ISSN 0003-6838, Applied Biochemistry and Microbiology, 2012, Vol. 48, No. 6, pp. 598–602. © Pleiades Publishing, Inc., 2012.
Original Russian Text © E.V. Filatova, S.G. Yakovlev, A.P. Bonartsev, T.K. Makhina, V.L. Myshkina, G.A. Bonartseva, 2012, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2012, Vol. 48, No. 6, pp. 662–667.

Prolonged Release of Chlorambucil and Etoposide from Poly-3-Oxybutyrate-Based Microspheres

E. V. Filatova[a], S. G. Yakovlev[a], A. P. Bonartsev[a, b], T. K. Makhina[a], V. L. Myshkina[a], and G. A. Bonartseva[a]

[a] *Bakh Institue of Biochemistry, Russian Academy of Sciences, Moscow, 119071 Russia*
e-mail: bonar@inbi.ras.ru

[b] *Faculty of Biology, Moscow State University, Moscow, 119992 Russia*

Received Aughust 26, 2011

Abstract—Microspheres were obtained on the basis of poly(3-oxibutyrate) (POB) with the inclusion of the Chlorambucil and Etoposide cytostatic drugs in a polymer matrix, and the morphology, kinetics of drug release from microspheres, and the interaction between microspheres and tumor cells in vitro were studied. Data on the kinetics of drug release suggests that a prolonged release occurs by drug diffusion from the polymer matrix at the initial stage and at the expense of hydrolytic degradation of the polymer at a later stage. A study of the biocompatibility and biological activity of biopolymeric microspheres showed that chlorambucil operates actively and strongly inhibits the growth of cultured cells for a short time (24 h). Etoposide acts weaker (the percentage of cell growth suppression during 48 h does not exceed 50%), but subsequently it has a basis for the creation of new dosage forms with prolonged action of Etoposide and chlorambucil for cancer therapy.

DOI: 10.1134/S000368381206004X

The current state of chemotherapy against oncological diseases shows a necessity for an improvement in the therapeutic efficiency of antitumor agents. A modern cytostatic agent has to meet the following criteria: to possess high efficiency at a constant predetermined concentration in tumor and at the same time to display low systemic cytotoxicity and prolonged activity. It is possible to achieve those conditions by developing macromolecular therapeutic systems with a controlled release of drug agents, i.e., by creating conjugates of drug substances with polymeric carriers.

Poly-3-oxybutyrate—a biodegradable plastic with good biocompatibility and an ability to completely degrade CO_2 and water in the human body—was used as a polymeric carrier in this work. At present, technical guidelines and regulations for medical poly-3-oxybutyrate production have been developed, and it is shown that the poly-3-oxybutyrate polymer is nontoxic, does not cause irritation or sensitizing, and meets the regulatory requirements for pharmaceutical use through parenteral and peroral administration [3, 4]. Poly-3-oxybutyrate is actively used for the creation and study of prolonged drug release systems for a wide range of drug substances [5, 6]. A gradual release of the drug substance from a biopolymeric matrix allows for the prolonged maintenance of a required active drug concentration in the body or locally in a specific organ or tissue. Thus, it eliminates the need for repeated multiple drug administration, decreases toxicity and side effects, and increases the stability of the drug and its efficiency owing to a constant administration rate. It is especially important for oncological therapeutic agents, which have high toxicity and a number of serious side effects in patients.

In the creation of controlled drug release systems, two anticancer drugs—chlorambucil and etoposide—were used. The traditional formulations of these drugs have a range of side effects; therefore, the use of biocompatible polymers, such as poly-3-oxybutyrate, could not only eliminate these disadvantages of traditional oncological drugs, but also allow one to control the time and dose of drug release. It was previously shown by us that the rate of drug release from a polymer base can be influenced by varying the molecular weight and size of polymer particles [8–10].

The aim of this work is the synthesis and study of the properties of prolonged chlorambucil and etoposide drug substances, creation of biopolymeric microspheres on the basis of a poly-3-oxybutyrate-containing cytostatic drug substance—chlorambucil or etoposide—study of microsphere drug release kinetics, and the analysis of biocompatibility and biological activity in vitro.

METHODS

Materials. Poly-3-oxybutyrate (Mm 236 kDa) of microbiological origin was used for the to create a microsphere. The *Azotobacter chroococcum* 7B strain was used as a POB producer [10]. Chlorambucil and etoposide (Sigma, Germany), chloroform (Ekos-1,

Russia), and polyvinyl alcohol (MP Biomedicals, United States) were used.

Poly-3-oxybutyrate production. The POB-producing *A. chroococcum* 7B strain was used, which can produce up to 80% POB of dry cell mass. To achieve super synthesis of POB, the *A. chroococcum* strain was cultivated on Berk culturing media under the conditions of an increased carbon source content (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 cultivation was performed for 48 h under anaerobic conditions at 28°C. The yield of dry biomass was 10 g/l of medium. The polymer content in *A. chroococcum* cells was 76% of dry mass.

Separation and purification of polymer from *A. chroococcum* biomass included POB solubilization in chloroform by shaking on a rocking platform at 37°C for 12 h, separation of POB from cell residues by filtration, and extraction of POB from the chloroform solution with isopropyl alcohol. After three times repeating the POB solubilization process in chloroform and extraction with isopropyl, the purification procedure was completed by air-drying POB at 60°C. The molecular weight of the polymer was established by viscometry: the POB solution viscosity in chloroform was measured at 30°C with a PT RHEOTEC viscometer (RheoTec, Germany). The molecular weight was calculated from the equation of Mark–Houwink–Kuhn, using the following coefficients: $[\eta] = 7.7 \times 10^{-5}M^{0.82}$, where η is viscosity and M is the molecular weight of POB [11].

Creation of POB microparticles. Microspheres were created using single-step emulsification of a drug and POB solution in chloroform, followed by evaporation of the solvent [10]. This method was adapted to encapsulate chlorambucil and etoposide. A drug and POB solution with a molecular weight of 236 kDa in a ratio of 1 : 4 in 8 ml of chloroform was gradually added to 100 ml of polyvinyl alcohol in distilled water (concentration 1.5% mass/volume) with agitation. Agitation was continued for 2 h, using an RZR 2021 mechanical top-driven mixer (Heidolph, Germany) at 1000 rpm. After the complete evaporation of the solvent, microspheres were separated by centrifuging (6 ml at 3000 g), using a 5702 R centrifuge (Eppendorf, Germany), and then three times washed with distilled water for the complete removal of emulsifier and drug substance from the surface of the microspheres. The microspheres were dried in a thermostat at 37°C.

Determination of microspheres and their POB content. The average diameter of the obtained microspheres was determined by microphotograpy using a Biomed 1 Var.2 optical microscope (Biomed, Russia) with a MYscope 300M digital ocular (Webbers, Taiwan).

The content of chlorambucil and etoposide in the microspheres was determined using spectrophotomerty after their solubilization in chloroform by measuring the level of light absorption on a DU-650 spectrometer (Beckman Coulter, United StatesA) (absorption maxima at 259 and 305 nm for chlorambucil and 288 nm for etoposide) in comparison with a control POB solution in chloroform and by creating a calibration curve using different concentrations of control POB and drug solutions in chloroform.

Kinetics of drug release from POB-based microspheres. The chlorambucil and etoposide release from a microsphere in vitro was studied at 37°C in a TS-1/80 SPU thermostat (Labtekh, Russia) in 25 M potassium phosphate buffer (pH 7.4) with the addition of a small amount of emulsifier (0.05% Triton X-10): four portions of 20-mg microspheres in 4 ml of buffer were mixed on a shaker (BioSan, United States) at 330 rpm. After certain time intervals (1, 2, 3 h, etc.), microspheres were separated from the buffer by centrifugation at 14100 g on a MiniSpin Plus centrifuge (Eppendorf, Germany); then a fresh buffer solution was added. The content of the drug substance in the buffer was determined on a DU-650 spectrophotometer (Beckman Coulter, United States) with the help of a calibration curve constructed using aqueous solutions of drug substances in different concentrations. The remaining content of drug substances in microspheres was also determined by spectrophotomerty after solubilization in chloroform.

Microscopy. An initial study of microparticle properties and morphology was performed by optical microscopy using a Biomed 1 Var. 2 microscope (Biomed, Russia) with a MYscope 300M digital ocular (Webbers, Taiwan). Microphoto pictures of the studied microparticles were obtained by scanning electron microscopy (SEM) in electron and ion beams using FEI-SMA-QUANTA 200 and SMA QUANTA FEG microscopes (FEI Company, United States).

Interaction of microspheres with a cell culture in vitro. Human breast cancer cell line MCF-7 was used to assess the biosafety of microspheres in vitro. Cells were cultivated according to the Freshni methods [12]. The cell survival rate after treatment with the studied drug was calculated by formula (1), where S is the cell survival rate, N_{exp} is the amount of viable cells in the experiment, and N_{contr} is the amount of viable cells in the control:

$$S = \frac{N_{exp}}{N_{contr}} \times 100\%. \quad (1)$$

A standard MTT assay (using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT) was used as the most representative in studies with tumor cell cultures [12]. Microspheres were dispergated in cultivation media and added to cell cultures in various concentrations. Prior to that, dry microspheres were sterilized at 100°C for 10 min. Microspheres were tested in parallel measurements at a concentration of 3 mg/ml. A suspension of biopolymeric microspheres that did not contain drugs (at a

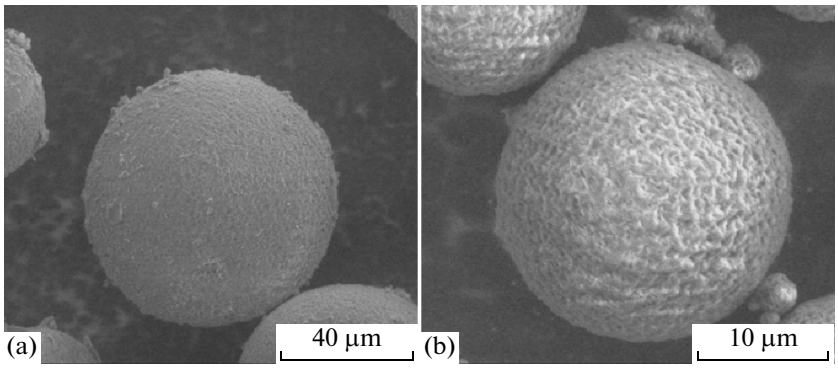

Fig. 1. Microphoto pictures of the obtained microspheres (electron microscopy): chlorambucil (a); etoposide (b).

concentration of 3 mg/ml) was used as a negative control. The measurements were taken 24 and 48 h after the beginning of cultivation.

RESULTS AND DISCUSSION

A single-step emulsification method was used to create POB-based microspheres with an encapsulated drug substance [10]. Microspheres with a mass ratio of chlorambucil to polymer of 12% were created. The average diameter was 24 μm. In addition, microspheres with a mass ratio of etoposide to polymer of 10% were created (with an average diameter of 28 μm).

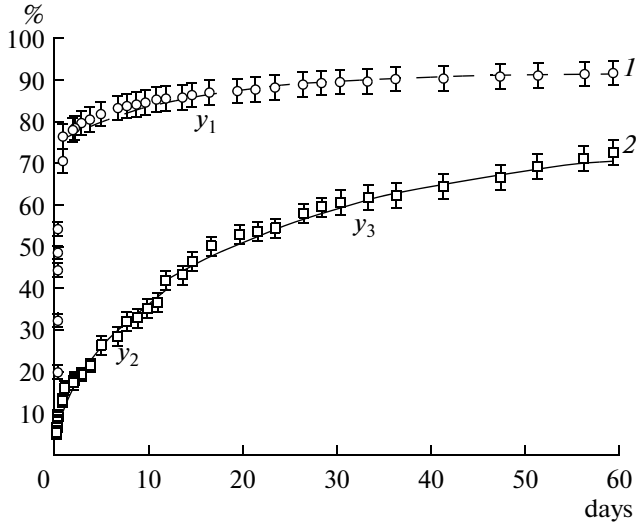

Fig. 2. Kinetics of drug release (%) from POB-based microspheres (0.025 M phosphate buffer, pH 7.4, at 37°C): *1*, microspheres with chlorambucil (the average diameter is 24 microns; inclusion, 12%); $y_1 = 1 + m_1\exp(-k_1x)$, $m_1 \sim -0.6$; *2*, microspheres with etoposide (the average diameter is 28 microns; inclusion, 10%); $y_2 = a_2xb_2 + c_2x$, $b_2 \sim 0.5$; $y_3 = 1 + m_3\exp(-k_3x)$, $m_3 \sim -0.6$.

Electron scanning microscopy was used for a detailed investigation of the microparticle properties and for the structure of their surface. On the microphoto pictures presented (Fig. 1), it is obvious that the surfaces of microspheres are uneven and polymer cords are clearly visible. Microparticles with chlorambucil have more even surfaces than ones with etoposide, which is possibly due to different hydrophobic properties of the substances and polymer base.

The kinetics of chlorambucil and etoposide release from a polymer matrix was investigated in vitro in 0.025 M phosphate buffer, pH 7.4, at 37°C (Fig. 2). The results show that the first stage of chlorambucil release is characterized by a high level of drug release (the so-called "burst effect"), which is probably due to low hydrophobic interactions between the drug and the polymer. At the next stage, diffuse processes between the drug and aqueous medium are predominant, and the kinetics profile is characterized by a degree function. At the later stages of drug release, it becomes constant and almost linear.

It is shown in Fig. 2 that the etoposide release is less active than the chlorambucil one and there is no obvious burst effect. This difference may be due to a higher affinity of etoposide to POB than that of chlorambucil because of hydrophobic interactions between the polymer and the drug, which elongated the time of the controlled release of the drug compared to more hydrophilic chlorambucil [13]. The release of etoposide lasts longer than that of chlorambucil, allowing one to reach a prolonged effect of the drug, which is also related to hydrophobic interactions between etoposide and POB [14].

The kinetic profile of etoposide release at the initial stages is approximated by a complex degree function with a degree close to 0.5 (Fig. 2), which corresponds to diffusion equation (2) [15,16], describing the release of a substance from polymeric microspheres, where M_t and M_0 are the amounts of drugs released

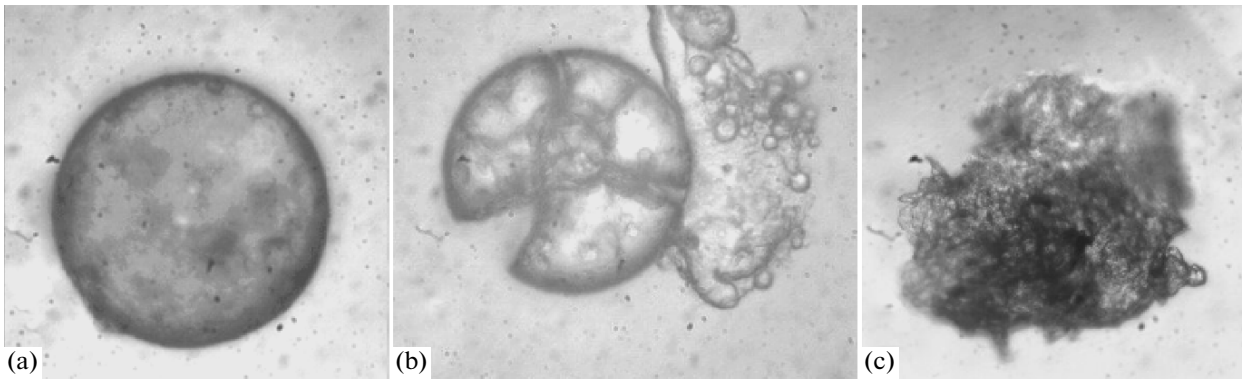

Fig. 3. Microphotography of hydrolytic degradation of biopolymer POB–based microspheres with encapsulated chlorambucil (optical microscopy): (a) 1 day, (b) 15 days, and (c) 30 days.

and initially loaded into the polymeric matrix (mg) at time t (sec); r is the microsphere radius (m, 24×10^{-6} and 28×10^{-6} m in our case); and D is the diffusion coefficient:

$$\frac{M_t}{M_0} = 6\left(\frac{Dt}{r^2\pi}\right)^{1/2} - \frac{3Dt}{r^2}. \quad (2)$$

While at the initial stage of etoposide release diffusion processes are prevalent, at later stages diffusion is replaced by hydrolytic degradation of the polymeric matrix, which explains the linearity of the drug release process. At these later stages of etoposide release (12 – 60 days), kinetic profiles are approximated by an exponential function (Fig. 2), which agrees with previously described relationship (3) [15, 16], where M_t and M_0 are the amounts of drugs released and initially loaded into the polymeric matrix (mg) at time t (sec); r is the microsphere radius (m), and D is the diffusion coefficient:

$$\frac{M_t}{M_0} = 1 - \frac{6}{\pi^2} e^{\left(\frac{-\pi^2 Dt}{r^2}\right)}. \quad (3)$$

During a chlorambucil release, because of its high hydrophilic properties, the main share of the drug (about 70%) is removed from the polymer matrix in 1 day. Therefore, the diffusion stage is fast (compared to an etoposide release) and almost completely equivalent to a burst effect (initial discharge of the drug), and all consequent stages are mainly determined by the degradation of the microparticle polymeric matrix; that is why this part of the kinetic profile (3–60 days) is described by an exponential function (Fig. 2).

Different degradation stages are clearly visible on pictures obtained by optical microscopy at different stages of the experiment (Fig. 3). The data shows that chlorambucil microsphere degradation is clearly noticeable on the 30th day of incubation in a phosphate buffer. By the 90th day, the microspheres lose their shapes and are presented by an amorphous polymeric aggregation. The etoposide microsphere degradation has the same steps and does not significantly differ from the degradation of chlorambucil microspheres.

At the last stage of the work, the interaction between a POB-based microspheres and encapsulated chlorambucil or etoposide with human breast cancer cell line MCF-7 was investigated. Cancer cells were incubated with clear (not carrying drugs) chlorambucil and etoposide polymeric microspheres for 48 h. The results were presented using a diagram of the cell survival rate as a function of the duration of microsphere incubation (Fig. 4).

The results indicate that polymeric microspheres not carrying drug substances are biocompatible with cancer cells and do not display cytotoxicity. POB-based microspheres with etoposide suppressed cell growth by 28% in 24 h and by 45% in 48 h of incubation. POB-based microspheres with chlorambucil inhibited cell growth even more efficiently. In 24 hours of chlorambucil microsphere incubation, the amount of tumor cells reduced fivefold, and in 48 h inhibition reached 96%.

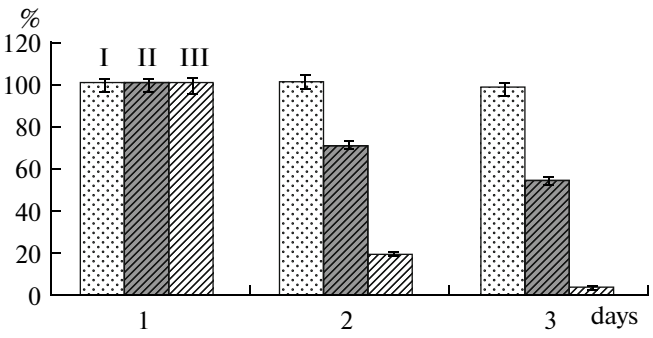

Fig. 4. Cell survival (%) diagram during incubation with microspheres containing (I) no drugs, (II) etoposide, and (III) chlorambucil.

Thus, the results obtained through a comparative analysis of the influence of etoposide and chlorambucil polymeric spheres on human breast cancer cell growth suppression showed that etoposide and chlorambucil polymeric spheres have different toxicity for cancer cell cultures and, to be specific, chlorambucil is more active and inhibits cell growth in a shorter incubation period (80% in 24 h) (Fig. 4). According to the kinetics data, the drug release level reached 80% in the first two days (Fig. 2). Etoposide has a weaker effect on cell growth (inhibition did not exceed 50% in 48 h), but it has a prolonged release effect from the polymeric matrix (according to an in vitro kinetic analysis (Fig. 2)). Chlorambucil can be used to perform an initial shock to a tumor with the consequent maintenance of a prolonged cytotoxic effect. Etoposide can be used for prolonged oncotherapy since a matrix system with this drug allows one to maintain the required therapeutic dose owing to its constant release.

***

Microspheres on the basis of biocompatible and biodegradable polymer poly-3-oxybutyrate were created with an inclusion of cytostatic drug substances—chlorambucil and etoposide. The kinetics of prolonged release of chlorambucil and etoposide from microspheres is investigated. The results indicate that their prolonged release is determined by the diffusion of drug substances from polymeric matrices at the initial stage and by the hydrolytic degradation of the polymer at later stages.

The main differences between prolonged chlorambucil and etoposide forms are identified based on an in vitro drug kinetic analysis and cell culture experiments. Chlorambucil is more active and has a stronger effect as an inhibitor of cell growth in a culture in a shorter time interval (24 h). Etoposide has a weaker effect (the percentage of cell growth inhibition in 48 h does not exceed 50%), but it has a prolonged release effect from the polymer matrix, which may be optimal for oncotherapy.

Thus, the results of this study can make a foundation for the creation and investigation of new prolonged formulations of chlorambucil and etoposide. Certain characteristics of prolonged chlorambucil and etoposide forms can be useful for the creation of an optimal strategy of oncological disease treatment in patients.

ACKNOWLEDGMENTS

The study was supported by the federal target programs "Investigation and Development of Scientific and Technological Complex of Russia in Priority Areas in 2007–2012" and "Human Capital for Science and Education in Innovation Russia" of the Ministry of Education and Science of the Russian Federation (state contracts nos. 16.512.11.2019, P548, and 16.740.11.0652); the equipment used belongs to the shared-use centers of the Moscow State University and Bakh Biochemistry Institute, Russian Academy of Sciences.

REFERENCES

1. Chen, G.Q. and Wu, Q., *Biomaterials*, 2005, no. 26, pp. 6565–6578.
2. Park, J., Ye, M., and Park, K., *Molecules,* 2005, vol. 1, no. 10, pp. 146–161.
3. Boskhomdzhiev, A.P., Bonartsev, A.P., Ivanov, E.A., Makhina, T.K., Myshkina, V.L., Bagrov, D.V., Filatova, E.V., Bonartseva, G.A., and Iordanskii, A.L., *Plast. Massy*, 2009, no. 8, pp. 13–18.
4. Boskhomdzhiev, A.P., Bonartsev, A.P., Makhina, T.K., Myshkina, V.L., Ivanov, E.A., Bagrov, D.V., Filatova, E.V., Iordanskii, A.L., and Bonartseva, G.A., *Biomed. Khim.*, 2009, vol. 55, no. 6, pp. 625–635.
5. Bonartsev, A.P., Livshits, V.A., Makhina, T.A., Myshkina, V.L., Bonartseva, G.A., and Iordanskii, A.L., *Express. Polym. Lett.*, 2007, vol. 12, no. 1, pp. 797–803.
6. Bonartsev, A.P., Iordanskii, A.L., Bonartseva, G.A., and Zaikov, G.E., *Polymers Res. J.*, 2008, vol. 2, no. 2, pp. 127–160.
7. *Gosudarstvennaya Farmakopeya Rossiiskoi Federatsii* (State Pharmacopoeia of the Russian Federation), Moscow: Nauch. Tsentr Ekspert. Sredstv Med. Primenen., 2008.
8. Bonartsev, A.P., Bonartseva, G.A., Makhina, T.K., Myshkina, V.L., Luchinina, E.S., Livshits, V.A., Boskhomdzhiev, A.P., Markin, V.S., and Iordanskii, A.L., *Appl. Biochem. Microbiol.*, 2006, vol. 42, no. 6, pp. 710–715.
9. Kosenko, R.Yu., Iordanskii, A.L., Markin, V.S., Bonartsev, A.P., and Bonartseva, G.A., *Khim.-Farmatsevt. Zh.*, 2006, vol. 41, no. 12, pp. 27–30.
10. Livshits, V.A., Bonartsev, A.P., Iordanskii, A.L., Ivanov, E.A., Makhina, T.A., Myshkina, V.L., and Bonartseva, G.A., *Vysokomol. Soed.*, 2009, vol. 51, no. 7, pp. 1243–1251.
11. Freshni, R.Ya., *Kul'tura zhivotnykh kletok: prakticheskoe rukovodstvo* (Animal Cell Culture: A Practical Guide), Moscow: Binom. Laboratoriya znanii, 2010.
12. Akita, S., Einada, Y., Miyaki, Y., and Fugita, H., *Macromoleculs,* 1976, no. 9, pp. 774–780.
13. Fundueanu, G., Constantin, M., and Ascenzi, P., *Acta Biomater.*, 2009, vol. 1, no. 5, pp. 363–373.
14. Yao, B., Ni, C., Xiong, C., Zhu, C., and Huang, B., *Bioproc. Biosyst. Eng.*, 2010, vol. 4, no. 33, pp. 457–63.
15. Arifin, D.Y., Lee, L.Y., and Wang, C.-H., *Adv. Drug Deliv. Rev.,* 2006, no. 58, pp. 1274–1325.
16. Baker, R., *Controlled Release of Biologically Active Agents,* New York: Acad. Press, 1987.