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New Poly-(3-hydroxybutyrate)-Based Systems for Controlled Release of Dipyridamole and Indomethacin

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Abstract—New poly-(3-hydroxybutyrate)-based systems for controlled release of anti-inflammatory and antithrombogenic drugs have been studied. The release occurs via two mechanisms (diffusion and degradation) operating simultaneously. Dipyridamole and indomethacin diffusion processes determine the rate of the release at the early stages of the contact of the system with the environment (the first 6–8 h). The coefficient of the release diffusion of a drug depends on its nature, the thickness of the poly-(3-hydroxybutyrate) films containing the drug, the concentrations of dipyridamole and indomethacin, and the molecular weight of the poly-(3-hydroxybutyrate). The results obtained are critical for developing systems of release of diverse drugs, thus, enabling the attainment of the requisite physiological effects on tissues and organs of humans.

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Poly-(-3-hydroxybutyrate) (PHB) and its copolymers obtained using biotechnological methods have become the subject of increasing interest due to their biodegradability and biocompatibility, which make it possible to use these polymers in medicine. The physicochemical and biological properties of PHB allow this polymer to be used as a material for implantable medical devices (e.g., membranes for treatment of periodontal disease and prevention of adhesions) and coatings (to be applied onto the surface of net endoprostheses, pacemakers, stents, vascular prostheses, etc.) [1].

Implantation of devices made from biodegradable materials, including PHB and its copolymers, into tissues of an organism may be associated with a series of undesirable processes; these include pathological inflammatory reactions, formation of thrombi, and the lack of correspondence between the rates of the implant replacement by the surrounding body tissues and the rate of its biodegradation (which may be higher or lower). The character of the inflammatory process accompanying polymer implantation determines to a considerable extent the intensity of the biodegradation of this polymer. The success of the integration of an implant into the surrounding tissues (if there is a contact between the implant and blood or intraperitoneal fluid) depends in its resistance to thrombus formation. The increased coagulability of peritoneal fluid favors the development of peritoneal adhesions, which is a serious pathology. The intensity of the cell proliferation associated with polymer implantation determines both the rate of formation of a connective tissue capsule enclosing the implant and, if the polymer contacts vascular tissues, the extent of the hypertrophy of the blood vessel walls. As is clear from the above, the processes constituting the organism's response to implantation of biodegradable polymers (i.e., inflammation, thrombus formation, and cell proliferation) need to be regulated [2].

The buildup of inflammatory and thrombogenic processes may be regulated by systemic administration of antiaggregant and anti-inflammatory preparations. In certain cases, however, this approach is not efficient, because the local concentrations of the drugs within the region of the implantation are either not sufficient for attaining the pharmacological effect or lack stability, whereas any further increase in the dose administered systemically entails side effects [3].

Systems of controlled release of drugs, based on polymer materials, make it possible to regulate the processes of inflammation, thrombus formation, and development of new tissue within the immediate vicinity of implantation of medical devices. In designing such systems, it is important to make the right choice of the drug. Dipyridamole (DP), a widely used antithrombogenic drug, is a phosphodiesterase inhibitor promoting intracellular accumulation of cGMP and cAMP, which inhibits both platelet aggregation and cell proliferation [4]. Indomethacin (IM), a nonsteroidal anti-inflammatory drug (NSAID), inhibits cyclooxygenase, thereby preventing the synthesis of prostaglandins (which are major mediators of inflammation), and cell proliferation [5]. It is noteworthy that DP and IM, as well as

PHB, are soluble in organic solvents (chloroform and methylene chloride), which simplifies the technology of creating polymer systems of controlled release.

The molecular weight (MW) of a polymer considerably affects the kinetics of the release of drugs introduced into its matrix [4]; for this reason, development of controlled release systems for drugs that have predefined characteristics requires a technology for synthesizing polymers with a particular MW. When PHB is obtained using biotechnological methods, the conditions of culturing of the PHB producer strains may influence the molecular weight of the polymer [6]. Thus, a technology for the biosynthesis of PHB with a defined MW is prerequisite to creating controlled release systems for the requisite characteristics of the kinetics of the drug release from the polymer matrix.

The use of such systems for controlled release of antithrombogenic and anti-inflammatory drugs is expected to (a) increase the resistance of medical devices contacting blood (e.g., coatings of stents and vascular prostheses) to thrombus formation, (b) regulate inflammatory processes and the rate of the implant biodegradation and capsulation (e.g., in the case of reticular endoprostheses for hernioplasty and membranes for treatment of periodontal disease), and (c) prevent the formation of adhesions (endoprostheses for hernioplasty and anti-adhesion membranes).

In this work, we sought to obtain and study PHB-based films incorporating DP and IM.

MATERIALS AND METHODS

The PHB producer strain used in this work (*Azotobacter chroococcum* 7B) was capable of synthesizing PHB in an amount of up to 80% of the dry weight of the bacterial cells. The strains were isolated from the rhizosphere of wheat (sod-podzol soil). A collection of strains of the genus *Azotobacter* were maintained on Ashbey's medium [6]. To achieve cellular PHB hyperproduction, the culture of the *Azotobacter* strain was grown on Burke's medium under conditions of an excess content of the source of carbon (g/l): $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.006; trisodium citrate, 0.5; $CaCl_2$, 0.1; $K_2HPO_4 \cdot 3H_2O$, 1.05; KH_2PO_4, 0.2; and sucrose, 40 [6, 7].

The process of isolation and purification of the polymer from the biomass of *A. chroococcum* 7B included the following stages: dissolution of PHB in chloroform by shaking at 37°C for 12 h (shaker), separation of the PHB solution from the cell residue by filtration, isolation of the PHB by isopropanol precipitation, and repeated dissolution of the PHB in chloroform followed by isopropanol precipitation and drying at 60°C.

The content of PHB in the cells was determined using the method of Zevenhuisen [8]. A suspension of the cells (20–100 mg of dry biomass) was centrifuged at 5000 *g* for 20 min. Thereafter, the cells were resuspended in 10 ml water and homogenized. 2M HCl was added to 2 ml of the suspension, and the mixture was heated at 100°C for 2 h (water bath); the insoluble residue (PHB granules) was separated by centrifugation at 8000 *g* for 20 min. Following the addition of 5 ml of chloroform to the residue, the tube was hermetically sealed and incubated overnight (28°C) under continuous shaking (shaker). Thereafter, the tube was centrifuged and the chloroform extract was dried in an air flow. Following the addition of concentrated sulfuric acid (5 ml per each 0.1 ml of extract), the mixture was heated at 100°C for 10 min (water bath) and allowed to cool. The amount of crotonic acid (formed as a result of acidic hydrolysis of PHB and subsequent hydroxybutyrate dehydration) was measured at 235 nm (against concentrated sulfuric acid) on a Beckman DU-650 spectrophotometer (Germany) in 1-cm cuvettes [8].

The MW of the polymer was determined viscometrically. Measurement of the changes in the viscosity of the PHB solution in chloroform were performed at 30°C. The MW was calculated using the Mark–Houwink–Kuhn equation; the value of the coefficient [η] was taken to be equal to $7.7 \times 10^{-5} \times M^{0.82}$ [9].

The chemical structure of the polymer, the type of its crystal lattice, and the extent of its crystallinity (0.74) were previously determined using the methods of differential scanning calorimetry, IR Fourier spectroscopy, and crystal X-ray structure analysis [10].

The traces of residual solvent were controlled by measuring the IR spectra on a Brucker IFS-48 IR spectrometer (Germany). The extent of the weight loss resulting from degradation was determined gravimetrically.

In experiments aimed at studying the kinetic characteristics of the drug release from the PHB matrix, two PHB batches were used differing in their MWs: 320 kDa (low-molecular-weight PHB) and 1470 kDa (high-molecular-weight PHB). The PHB films were 10, 20, or 40 μm thick, containing 3.3, 10, or 30 wt %, respectively, of DP or IM. Systems with a predefined content of the drugs were prepared by evaporating chloroform on a glass substratum. In addition to films, a polypropylene net was studied, which was modified by applying onto its surface a polymer composition containing PHB (320 kDa) and DP (10 wt %).

The rate of the drug release was recorded by UV spectrometry (DU-650) within the region of maximum absorption of aqueous solutions of DP and IM (at 293 and 256 nm, respectively). The release was performed in phosphate-buffered saline (pH 7.4) at 37°C for 18 h.

RESULTS AND DISCUSSION

Effect of the conditions of culturing on the molecular weight of the poly-(3-hydroxybutyrate) synthesized. In experiments addressing the effects of the conditions of the culturing on the MW of the polymer synthesized, we varied the concentration of the supplemental source of carbon.

As demonstrated previously, the addition of organic acids to a sucrose-containing medium decreases the MW of the polymer synthesized [6]. For this reason, we designed experiments in which we varied the concentration of sodium acetate in the culture medium. The results obtained are summed up in Table 1. On increasing the concentration of sodium acetate in the medium from 0 to 5 g/l (the content of sucrose, which served as the primary source of carbon, remained constant and was equal to 40 g/l), we observed a decrease in the MW of the PHB synthesized by the cells of *A. chroococcum* 7B.

It is conceivable that an increase in the intracellular concentration of the acetyl groups stimulates the activity of acetoacetyl-CoA reductase (EC 1.1.1.36), which, in turn, elevates the content of hydroxybutyryl-CoA. At high concentrations of acetate, the numbers of polymerization centers and initial fragments of polymer chains increase, which results in the synthesis of PHB with a low MW [11].

Thus, the method used in this work makes it possible to synthesize PHB with a defined MW.

Studies of the kinetics of drug release from a poly-(3-hydroxybutyrate) matrix. Figure 1 shows typical kinetic curves of DP and IM release from PHB films (each graph is a time dependence of the relative amount (%) of the drug released). As is evident from the figure, most of the systems lack constant limiting values of the concentrations, which would be observed if the release were underlain solely by diffusion mechanisms. These kinetic curves are characterized by the presence of an initial nonlinear (with respect to time) segment and a terminal linear segment within which the rate of release is near-constant. Our analysis of the curves presented in Fig. 1 demonstrates that the mechanism of release is determined by a superposition of two processes: (1) DP and IM desorption proper (diffusion mechanism) and (2) hydrolytic PHB degradation (which becomes most obvious when the first, diffusion-related stage has been completed). As a result of this degradation, the release of the drugs is linear over the last 8–10 days.

To analyze the kinetics of the release, we subtracted the linear input of the hydrolytic degradation from the common current values of the concentration of the drug

Table 1. Effect of a supplemental carbon source (sodium acetate) on the molecular weight (MW) of PHB synthesized by *A. chroococcum* 7B

Acetate, g/l	Biomass yield, g/l	Intracellular PHB content, %	Viscosity-average MW, kDa
0	5.2	79.0	1470*
2	5.1	78.5	1140
3	4.5	73.4	460
5	4.3	70.2	320*

* Molecular weights of the PHB batches used for creating systems for controlled release of dipyridamole and indomethacin.

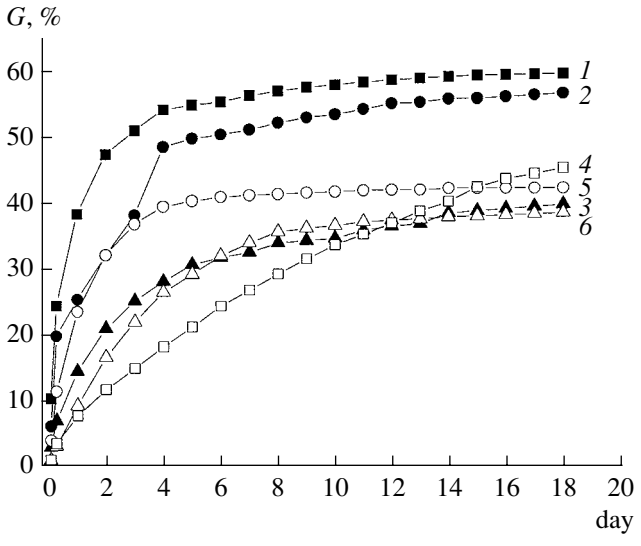

Fig. 1. Kinetic curves of drug release: IM 10% (*1–3*) and DP 10% (*4*, *5*) and 30% (*1–4*) from PHB (MW = 320 kDa) films with a thickness of 10 μm (*1–4*) and 20 μm (*6*) or a polypropylene surgical net coated with a 20 μ layer of PHB containing 10 wt % DP (*5*).

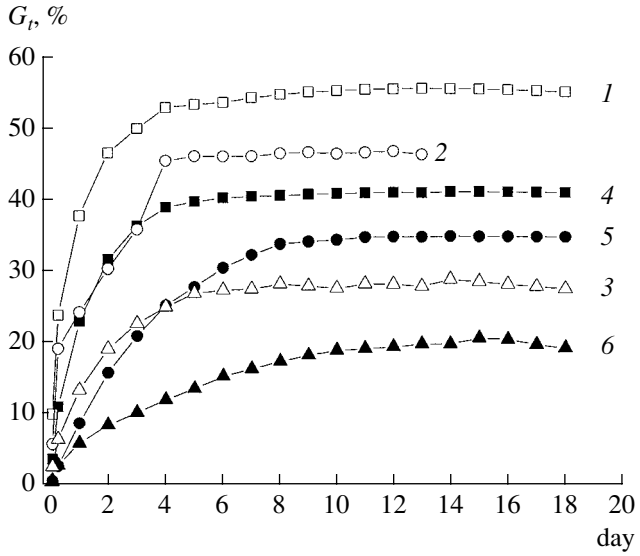

Fig. 2. Kinetic desorption curves of indomethacin (*1–3*) and dipyridamole (*4–6*) following the diffusion mechanism. The samples of PHB (MW = 320 kDa) used had a thickness of 10 (*1*, *4*), 20 (*2*, *5*), and 40 (*3*, *6*) μm.

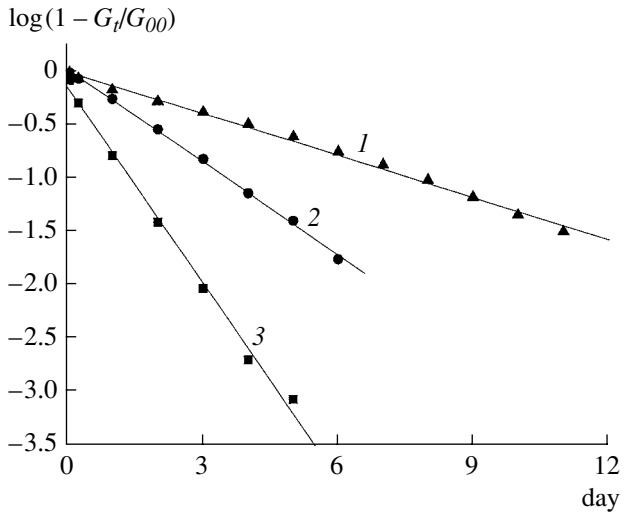

Fig. 3. Graphical solution of the diffusion equation for determining the coefficient of dipyridamole diffusion in PHB (MW = 320 kDa) films with a thickness of 10 (*1*), 20 (*2*), and 40 (*3*) μm.

released (such as those shown in Fig. 1). The result of this data processing characterizing the diffusion process proper is depicted in Fig. 2. Figure 2 shows that thin PHB films (10 μm thick) have higher limiting values of the freely diffusing component (DP or IM) than their thicker counterparts. This result may be accounted for by the observation that thin films preclude organization of perfect crystalline structures, this being the reason why the sorption capacity of the low-molecular-weight component in such polymer systems increases [12].

Table 2. Diffusion parameters of the system PHB–drug (DP or IM)

System	Concentration, %	Thickness, μm	Diffusion coefficient $\times 10^6$, m^2/s
PHB (320 kDa)–DP	3.3	10	0.19
		20	0.76
		40	2.60
PHB (1470 kDa)–DP	3.3	10	0.28
		20	0.39
		40	1.30
PHB (320 kDa)–DP	10	10	0.17
		20	0.32
		40	0.58
PHB (320 kDa)–DP	30	20	0.18
Surgical net (polypropylene)–DP	10	20	0.27
PHB (320 kDa)–IM	3.3	40	1.50
PHB (320 kDa)–IM	10	10	0.20
		20	0.38
		40	2.46
PHB (320 kDa)–IM	30	20	0.82

The analysis of the kinetic curves in the diffusion equation 1 plots makes it possible to calculate the diffusion coefficients of the drugs and, consequently, give a quantitative characterization of the systems for controlled drug release:

$$\partial G/\partial t = D[\partial^2 G/\partial x^2] + k, \quad (1)$$

where D is the diffusion coefficient (of DP or IM), cm^2/s; k is the constant of the polymer hydrolysis, s^{-1}; G is the concentration (of DP or IM), %; and x and t are the coordinate position (cm) and time (s) of the diffusion, respectively.

The solution of this equation for the condition $G_t/G_{oo} > 0.5$ has the classic appearance

$$G_t/G_{oo} = 1 - (8/\pi^2 \exp(-Dt/L^2), \quad (2)$$

where L is the thickness of the PHB film, cm (the other designations being the same as in Eq. 1).

If the logarithm of this equation is taken, the diffusion coefficients may be determined by solving the graphical equation in $\log(1 - G_t/G_{oo}) - t$ plots:

$$\log(1 - G_t/G_{oo}) = \log(8\pi^2) - Dt/L^2. \quad (3)$$

Examples of such solutions are shown in Fig. 3 for DP diffusion from films of variable thickness. The values of the diffusion coefficient, calculated using equation (3), are listed in Table 2.

Diffusion coefficients are known to characterize the mobility of polymeric segments, the morphology of PHB, and the intensity of the interactions of the drug with functional groups (in this case, ester groups) of the polymer. The rate of the diffusion-mediated release is higher for IM than DP, all other conditions (i.e., the film thickness and drug concentration) being the same. The maximum sorption capacity of PHB is also higher for IM than DP, regardless of the film thickness, as Fig. 4 demonstrates. It is exactly this amount of the drug that is contained within PHB in a nonimmobilized form capable of free diffusion from the matrix. Thus, the nature of the drug considerably affects the rate of its release, which is particularly important in the case of combined systems releasing two or more drugs.

The rate of the drug release also depends on the MW of PHB. With films that were 20 and 40μm thick, the diffusion coefficient of DP was two times greater in the case of the low-molecular-weight PHB (320 kDa) as compared to the high-molecular-weight species (1470 kDa). It is conceivable that the higher rate of the drug release from the matrix of the low-molecular-

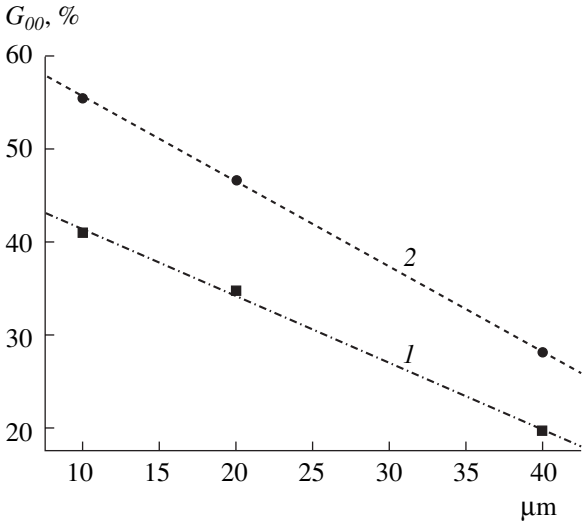

Fig. 4. Dependence of the maximum concentration of the freely diffusing component (G_{oo}) on the thickness (L, μm) of a PHB (MW = 320 kDa) film for dipyridamole (*1*) and indomethacin (*2*).

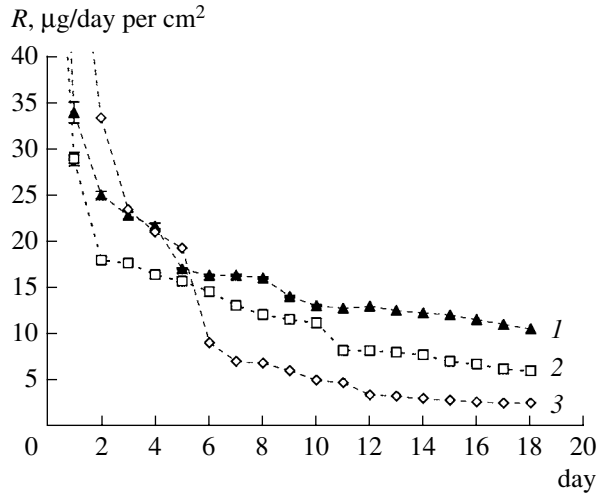

Fig. 5. Rate of release (R, μg/day per cm^2) of dipyridamole (*1*, *2*) and indomethacin (*3*) from a matrix of PHB (MW = 320 kDa): *1*, 30 wt % DP; *2*, 10 wt % DP; and *3*, 10 wt % IM.

weight PHB is accounted for by the greater mobility of its polymeric segments. However, the relationship was reversed when we examined 10-μm films. This observation may be underlain by the fact that the organization of the polymer molecules in thin films is lower than that observed in thick films [12].

In recent years, systems for controlled release of DP and IM based on other biodegradable polymers (e.g., polylactides and copolymers thereof) have been the subject of active development and investigation [13, 14]. Judging by the reported evidence, these systems are pharmacologically efficient. The results of our studies (the kinetics of drug release from PHB matrices and the underlying mechanisms) are comparable with these data. Moreover, our observation that the release of DP and IM from the PHB matrix occurs at a uniform rate and for a considerable period of time (Fig. 5) makes it possible to use these systems for long-term regulation of processes involving inflammation, thrombus formation, and tissue proliferation in the immediate vicinity of the implantation zone. The possibility to regulate the rate of drug release from the matrix by changing the MW of the PHB offers an opportunity to design PHB systems for controlled drug release with predefined characteristics.

In conclusion, we propose new polymer systems (PHB-based) for controlled release of anti-inflammatory and antithrombogenic drugs. The release occurs via two mechanisms (diffusion and degradation) operating simultaneously. The diffusion of dipyridamole and indomethacin, which determines the rate of the release at the early stages of contact of the system with the environment (the first 6–8 days), is examined in detail. The coefficients of diffusion are shown to depend on the nature of the drug, the thickness of the PHB films containing the drug, the concentrations of DP and IM, and on the MW of the PHB. The results obtained are critical for developing systems of release of diverse drugs enabling the attainment of the requisite physiological effects on tissues and organs of human beings.

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