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Microspheres Based on Poly(3-hydroxy)butyrate for Prolonged Drug Release[1]

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Abstract—The kinetics of the controlled release of the antiproliferative drug dipyridamole from microspheres based on the biocompatible and biodegradable polymer poly(3-hydroxy)butyrate is studied. As carriers for dipyridamole, microspheres prepared from a solution of poly(3-hydroxy)butyrate by single emulsion method are used. Under in vitro conditions, the kinetic curves describing the release of dipyridamole from microspheres with diameters of 19, 63, and 92 μm show two characteristic regions: the region of fast drug release within a short time period and a well-pronounced continuous linear region. For microspheres with a diameter of 4 μm, the linear region is missing. Analysis of the kinetic curves illustrating controlled drug release together with the measurements on polymer degradation shows that their kinetic profiles depend on the diffusion-controlled process and hydrolytic degradation of poly(3-hydroxy)butyrate. The diffusion kinetic equation describing both linear and nonlinear regions of dipyridamole released from the microspheres involves the sum of two terms: desorption from the sphere via the diffusion-controlled mechanism and drug release via the zero-order reaction. The linear region of the drug release curve is explained by the zero-order hydrolysis of poly(3-hydroxy)butyrate. The diffusion coefficients and kinetic constants are calculated. For bigger microspheres, the existence of the continuous linear region in the corresponding kinetic curves makes it possible to use microsystems based on poly(3-hydroxy)butyrate and dipyridamole as novel systems for local prolonged drug delivery.

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INTRODUCTION

In the past three decades, intensive studies have focused on polymer systems for controlled release of biologically active compounds [1–3]. Controlled prolonged delivery of biologically active compounds in desired doses makes it possible to circumvent many existing limitations of traditional drug delivery such as peroral, injection, aerosol, etc. Usually, the existing limitations concern increased toxicity and instability of drugs, irregular rate of drug delivery, inefficient consumption of the target compound, etc. The use of polymer systems for controlled drug release as macroimplants and microparticles allows controlled and targeted delivery of drugs, and these advantages are particularly important for the therapy against chronic deceases. The use of drug-containing polymer systems also makes it possible to expand the time of controlled drug release from several minutes (nanoparticles) to several months (matrices and containers), and this opportunity improves the efficiency of biologically active compounds. Even though polymer systems show evident advantages for controlled drug release, one should also consider their limitations, among which are the toxicity of the polymer matrix, biological incompatibility, undesirable secondary products of biodegradation, inevitable implantation and further removal of the polymer systems (for stable polymers), as well as high cost of polymer systems for controlled drug release as compared with traditional drug systems [4].

One of the promising and efficient drugs used for therapy against cardiovascular [5] and oncological diseases [6] is the class of cell proliferation inhibitors (CPIs). However, introduction of some CPIs is associated with undesirable secondary effects characteristic of toxic compounds, and this factor prevents wide use of the above inhibitors. It was shown earlier that the antithrombogenic drug dipyridamole (DPD) is capable of efficient inhibition of cell proliferation [7]; however, in contrast to many other CPIs, this drug shows a smaller amount of negative effects [8]. Due to the combination of its high efficacy and low toxicity, DPD was selected as the test compound for the development

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and study of polymer systems for the prolonged drug release.

Using DPD as the target drug, W. Zhu et al. [9] were the first to propose a DPD-containing system for local prolonged drug release. This system uses microspheres based on the copolymer of polylactide and polyglycolide, and this system was shown to operate for 35 days. The release of the desired biologically compound proceeded in two stages: the initial stage is diffusion-controlled drug release from the polymer matrix, and the second stage involves enzyme-induced polymer degradation.

However, the use of polylactides, polyglycolides, and their copolymers has certain complications due to chronic tissue inflammation as a response to implantation of the above polymers [10, 11]. To reduce the inflammatory response, researchers proposed applying either biocompatible coatings [12] or biologically active compounds [13–17].

As an alternative to polylactides, polyglycolides, and their copolymers, we used the bacterial poly(3-hydroxy)butyrate (PHB) [18, 19]. In recent years, PHB and its copolymers prepared by biotechnological processing have attracted keen interest as biodegradable and biocompatible polymers for various medical applications. The physicochemical and biological characteristics of PHB allow this polymer to be used for diverse implantable medical systems: vascular implants, periodontal membranes, prostheses for osteosynthesis and regeneration of cartilaginous tissues, as well as for the deposition of biocompatible coatings on various medical articles (cellular prosthetic implants, stents, vascular implants), etc. [20]. Moreover, PHB can offer the advantages of encapsulation and further prolonged release of various chemical compounds, including pharmacologically active compounds and this knowledge makes it possible to use this polymer for novel polymer systems for controlled drug release [21].

Nowadays, the high biocompatibility of PHB has been proved by abundant experimental evidence. In vitro experiments on cell growth on PHB-based supports have demonstrated fair survival and proliferation of various cells; these experiments have also demonstrated a low inflammable tissue reaction towards the implanted material [22–30].

The high biocompatibility of PHB is provided by the following factors: bacterial PHB is characterized by its stereoselectivity [31]; as oligomers (up to 150 residues of 3-hydroxybutyric acid), PHB is present in tissues and blood of mammals [31–35]; 3-oxybutyric acid is an intermediate product of the biodegradation of PHB, which is normally present in blood and intercellular fluid [36, 37]; the rate of enzymatic degradation of PHB is appreciably slower that the enzymatic degradation of polylactides, and this factor reduces the local concentration of the polymer degradation products [28].

The objective of this work is the development and characterization of systems for controlled drug release that are based on PHB microspheres, and this study addresses the preparation of novel polymer drug systems. In the future, the use of DPD as a biologically active compound in the proposed systems for controlled drug release makes it possible to advance a new drug injection system that, as compared with the traditional one, provides local long-term antiproliferative action.

EXPERIMENTAL

In this work, we used the Azotobacter chroococcum 7B producing strain for the preparation of PHB; this strain is capable of synthesizing up to 80% of PHB with respect to dry cell weight. To provide overproduction of PHB, the Azotobacter culture was cultivated in Burk's medium in the presence of excess amounts of carbon source (g/l): $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.006; sodium citrate, 0.5; $CaCl_2$, 0.1; $K_2HPO_4 \cdot 3H_2O$, 1.05; KH_2PO_4, 0.2; sucrose, 40. The process was conducted for 48 h under aerobic conditions at 28°C. The yield of the dry biomass was 10 g/l. The content of polymer in the Azotobacter cells was 76% with respect to the dry weight of cells. Recovery and purification of the polymer from the Azotobacter chroococcum biomass included the dissolution of PHB in chloroform by stirring at 37°C for 12 h; PHB solution was separated from cells by filtration; PHB recovery from its chloroform solution was accomplished by its precipitation with isopropanol. After triple dissolution of PHB in chloroform and precipitation with isopropanol, the polymer was dried in air at 60°C.

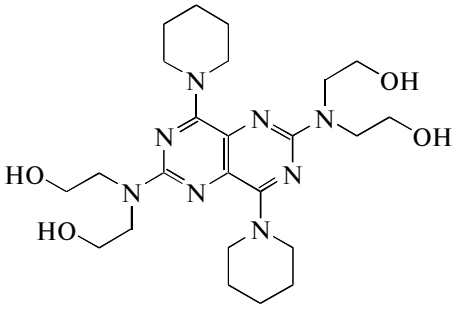
The molecular mass of the polymer was estimated by viscometry: the viscosity of the PHB solution in chloroform was measured at 30°C on an RT RHEOTEC viscometer (RheoTec, Germany); the molecular mass was calculated using the MarkKuhnHouwink equation, and the characteristic viscosity of the polymer solution in chloroform was $[\eta] = 7.7 \times 10^{-5} \times M^{0.82}$ [38]; this value was 485×10^3.

The chemical structure, type of crystalline lattice, and degree of crystallinity (0.74) of PHB were estimated in earlier studies by differential scanning calorimetry, IRFourier spectroscopy, and X-ray analysis [39].

In this work, we prepared DPD-loaded microspheres using single emulsion method [9]. The chemical structure of the DPD molecule is presented below.

Table 1. Conditions for preparation of PHB-based microspheres loaded with DPD

Weight of components in solution, mg		Volume of solvent, ml	Concentration of PVA, wt %	Rotation speed, rpm	Diameter of microspheres, μm	Content of DPD in PHB-based microspheres, wt %
DPD	PHB					
24	96	8	1.2	2000	3.6 ± 2.4	4.8 ± 0.4
24	96	9	0.6	1000	18.7 ± 2.9	5.2 ± 0.4
24	96	5	0.4	600	62.7 ± 6.6	4.9 ± 0.4
47	100	4	0.6	500	91.7 ± 15.4	11.0 ± 0.5



DPD and PHB (1 : 4) were dissolved in chloroform (4–9 ml), and this mixture was added to 300 ml of PVA aqueous solutions (0.4–1.2 wt %) under stirring. The cocktail was mixed for 2 h on an RZR 2021 mechanical overhead stirrer (Heidolph, Germany) at 600–2000 rpm or on a SilentCruisher M homogenizer (Heidolph, Germany) at 20000 rpm. Once the organic solvent was fully evaporated by heating at 45°C, the fractions of microspheres with uniform size distribution were obtained by filtration through glass filters with various pore diameters (16 and 40 μm). The separated microspheres were repeatedly separated by centrifugation (for 6 min at 4400 rpm) on a 5702 R centrifuge (Eppendorf, Germany); then, the microspheres were washed three times with distilled water for complete removal of the emulsifying agent and DPD from the surface of microspheres. Then, the microspheres were dried in a thermal chamber at 60°C, and the resultant powder was carefully ground in a mortar.

The content of DPD in the microspheres was estimated after their dissolution in chloroform by measuring light absorption on a DU-650 spectrophotometer (Beckman Coulter, United States); the absorption maxima were seen at 293 and 415 nm; the samples were compared with the reference solutions of PHB and DPD in chloroform.

As follows from Table 1, the stirring rate appears to be the key factor that controls the dimensions of microspheres.

The controlled release of DPD from the microspheres was performed at 37°C in a TC-1/20 thermostat (Russia) in phosphate buffer (pH 7.4) containing minor amounts of emulsifying agent (0.05% by volume, Triton X-10): four batches of microspheres (5 mg) in 4 ml of the buffer solution were mixed in weighing cups at 50 rpm on an MS-01 magnetic stirrer (Elmi, Latvia). The kinetics of DPD release within specified time intervals was studied as follows: the microspheres were separated from the buffer by centrifugation at 14000 rpm on a 5702 R centrifuge (Eppendorf, Germany), and 4 ml of fresh buffer was added. The content of DPD in the buffer was estimated spectrophotometrically in comparison with the phosphate buffer. Extinction coefficients of DPD at 293 and 415 nm [$\varepsilon_{293} = 54245$ and $\varepsilon_{415} = 15340$ l/(mol cm)] were calculated from the calibration curves. The residual content of DPD in the microspheres was found by their dissolution in the weighed portions of the solvent and solvent evaporation; spectrophotometric estimation of the DPD concentration was performed in comparison with reference solutions of known concentrations.

The mean diameter and standard deviation of different batches of microspheres were calculated from the corresponding microscopic images collected on a Biomed 1 Version 2 microscope (Biomed, Russia) equipped with a MYscope 300M ocular (Webbers, Taiwan).

Table 1 lists the experimental conditions (stirring rate, volume and concentration of initial solutions) for the preparation of PHB-based microspheres of different diameters containing given weight fractions of the encapsulated DPD.

RESULTS AND DISCUSSION

Figure 1 shows microscopic images of spherical PHB-based microparticles of different diameters containing encapsulated DPD. The microparticles have a regular spherical shape without any visible defects. They are colored throughout the whole volume and contain no visible inclusions (dense regions) or cavities; this allows us to conclude that DPD is uniformly distributed throughout the whole space of the microspheres.

Figure 2 presents the kinetic profiles of DPD release into phosphate buffer from PHB-based microspheres of different diameters. In Fig. 2, curves *3* and *4*, corresponding to microspheres with diameters of 63 and 92 μm, have two characteristic regions: the initial region of fast desorption of drugs and a well-pro-

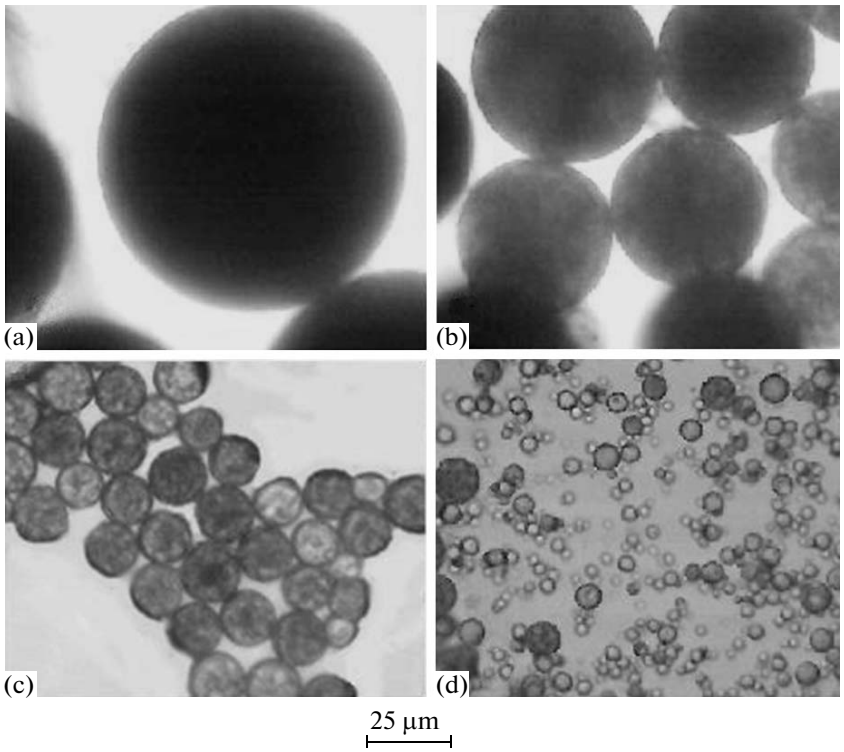


Fig. 1. Microscopic images of PHB-based microspheres in the initial state in phosphate buffer: diameters of microspheres are 92 ± 13 (a), 63 ± 7 (b), 19 ± 3 (c), and 4 ± 2 μm (d).

nounced continuous linear region corresponding to the kinetics of zero-order release. Once the release from the 63 μm microspheres has completed, the kinetic curves at long times change their slopes. For smaller microspheres (19 μm), the curves also show a short linear region at 6–21 h (curve *2*); for the smallest microspheres (4 μm), this region is missing (curve *1*).

A similar kinetics of drug release has been described for microspheres based on the copolymers of polylactides, polyglycolides, and 5-fluorouracil [40]. The only difference is that, in addition to the above stages of drug release (linear and nonlinear), the system shows a third time interval where, at the final stage, a burst release is observed. In our recent works, the kinetic profiles of DPD release from PHB films were studied. These curves also show two similar regions of drug release but without the final stage of the burst release [21]. The thickness of the PHB films (10, 20, and 40 μm) is close to the diameter of the microparticles studied in this work.

At the present time, the adopted knowledge is that controlled drug release in polymer systems is governed by diffusion processes. These processes primarily occur because, in a multicomponent system (for example, a microparticle containing drug, water and in vitro low-molecular-mass components of phosphate buffer or in vivo components of biological media), gradients of the chemical potentials of the above components arise. In this case, the diffusing compounds can flow in opposite directions. For example, drug flow is directed from the microsphere to the surrounding medium, whereas water flow is directed oppositely. With allowance for the fact that the diffusion phenomena can be aggravated by structural relaxation, chemical (catalytic) reaction of macromolecules, transformations in the polymer matrix, as well as by certain other processes, it seems evident why the profiles of the kinetic curves can be predicted only if the whole set of various chemical and physical processes is known.

Siepmann et al. have observed three stages with different mechanisms of drug release when 5-fluorouracil is released from microparticles based on random copolymers of polylactide and polyglycolide under in vitro conditions [40–42]: at the early stages, the diffusion-controlled process dominates, when the drug release rate is linear and high (in the literature, this stage is called the burst effect); according to Siepmann et al., the central part of the kinetic profile represents the combination of drug diffusion and degradation of macromolecules, and this behavior is described by the linear curve; at the final stage, the breakdown of the polymer network leads to another burst of the drug component (see Fig. 12 in [40]).

In our recent works on the in vitro release of 5-nitrofurfurilidene semicarbazone (as a model antiseptic) [43], DPD, or indomethacine [21] from PHB-based films, the kinetic profiles of all three sys-

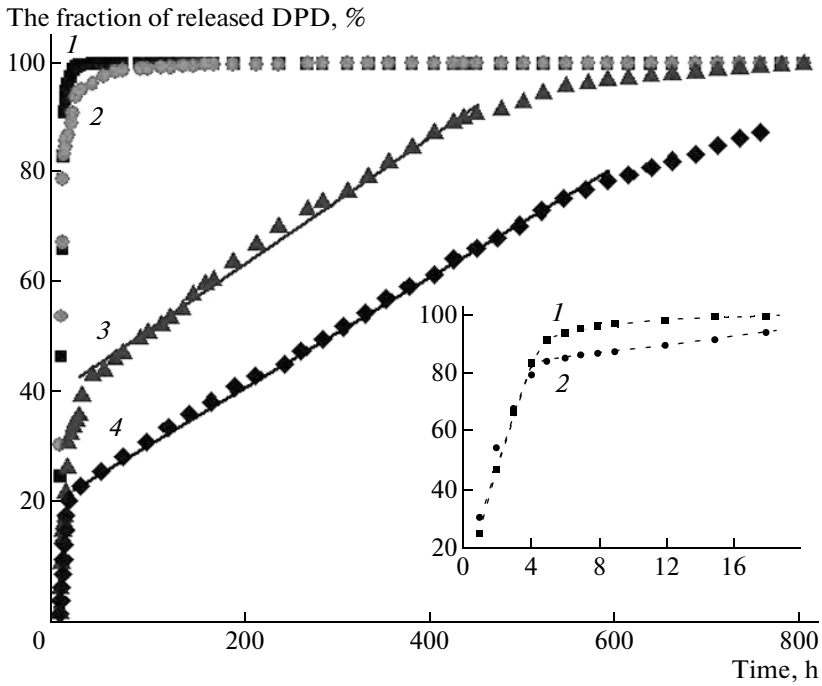


Fig. 2. Kinetic profile illustrating DPD release from PHB-based microspheres in phosphate buffer at 37°C. Diameters of microspheres are 4 (*1*), 19 (*2*), 63 (*3*), and 92 μm (*4*) (inset: initial portion of curves *1* and *2*). Straight lines show the linear regions in kinetic curves *3* and *4*.

tems was shown to have the initial diffusion-controlled stage, which gradually transforms into the linear stage, but the final third stage corresponding to the breakdown of the polymer network is missing. Therefore, the kinetic profiles describing drug release from PHB-based films or microspheres appear virtually the same. By adopting the proposed three-stage scenario of drug release, we would like to mention that PHB with its high hydrolytic stability (as compared with polylactides, polyglycolides, and their copolymers) does not experience the final stage of network breakdown, and no final burst release of DPD from films and microspheres is observed (Fig. 2). Moreover, we believe that the existence of the linear regions in the kinetic curves describing drug release in films [21, 43] and microspheres can be explained not only by the combination of diffusion and hydrolytic degradation but also by the predominant zero-order reaction, which reflects the onset of the in vitro hydrolytic degradation of PHB macromolecules.

Similar to [21, 43], the diffusion kinetic equation describing the linear and nonlinear stage of DPD release from spherical particles can be represented as the sum of two contributions: desorption from the sphere via the diffusion-controlled mechanism [the first term in Eq. (1)] and release via the zero-order reaction (the second term):

$$\partial G_t/\partial t = D[\partial^2 G_t/\partial z^2 + (2/z)(\partial G_t/\partial z)] + k. \quad (1)$$

Here, D is the constant (or integral) diffusion coefficient of the drug in the polymer microsphere (cm^2/s); $G_t(z, t)$, $\partial G_t/\partial z$, and $\partial^2 G_t/\partial z^2$ stand for the current drug concentration (%) and its first and second derivatives with respect to the diffusion coordinate (z), respectively; $\partial G_t/\partial t$ is the first derivative of the drug concentration with respect to the diffusion time (t), and k is the constant of the hydrolytic degradation of PHB (s^{-1}). The transition from Eq. (1) to the traditional diffusion equation, which is valid for a spherical particle and hence for microspheres, is performed by the introduction of new variables, $C_t \equiv G_t - kt$ and $u = C_t z$:

$$\partial u/\partial t = D[\partial^2 u/\partial z^2]. \quad (2)$$

Boundary and initial conditions for the solution of Eq. (2) are written in the standard way, which is valid for desorption from spherical objects [44]: at $t = 0 \rightarrow C_0 = \text{const}$ when $0 < z < R$; at $t > 0$ and $z = R \rightarrow C_0 = 0$, at $t > 0$ and $z = 0 \rightarrow u = C_t z = 0$.

In the absence of any complications at the microsphere(aqueous medium) interface (in the case under study, phosphate buffer), in particular, in the absence of concentration polarization, drug release from spherical objects is described by Eq. (2), which corresponds to the diffusion-controlled mechanism of desorption of low-molecular-mass compounds [44].

At relatively short times or at the initial stage of the kinetic desorption curve when $M_t/M_\infty \leq 0.5$–0.6, Eq. (2) can be approximated as

$$M_t/M_\infty = 6[(Dt/\pi^2 R^2)^{0.5}], \quad (3)$$

where M_t, M_∞ stand for the overall weights of the desorbed drug component at time t and at infinite time ($t \to \infty$), respectively; R is the average radius of the microsphere; all other designations are similar to those in Eq. (1).

A graphical solution of Eq. (3) in coordinates of $(M_t/M_\infty) - t^{0.5}$ makes it possible to use this equation as the criterion for the diffusion-controlled mechanism of drug release and to quantitatively estimate the diffusion coefficient in the polymer matrix. Figure 3 shows the initial fragments of kinetic curves of DPD release from PHB-based microspheres of different diameters. The above curves are given in the following coordinates: relative weight of the released drug from the DPD-containing microspheres (M_t/M_∞) and square root of time. As follows from Eq. (3), the diffusion coefficients can be easily estimated from the following relationship:

$$D_\beta = \pi^2 R^2 (\tan\beta)^2/36. \quad (4)$$

Here, tanβ is the tangent of the slope of the linear region of the kinetic curve in (M_t/M_∞)–$(t)^{0.5}$ coordinates. Table 2 lists the diffusion coefficients for DPD calculated using Eq. (4) for microspheres of different diameters. Similar to [45], for the PHB–DPD system under study, one can observe a dramatic dependence of the diffusion coefficients on the radius of microparticles.

Returning to the analysis of the kinetic curves in terms of diffusion equation (2), let us mention that, for microspheres in phosphate buffer at relatively high exposure times or, more correctly, at $M_t/M_\infty \geq 0.5$, the solution of this equation appears as

$$M_t/M_\infty = 1 - (6/\pi^2)\exp[-D_\alpha \pi^2 t/R^2]. \quad (5)$$

In this case, the diffusion coefficient is also estimated by the graphical solution of Eq. (5) in semilog coordinates of $\ln(M_t/M_\infty)$–t as

$$\ln[1 - (M_t/M_\infty)] = \ln(6/\pi^2) - D_\alpha \pi^2 t/R^2. \quad (6)$$

Figure 3 gives examples of this solution for microspheres of different diameters. The linear regions of the kinetic curves in semilog coordinates make it possible to calculate the tangent of the slope angle tanα and the integral diffusion coefficient

$$D_\alpha = \tan\alpha R^2/\pi^2. \quad (7)$$

In the case of molecular diffusion described by Fick's law, one can expect that the diffusion coefficients calculated by the two independent methods at the initial and final fragments of the kinetic curve of drug desorption by Eqs. (4) and (7) should be equal, $D_\alpha = D_\beta$. Indeed, as follows from Table 2, the diffusion coefficients calculated at different portions of the kinetic curves are close, and this indicates the classical diffusion mechanism, which controls the kinetics at the initial stage of drug release.

The diffusion is coupled to the linear kinetics of DPD release (Fig. 2). The contribution from the linear kinetic process becomes most pronounced when the diffusion-controlled stage is completed. In this case, a constant rate of drug release slightly depends on the diameter of particles. As follows from Fig. 2, this pro-

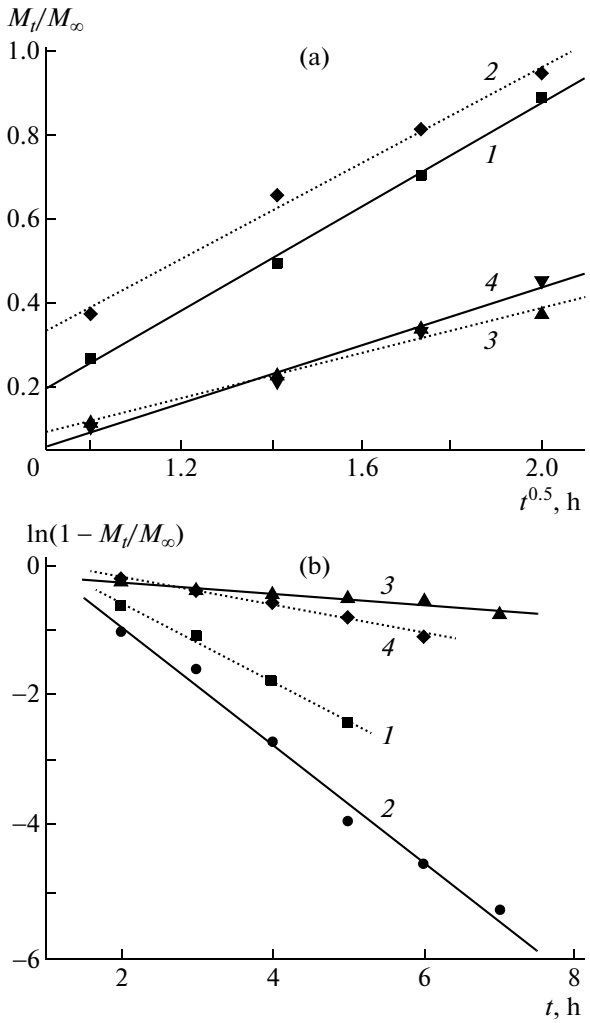


Fig. 3. Initial (a) and final (b) regions of the kinetic curves illustrating DPD release from PHB-based microspheres. Diameter of microspheres are 4 (*1*), 19 (*2*), 63 (*3*), and 92 μm (*4*). Data are given in the coordinates of Eq. (3) and in the semilog coordinates of Eq. (6).

Table 2. Diffusion coefficients of DPD upon its release from PHB-based microspheres, which control the diffusion stage of drug release [Eqs. (4) and (7)]

Diameter of microsphere $d \times 10^3$, cm	Diffusion coefficient $D \times 10^{11}$, cm^2/s	
	calculation by Eq. (7)	calculation by Eq. (4)
0.4	0.1	0.08
1.9	1.5	2.0
6.3	2.8	2.6
9.2	21.1	16

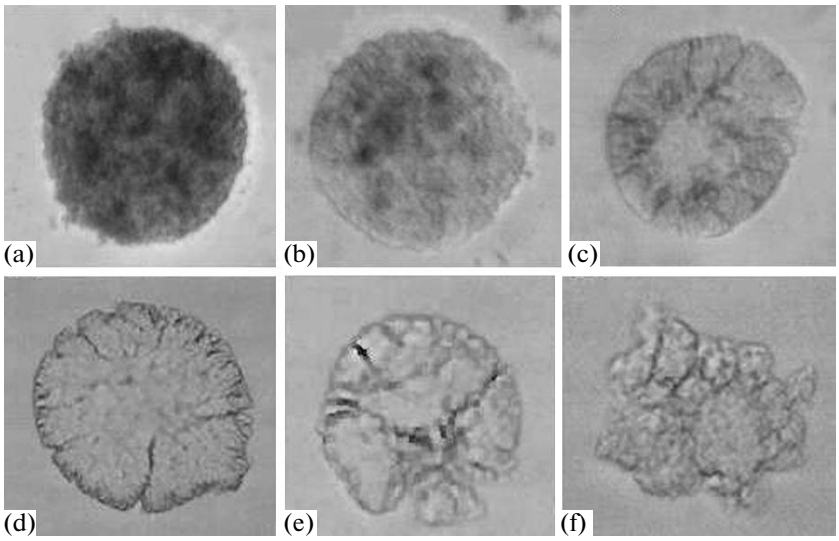


Fig. 4. Hydrolytic degradation of the PHB-based microspheres with a diameter of 60 μm containing encapsulated DPD after their incubation in phosphate buffer (pH 7.4) at 37°C for 1 (a), 5 (b), 12 (c), 17 (d), 20 (e), and 25 days (f).

cess is vividly illustrated for the biggest particles. The slopes of the linear regions are close and correspond to the constant of hydrolytic degradation of PHB; this case is similar to that observed for the PHB-based films. The rate of hydrolytic degradation of PHB estimated through the zero-order equation is independent of the size of the sample, and Fig. 2 proves this.

The profiles of the kinetic curves and the low rate of DPD release agree fairly well with the kinetics of weight loss of the biopolymer measured under the same conditions for the PHB-based films of the same thickness (40 μm) of the diameter of microspheres [46]. Except for the initial time interval when weigh loss proceeds at a high rate (burst effect [47]), the main weight loss in time is described by the linear law or according to the zero-order kinetic equation. Slow weight loss of the samples within the same time interval as the linear DPD release attests to the hydrolytic degradation of PHB macromolecules. The process of degradation is vividly proved by the sequence of microscopic images corresponding to microparticles at different exposure times in phosphate buffer (Fig. 4). Therefore, the weight loss of PHB, similar to shape loss and degradation of microspheres containing incapsulated DPD, indicates a marked contribution from the hydrolytic process to DPD release.

CONCLUSIONS

In this work, we reported on the preparation of microspheres of different diameters (from 4 to 92 μm) from biodegradable PHB and the biologically active compound DPD. In phosphate buffer at 37°C, the above spheres demonstrate prolonged drug release, which manifests itself in prolonged (more than one month) release of DPD at a constant rate. Analysis of the kinetic curves of drug release, measurements of weight loss of PHB samples, and direct microscopic observations illustrating loss in shape shows that the kinetic profile depends on the combination of the diffusion process and hydrolytic degradation of the biopolymer. The diffusion constants are measured, and their correlation with the dimensions of microparticles is shown. For bigger microspheres, the kinetic curves demonstrate the existence a continuous linear region, and this observation allows the use of the PHB–DPD microsystems for local controlled drug delivery. Introduction of DPD to PHB-based microspheres seems promising for new drug injection systems for local long-term antiprolifetive and antithrombogenic effects, for example, revention of restenosis (narrowing of blood vessels), which is often observed after vascular surgery.

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