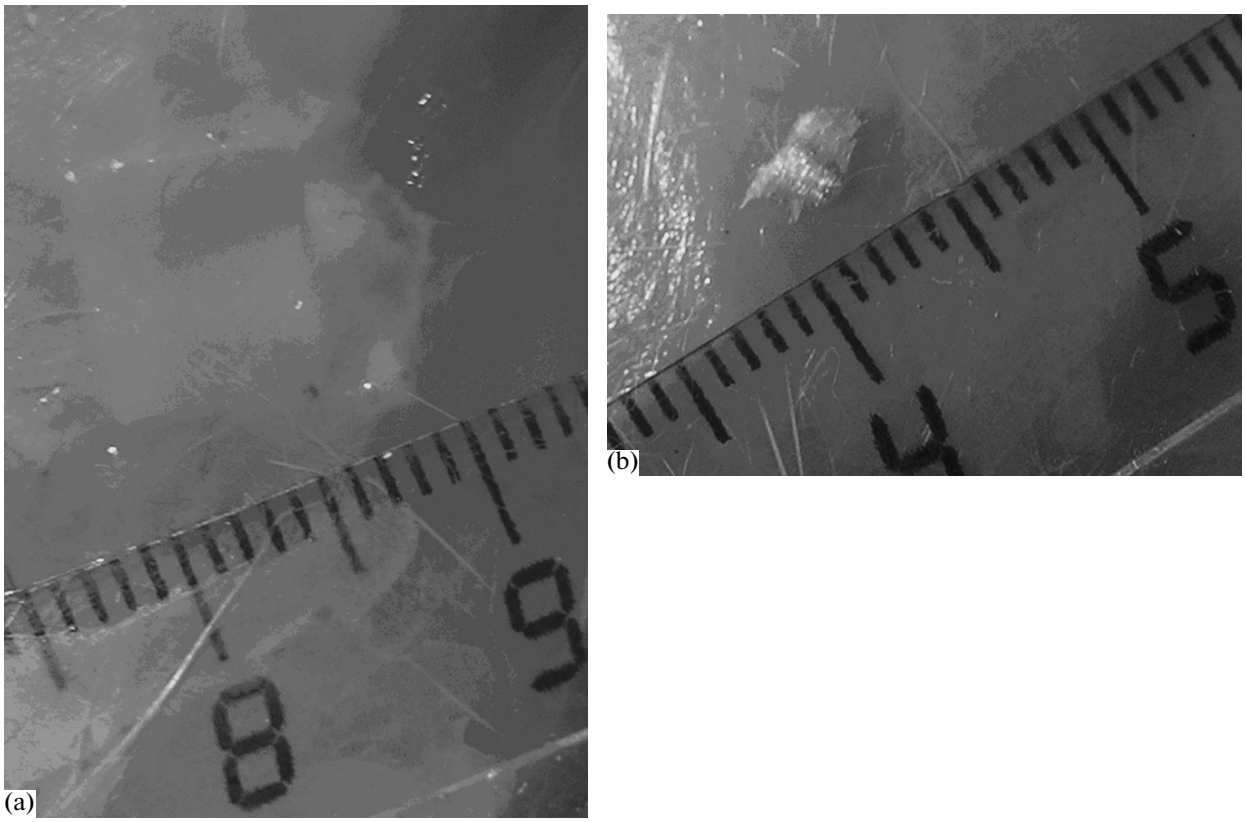

Fig. 2. A photograph of partially degraded 450 kDa PHB film subcutaneously implanted to rats *in vivo*: (a)—two weeks after implantation, (b)—six months after implantation.

cleave these polymers. Earlier it was demonstrated that implantation of PHB-based devices is accompanied by expression and production of nonspecific esterases, particularly, lipase-1 and lipase-2, by macrophages and FBGC [20]. Contribution of nonspecific esterases, macrophages, and FBGC to biodegradation of PBA was demonstrated in various studies [21–23].

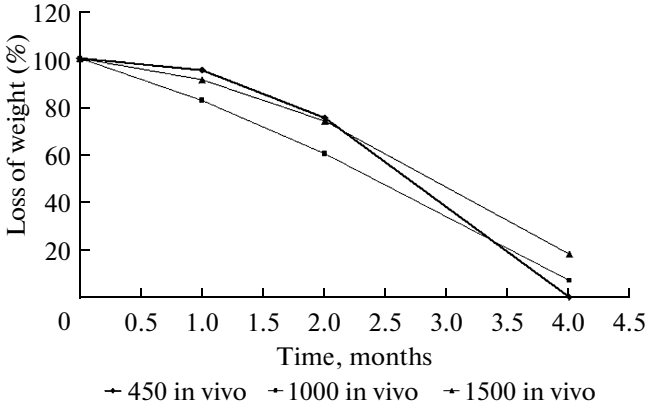

Fig. 3. Comparative weight loss of PHB films (450, 1000, and 1500 kDa) in vivo after subcutaneous implantation toWistar rats.

Change in molecular weight of PHB and PHBV

In the study on in vitro enzymatic degradation films were incubated in Tris buffer, pH 7.7, containing addition of lipase. Figure 4 shows that incubation for 83 days in the presence of lipase caused significant decrease of molecular weights of PHB and PHBV compared with control (incubated without lipase). For example, in control samples molecular weight of the polymer decreased from 159 to 132 kDa (by 16.98%) and in the presence of lipase to 104 kDa (by 34.6%); in the case of 329 kDa polymer the decrease was 19.45% in control (from 329 to 265 kDa) and 53.5% in the presence of lipase (153 kDa); in the case of 463 kDa polymer the decrease was 33.91% in control (from 463 to 306 kDa) and 70.4% in the presence of lipase (137 kDa); molecular weight of the PHBV polymer in control decreased by 47.82% (from 1056 to 551 kDa) and by 75.09% (263 kDa) in the presence of lipase. Comparing these results with the results described in the previous sections one may conclude that the decrease in molecular mass does not cause the decrease of total mass of the films studied. This effect may be attributed to rather low size of lipase, which

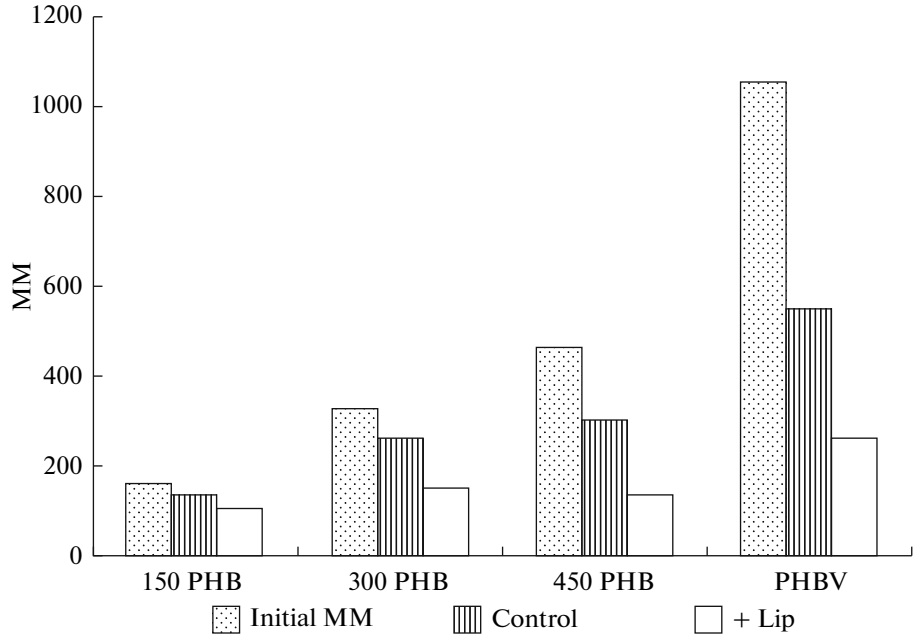

Fig. 4. Changes in molecular weight of PHB (150, 300, and 450 kDa) and PHBV after their incubation in Tris buffer, pH 7.7, with (+Lip) and without lipase at 37°C.

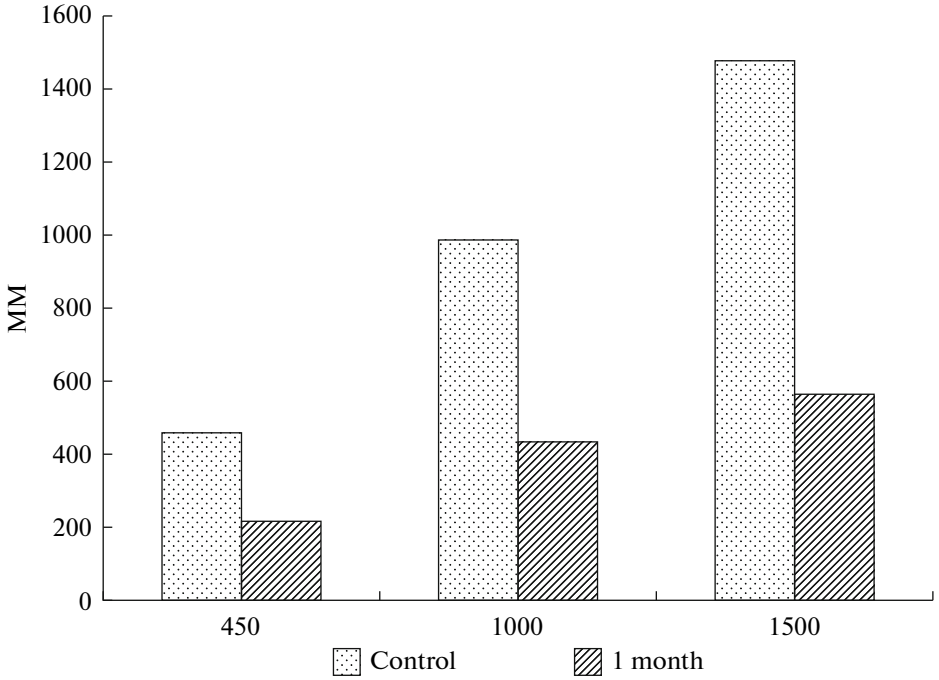

Fig. 5. Changes in molecular weight of PHB (450, 1000, and 1500 kDa) 1 month after their implantation to rats.

can penetrate inside the volume of polymer matrix of PHB and PHBV and thus increase area of the enzymatic attack. Consequently, total reaction rate of enzymatic hydrolysis also increases, but reaction products cannot be desorbed from the polymeric volume of the films due to their poor solubility in water and steric hindrance.

We have also investigated changes in molecular weight of PHB 450, 1000, and 1500 in vivo one month after implantation (Fig. 5). We also observed the decrease in molecular weights of the implanted films. The decrease in molecular mass of two PHB samples (215 and 436 kDa, respectively) was markedly higher than *in vitro* conditions in the buffer.

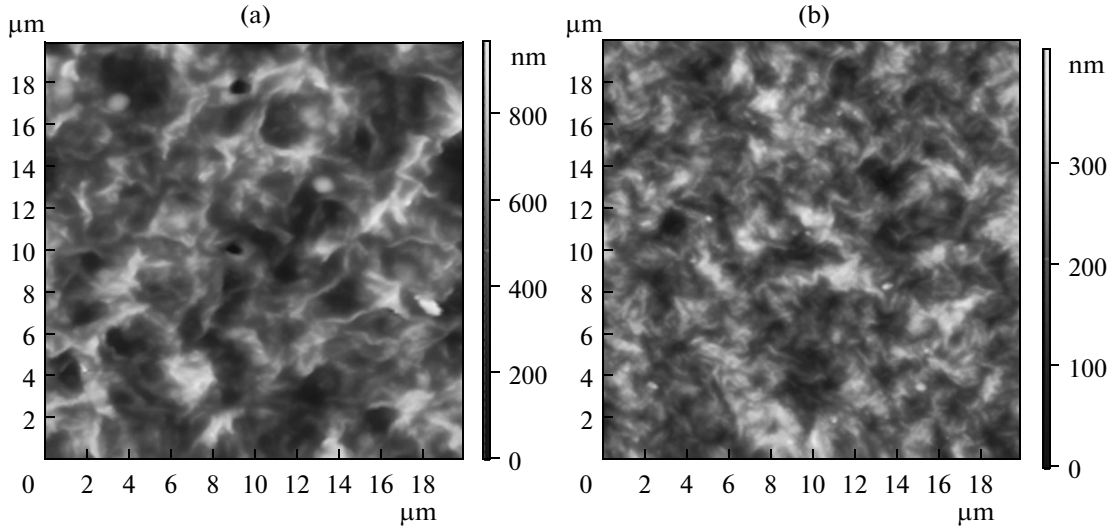

Fig. 6. The effect of lipase treatment on the surfaces the PHB film during 83 days. (a)—the sample surface (150 kDa) exposed to air during film preparation; (b)—the opposite surface contacting with glass.

Analysis of the surface of PHB films by atomic force microscopy

Morphology, structure and roughness of surfaces of the PHB films subjected to various corrosive (phosphate buffer, NaOH solution) and biologically active (lipase) media were investigated by the method of atomic force microscopy (AFM). Figure 6 shows a control sample of 150 kDa PHB after formation of the polymer film on a glass from chloroform solution. Such method of film preparation suggests possible difference in structure/morphology of surfaces exposed to the glass or air. Indeed, Fig. 6 shows that the side exposed to air is rough (Fig. 6a) and contains many pores of 500–700 nm in depth. The surface has ledges of 200–400 nm in width and 1–2.5 μm in length, which possibly represent crystalline areas. The opposite surface of the film faced to glass (Fig. 6b) has less relief morphology characterized by a pore depth of less than 100 nm.

Differences between two sides become especially demonstrative, when parameters of roughness are compared. Two parameters of roughness have been calculated to describe film surfaces. These include the average roughness $R_a = \frac{1}{N}\sum_{n=1}^{N}|r_n|$ and the root mean square roughness $R_q = \sqrt{\frac{1}{N}\sum_{n=1}^{N} r_n^2}$. These parameters were calculated by three scan areas of 20 × 20 μm^2 (512 × 512 points). In addition several scans of better resolutions (e.g. 5 × 5 μm^2, 512 × 512 points) were obtained for each sample for more detailed description of the polymer surface.

Thus, analysis of roughness of both surfaces of the same film indicates that the average roughness and the root mean square roughness of surfaces exposed to air and glass differ by one order of magnitude. Such differences are related to conditions of solvent (chloroform) desorption from the forming PHB films. In the case of chloroform evaporation from the surface to surrounding air environment solvent flow forms additional channels, pores, which are formed during hardening and crystallization of samples. At the same time, PHB morphology on the opposite surface is less subjected to the effect of solvent transport and is determined by surface energetic (surface tension) on the border glass—PHB. Thus, morphology, pore size and roughness of the surface exposed to air are determined by conditions of solvent evaporation such as temperature, the rate of chloroform desorption, formation of crystalline areas preventing chloroform diffusion into polymer. On the contrary, in the case of fixed chemical composition of the glass matrix (scaffold) and constant chemical composition of PHB the surface energy of the polymer side exposed to the glass exhibits less

Roughness parameters of PHB films

Sample	Contact surface	R_a, nm	R_q, nm
initial	air	130 ± 10	165 ± 10
initial	glass	15 ± 2	20 ± 1
After contact with buffer, 37°C	air	135 ± 5	166 ± 7
After contact with buffer, 37°C	glass	46 ± 2	59 ± 1
After contact with lipase, 37°C	air	127 ± 7	161 ± 11
After contact with lipase, 37°C	glass	48 ± 2	60 ± 2

dependence on the above mentioned factors. Thus, in the future we will try to use the PHB surface faced to the glass as a standard.

Long-term incubation of the PHB sample in Tris buffer for 83 days caused a threefold increase in roughness of the surface previously exposed to the glass without significant changes in roughness and morphology of the opposite surface (table). Lipase effect on the surface of the PHB films was investigated in the samples treated with lipase in Tris buffer for 83 days at 37°C (Fig. 6).

Both surfaces of lipase-treated and control films were differed by morphology (Fig. 6) and roughness (table). However, comparison of these characteristics obtained for lipase-treated and control (treated with Tris buffer only) did not reveal significant differences in the analyzed areas (scan sizes from 5×5 μm^2 to 20×20 μm^2). Table shows that the values of the roughness parameters for films contacted with lipase solution and buffer solution at 37°C are close. Hence, we can make pilot conclusion that initial step of PHB hydrolysis in the absence and in the presence of lipase preferentially occurs in a voluminous area. Less porous structure of the polymer surface originally exposed to the glass (see Figs. 2b and 6b) undergoes larger changes as it represents a diffusion barrier for enzyme penetration into the PHB volume. Here surface hydrolysis also takes place (in addition to voluminous processes). On the contrary, the surface exposed to air and characterized by a highly porous morphology does not form such barrier and therefore there is a unilateral diffusion of lipase molecules into a volume of the polymeric sample. Kinetic aspect of the enzymatic hydrolysis will be considered in our future studies.

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REFERENCES

1. Bonartsev, A.P., Iordanskii, A.L., Bonartseva, G.A., and Zaikov, G.E., *Polymers Res. J.*, 2008, vol. 2, pp. 127–160.
2. Williams, D., *Medical Device Technology*, 2005, vol. 16, pp. 9–10.
3. Chen, G.Q. and Wu, Q., *Biomaterials*, 2005, vol. 26, pp. 6565–6578.
4. Lenz, R.W. and Marchessault, R.H., *Biomacromolecules*, 2005, vol. 6, pp. 1–8.
5. Anderson, A.J. and Dawes, E.A., *Microbiol. Rev.,* 1990, vol. 54, pp. 450–472.
6. Qu, X.H., Wu, Q., Zhang, K.Y., and Chen, G.Q., *Biomaterials*, 2006, vol. 27, pp. 3540–3548.
7. Miller, N.D. and Williams, D.F., *Biomaterials*, 1987, vol. 8, pp. 129–137.
8. Fostera, L.J.R., Sanguanchaipaiwonga, V., Gabelisha, C.L., Hookc, J., and Stenzel, M., *Polymer*, 2005, vol. 46, pp. 6587–6594.
9. Gogolewski, S., Jovanovic, M., Perren, S.M., Dillon, J.G., and Hughes, M.K., *J. Biomed. Mater. Res.*, 1993, vol. 27, pp. 1135–1148.
10. Freier, T., Kunze, C., Nischan, C., Kramer, S., Sternberg, K., Sass, M., Hopt, U.T., and Schmitz, K.P., *Biomaterials*, 2002, vol. 23, pp. 2649–2657.
11. Kunze, C., Bernd, E.H., Androsch, R., Nischan, C., Freier, T., Kramer, S., Kramp, B., and Schmitz, K.P., *Biomaterials*, 2006, vol. 27, pp. 192–201.
12. Bonartseva, G.A., Myshkina, V.L., Zagreba, E.D., and Nikolaeva, D.A., *Patent no. 2201453* of 18.10.2001.
13. Bonartseva, G.A., Zagreba, E.D., Myshkina, V.L., and Furina, E.K., *Patent no. 2194759* of 18.10.2001.
14. Bonartseva, G.A., Myshkina, V.L., Nikolaeva, D.A., Rebrov, A.V., Gerasin, V.A., and Makhina, T.K., *Mikrobiologiya*, 2002, vol. 71, pp. 258–263.
15. Zevenhuisen, L.P. and van Leeuwenhoek, A.J., *Microbiol. and Serol.*, 1981, vol. 47, pp. 481–497.
16. Akita, S., Einada, Y., Miyaki, Y., and Fugita, H., *Macromol.*, 1976, vol. 9, pp. 774–780.
17. Rebrov, A.V., Dubinskii, V.A., Nekrasov, Yu. P., Bonartseva, G.A., Stamm, M., and Antipov, E.M., *Vysokomolekulyarnye Soedineniya*, 2002, vol. 44, pp. 347–351.
18. Sudesh, K., Abe, H., and Doi, Y., *Prog. Polym. Sci.*, 2000, vol. 25, pp. 1503–1555.
19. Saito, T., Tomita, K., Juni, K., and Ooba, K., (1991) *Biomaterials*, 1991, vol. 12, pp. 309–312.
20. Lobler, M., Sass, M., Kunze, C., Schmitz, K.P., and Hopt, U.T., *Biomaterials*, 2002, vol. 23, pp. 577–583.
21. Shishatskaya, E.I., Volova, T.G., Gordeev, S.A., and Puzyr, A.P., *J. Biomater. Sci. Polym. Ed.*, 2005, vol. 16, pp. 643–657.
22. Malm, T., Bowald, S., Karacagil, S., Bylock, A., and Busch, C., *Scand. J. Thorac. Cardiovasc. Surg.*, 1992, vol. 26, pp. 9–14.
23. Hoshino, A. and Isono, Y., *Biodegradation*, 2002, vol. 13, pp. 141–147.