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Low Molecular Weight Poly(3-hydroxybutyrate) Microparticles Synthesized by Piezoelectric Spray Drying for the Sustained Release of Paclitaxel

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Abstract—Biocompatible and biodegradable polymer microparticles are contemporary medicines able to eliminate the side effects and unsatisfactory pharmacokinetics of already existing preparations. In this work we have developed a high-tech scalable method for the synthesis of paclitaxel-loaded poly(3-hydroxybutyrate) (PHB)-based microparticles. These particles were synthesized on a B-90 Buchi nano spray dryer by piezoelectric spray drying in an inert atmosphere. A regular spherical shape, narrow size distribution, and satisfactory results for the release of paclitaxel from the polymeric matric of microparticles in vitro make this polymeric medicinal form promising for its further application in pharmaceutics. Nanoparticles with a similar composition synthesized via the laboratory one-stage emulsification method were used for comparison. This study is the first stage in the creation of a sustained-action anticancer paclitaxel preparation.

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INTRODUCTION

Nowadays, chemotherapy remains the basic method for treating malignant tumors of different natures. One of the most widely applied anticancer medicines is paclitaxel representing is a cytostatic agent, which is classified among tyaxanes and was extracted from Pacific yew (*Taxus brevifolia*) bark for the first time. In its traditional medicinal form, with Cremophore representing polyoxyethylated castor oil as an active medicinal-substance solubilizer, the preparation is characterized by therapeutic efficiency, but is highly toxic and may have serious side effects [1, 2].

In general, most of the chemotherapeutic preparations in traditional medicinal forms are characterized by a rather incomplete fulfilment of therapeutic potential on the one hand and the retention of their negative side effects on the other hand. They frequently demonstrate low bioaccessibility in combination with high toxicity, due to which a preparation must be used in inefficient doses because of the risk of serious side effects. Moreover, several preparation infusions are usually required to attain the essential result of such treatment and maintain the pharmacokinetic parameters at a required level [3]. One of the approaches to the solution of this problem is the use of systems with the sustained release of a medicinal substance from polymeric micro- and nanoparticles [4, 5]. The bonding of a preparation in polymeric microparticles and its sustained release at a constant rate opens up opportunities for an increase in the efficient dose and provides the long-term maintenance of a required active substance concentration in a tumor. This provides the possibility of eliminating to some extent system side effects during the posttherapeutic period. It also eliminates the need for additional multiple-dose introduction of a preparation, simplifies its application, improves its pharmacokinetics and bioaccessibility, and reduces its local side effects. Nevertheless, the currently developed and studied medicinal forms based on polymeric micro- and nanoparticles also frequently have essential shortcomings, in particular, toxicity due to the low biocompatibility of polymers used; unsatisfactory release kinetics of a medicine; and the biodegradation of microparticles, whose control is difficult [6, 7].

For this reason, biodegradable and biocompatible polymers of microbiological origination, such as poly(3-hydroxybutyrate) and its copolymers, are increasingly frequently studied with the purpose of developing new systems for the efficient delivery of

Table 1. Spray drying process parameters

Process parameter	Parameter value
Drying gas flow rate	120 L/min
Inlet drying gas temperature	61°C
Inner instrument pressure	53 mbar
Spray-hole diameter	7 μm

chemotherapeutic preparations [8, 9]. It has recently been shown that PHB has high biocompatibility and capability for both hydrolytic and enzymatic degradation in vitro and biodegradation in mammalian tissues in vivo [10, 11]. Moreover, PHB has been shown to be able to form copolymers and composites with other polymers, this providing the possibility to change the physicochemical properties of a polymer within broad ranges and thereby influence the character of its interaction with a preparation [11–13]. This in turn enables control over the kinetics of medicine release from polymeric microparticles, thereby changing their pharmacokinetic properties. Such an approach will open up the opportunity to create new polymeric medicinal forms based on polymeric micro- and nanoparticles with a broad spectrum of pharmacokinetic characteristics, which must necessarily be taken into account when selecting a method of therapy for one or another disease.

Until quite recently, one-or two-stage emulsification was the most widely applied method for preparing such micro- and nanoparticles. The most frequently used method was the one-stage "oil–water phase" (O/W) emulsification, which consisted of the emulsification of a combined polymer–medicine solution in an organic solvent (oil phase) in an aqueous phase with an emulsifier, the further gradual evaporation of an organic solvent under vigorous stirring or ultrasonic treatment, and the precipitation and drying of biopolymeric microparticles [14]. However, such methods are poorly scalable for the production of preparations out of laboratory conditions. An alternative to such an approach is the synthesis of polymeric particles by spray drying [15].

Hence, the objective of this work was to create and study a sustained paclitaxel release system based on PHB microparticles synthesized by piezoelectric spray drying and to compare them with similar composition nanoparticles obtained by the laboratory direct membrane-emulsification method.

MATERIALS AND METHODS

Materials

To prepare the particles, we used poly(3-hydroxybutyrate) (PHB) representing a low-molecular biopolymer (~6000 Da), which was microbiologically synthesized using the *Azotobacter clorococcum 7B*–producing strain [16], and paclitaxel (LC labs, United States). Moreover, we used chloroform (Ekos-1, Russia) as a solvent and Tween-80 (Sigma-Aldrich, Germany) as an additional substance in the work. Gases N_2 and CO_2 of extra-pure grade were used to create an oxygen-free atmosphere inside the instrument for the preparation of microparticles. A 25-mM potassium phosphate buffer solution with sodium azide (Sigma-Aldrich, Germany) was also used.

Methods

Biosynthesis of poly(3-hydroxybutyrate) was performed by an original method developed in our laboratory and described in the earlier work [17]. In brief, the *Azotobacter chroococcum 7B* culture was grown in a Burk's medium with saccharose as a primary carbon source. Cultivation was performed for 72 h. At the end of this period, cells were precipitated by centrifugation in the further extraction of the polymer with chloroform.

Hydrolytic molecular weight reduction. The molecular weight of the synthesized polymer was reduced by acidic hydrolysis of the extracted polymer in a chloroform solution [18]. A PHB solution was titrated with a chloroform solution of hydrochloric acid under continuous stirring. The samples taken in specified time intervals were used for the viscosimetric determination of the molecular weight of the contained polymer with the Mark–Kuhn–Houwink equation. Hydrolysis was stopped by neutralizing the solution after a molecular weight of 6000 Da was attained.

Preparation of microparticles. Microparticles were prepared by ultrasonic piezoelectric membrane spray drying in an oxygen-free atmosphere [19]. At the first stage, PHB and paclitaxel were dissolved in chloroform at a ratio of 90% of biopolymer to 10% of medicine. The final concentration of this composition was 50 mg/mL. However, it has been revealed that only water-insuspendable aggregated particles were obtained without the addition of any surfactants. Just for this reason, it was decided to add Tween-80 at a ratio of 1 : 1000 with respect to the composition. The resulting solution was filtered out through a glass filter (grade 3) and loaded into a B-90 Buchi nano spray dryer (Switzerland). The selected volume of a single loaded portion was 50 mL. Gases N_2 and CO_2 of extra-pure grade were used to create an oxygen-free medium. The parameters selected for the instrument were as follows (Table 1).

It is noteworthy that the drying air temperature was selected equal to the solvent (chloroform) boiling temperature. After spray drying, the particles were gathered with a Buchi special electrostatic particle collector and allowed to stand for a night under vacuum for the removal of residual chloroform.

For control, paclitaxel-free particles were synthesized.

It is important to note that this method enables the synthesis of minimum-size microparticles by spray drying.

Preparation of nanoparticles. Paclitaxel-loaded nanoparticles were prepared by direct membrane emulsification. A paclitaxel–PHB solution in chloroform was passed with an Avanti miniextruder (Polar Lipids Inc.) through Omnipore membrane filters (Millipore, Ireland) with a pore diameter of 100 nm into an aqueous medium (1.5% polyvinyl alcohol solution). The resulting emulsion was added to a 1.5% polyvinyl alcohol solution (40 mL) with stirring at a speed of 26000 rpm on a SilentCrusher M homogenizer (Heidolph, Germany). The mixture was centrifuged for 15 min at 14000 rpm on MiniSpin microcentrifuge (Eppendorf, Germany) and washed with distilled water from residual polyvinyl alcohol. The purified solution of nanoparticles was lyophilized with an ALPHA 1-2LD plus laboratory freeze dryer (Martin Christ, Germany).

This method enables the synthesis of minimum-sized particles from PHB by emulsification.

Characterization of micro- and nanoparticles. The size and morphology of particles were studied on a JSM-6380LA scanning electron microscope (SEM) (JEOL, Japan) and a Quanta 200 3D scanning ion-electron microscope (FEI Company, United States). The diameters of particles were determined using the ImageJ 1.46 software. The hydrodynamic diameter and the ζ potential were determined by dynamic light scattering (DLS) in an aqueous solution on a Zetasizer Nano ZS particle analyzer (Malvern Instruments, United Kingdom). The loaded portion of paclitaxel-loaded particles was determined as follows: a precisely weighed portion of several milligrams of particles was dissolved in chloroform, and then the paclitaxel content in a sample was spectrophotometrically measured. Absorption spectra were recorded on a UV-1601PC ultraviolet spectrophotometer (Shimadzu, Japan) (paclitaxel absorption peaks at 242 and 278 nm) and then the data were compared with the control PHB solutions in chloroform and the calibration curve of paclitaxel solutions in chloroform at different concentrations [20].

In vitro drug release. The experimental study of the sustained release of paclitaxel from micro- and nanoparticles in vitro was performed at 37°C in a 25 mM potassium phosphate buffer solution (pH 7.4) with a small emulsifier addition (0.1%, Tween 80). Six samples of microparticles and six samples of nanoparticles with 20 mg of particles in 4 mL of a buffer solution each were placed into a TS-1/20 thermostat (Russia) at 37°C on a OC-10 Biosan orbital shaker (Latvia). In other words, the release of paclitaxel from solid polymeric micro/nanoparticles into the liquid phase of an aqueous dispersion of micro/nanoparticles was studied. At certain moments, buffer solution samples with released paclitaxel were separated from particles by centrifuging the mixture at 14000 rpm (5702 R centrifuges, Eppendorf, Germany) and spectrophotometrically analyzed for the medicinal substance. The data were used to plot kinetic release profiles. The removed medium was replaced by an equal volume of fresh medium after each sampling. The UV analysis of samples was performed as described earlier on a UV-1601PC ultraviolet spectrophotometer (Shimadzu, Japan). The residual paclitaxel content in micro- and nanoparticles was also determined spectrophotometrically by dissolving them in chloroform.

Differential scanning calorimetry measurement of the crystallinity degree of micro- and nanoparticles. The thermophysical properties of microparticles synthesized by electrodynamic spray drying and nanoparticles obtained by direct membrane emulsification were measured by differential scanning calorimetry (DSC) on a DSC 204 F1 Phoenix calorimeter (Netzsch, Germany). A precisely weighed portion of particles with a mass of 1–4 mg was placed in a 25-μL crucible. The samples were heated from 25 to 200°C at a heating rate of 10 K/min in a nitrogen atmosphere. To provide the precise calibration of temperature and enthalpy within a temperature range from –100 to 600°C, a Netzsch calibration kit (high-purity samples of KNO_3, In, Bi, Sn, Zn, CsCl, Hg, and C_6H_{12}) was used according to the producer's instructions. The initial and peak temperatures of change in heat capacity were denoted as melting points T_m^{init} and T_m^{peak}. The crystallinity of the structures (X_s) can be calculated as follows [21]:

$$X_s = (\Delta H_m + \Delta H_r)/\Delta H_{0m}(\text{POB}) \times 100\%,$$

where ΔH_r and ΔH_m are the enthalpy changes due to the recrystallization and melting of a studied sample, respectively, and ΔH_{0m} (POB) is the theoretical thermodynamic melting enthalpy, which would be obtained for 10% crystalline poly-3-oxybutirate (POB) samples (146.6 J/g) [22]. All calculations were performed for the first heating cycle [21, 23].

RESULTS AND DISCUSSION

Biosynthesis of the Low-Molecular Polymer

Poly-(3-hydroxybutyrate) being a polyester of oxybutyric acid is a biocompatible polymer, which can degradate in an organism to nontoxic products under the action of hydrolytic enzymes. It is very applicable for the creation of a broad spectrum of biomedical devices. Moreover, in contrast to analogues such as polylactides and their copolymers, PHB is a completely biosynthetic product, and this causes its higher purity and, consequently, lower injury for an organism [24]. To perform the biosynthesis of this polymer, the *Azotobacter chroococcum 7B* strain able to accumulate PHB up to 85% of the dry biomass weight was bred in our laboratory. The polymer obtained by extracting

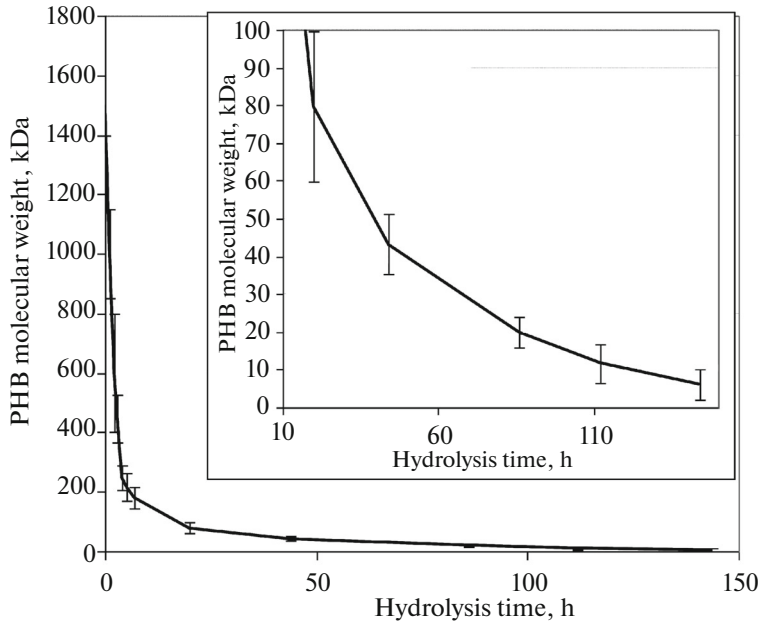
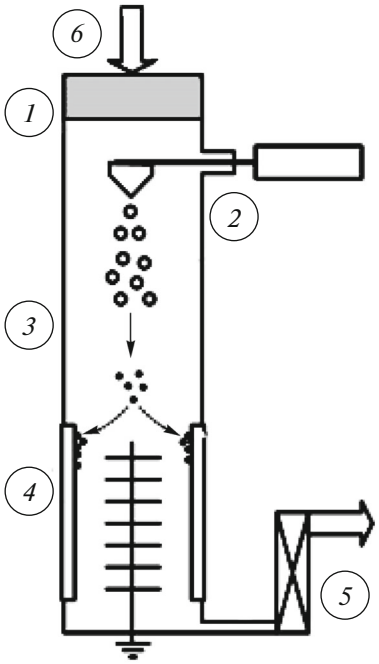


Fig. 1. Hydrolytic decomposition of high-molecular PHB. The initial time period is magnified in the embedding.

the biomass with chloroform had a high molecular weight of 1500 kDa.

However, such a high molecular weight is not suitable to provide the technological process of the creation of microparticles. The thing is that the structure creation process, which will be described below, implies work with nonviscous solutions of substances, while PHB solutions of more than 150 kDa have an increased viscosity. To obtain a low-molecular polymer, the stock PHB solution was subjected to acidic hydrolysis. Hydrochloric acid was selected as a hydrolytic agent. The results of the viscosimetric measurement of the molecular weight are shown in Fig. 1. The high polymeric chain hydrolysis rate is due to the fact that the ester bonds of PHB in the state of a solution are open, while the polymeric chain of PHB in a solid condensed state is packed in the form of lamellar structures [25]. Hence, the polymer with specified characteristics required for the creation of microstructures has been synthesized.



Scheme 1. Operational principle of a B-90 Buchi nano spray dryer: (*1*) heating, (*2*) formation of drops, (*3*) drying chamber (aerodynamic tube), (*4*) collection of particles, (*5*) outlet filter, (*6*) gas drying [26].

Characterization of Micro- and Nanoparticles

In this work, paclitaxel-loaded particles synthesized by spray drying [15] were studied. They were created by a B-90 Buchi nano spray dryer (Switzerland). In brief, this device has a spraying head placed inside an aerodynamic tube. The head has an injector with a membrane, which has holes of certain diameter (7 μm in this case). A substance solution is fed to the resonating membrane with a peristaltic pump. The vibration process required for spray solution drops from the head is provided by inverse piezoeffect. Drops of a specified diameter equal to the hole size further pass through the aerodynamic tube, where they are dried. They are already solid particles when approaching the end of the tube equipped with a collector for synthesized microparticles, where they are precipitated onto a metallic cylinder due to static electricity supplied to

Table 2. Size and physicochemical properties of synthesized micro- and nanoparticles

Parameter	Nanoparticles	Microparticles
Preparation method	Two-stage emulsification	Electrodynamic drying
Diameter from SEM data	216 ± 52 nm	2.71 ± 1.01 μm
Hydrodynamic diameter	351 ± 74 nm	7.86 ± 0.45 μm
ζ potential, mV	–9.43	–42
Medicinal substance inclusion, %	10.5 ± 1.2	12.6 ± 0.7
Melting temperature T_m^{init}/T_m^{peak}, °C	132.0/157.3	133.9/155.2
Crystallinity	65.0	65.0

it (Scheme 1). To adapt this method and instrument for operation with a chloroform solution of PHB and paclitaxel, a system of inert gases N_2 and CO_2 was used.

Hence, paclitaxel-loaded microparticles of PHB (molecular weight, 6000 Da) with a diameter of 2.71 ± 1.01 μm (Table 2) were synthesized. Control particles free from the medicinal substance were also prepared. Their diameter was also approximately equal to 3 μm. Microparticle photos obtained on a scanning electron microscope are shown in Fig. 2. Microstructures have a regular spherical shape with a relatively smooth surface. The particle size distribution obtained with ImageJ 1.46 software is shown in the histogram (Fig. 3). Here, it can be seen that the size of particles has a narrow distribution, which indicates their uniformity. Paclitaxel-loaded microparticles have demonstrated good dispersion ability. The hydrodynamic diameter and the surface charge (ζ potential) were 7.86 μm and –42 mV for paclitaxel-loaded microparticles and 7.48 μm and –41 mV for paclitaxel-free particles (Table 2).

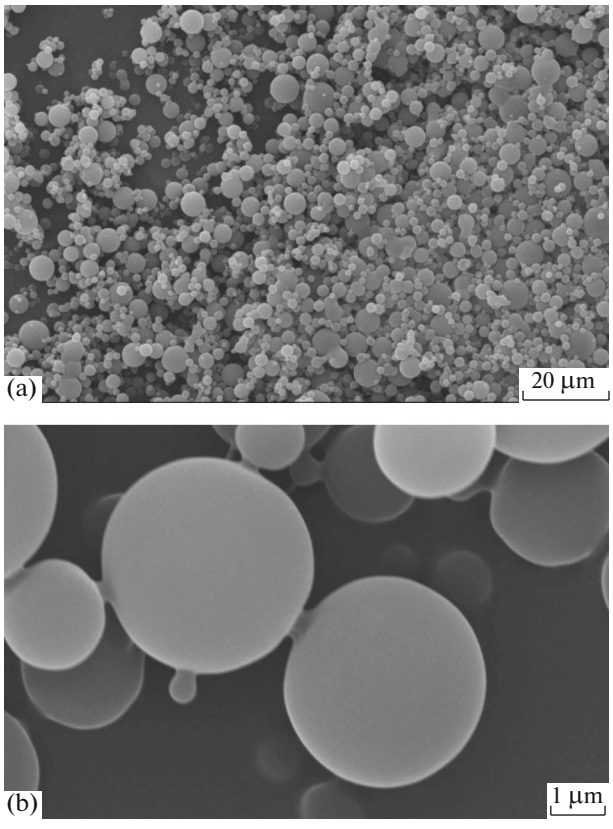


Fig. 2. SEM photos of microparticles prepared from PHB (6000 Da) with encapsulated paclitaxel.

It has been demonstrated that the particle size and other encapsulation characteristics depend on the nature of the polymer and some other microparticle formation factors and conditions [27]. For example, it has been revealed that the most important factor responsible for the size of microparticles is the overall concentration of sprayed substances in a solution. Moreover, instrument parameters such as the inlet temperature, the head temperature, the pressure, and spraying rate, etc., may have an effect on the size and shape of particles.

One of the most important conditions influencing the size of polymeric microparticles is also the polymer concentration in an organic solvent. The thing is that a drop with a fixed size (this size is greater than the diameter of a membrane hole) dries to a final size, which is governed by the amount of polymer contained in it. The lower the substance content in a drop is, the smaller the size of the particles is. However, when the polymer concentration increases, the viscosity and density of a polymer solution also grow, and there comes a moment when the creation of particles becomes impossible due to the fact that the solution has become too viscous and dense. Empirically, the maximum concentration of polymer of this molecular weight in a solution, at which electrodynamic drying is possible, is 50 mg of substance per 1 mL of chloroform. A drop dried in the aerodynamic tube contains the maximum substance amount just at this concentration and, consequently, the process efficiency grows, but microparticles retain a rather small size sufficient for their endocytic penetration into a cell in this case [27].

The inclusion of paclitaxel into microparticles was detected by means of UV spectroscopy. The substance

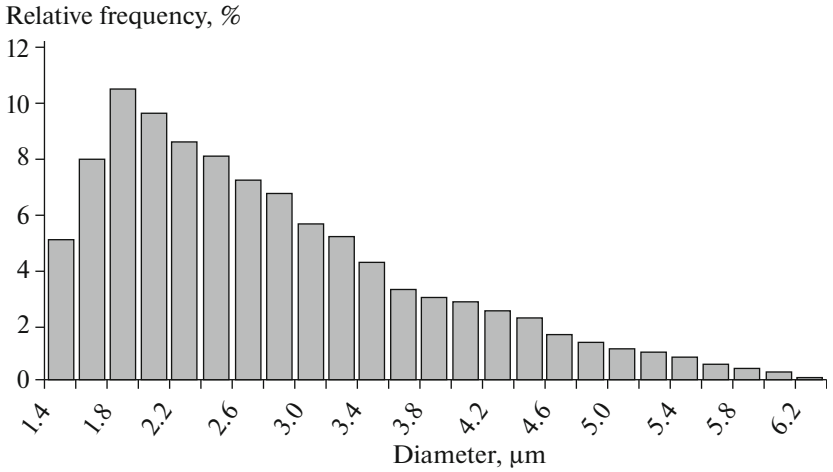


Fig. 3. Particle size distribution.

content was equal to its theoretical value of 12.6 ± 0.7 wt %.

The other varied instrument parameters did not make any appreciable contribution to the morphology of microparticles and the inclusion of paclitaxel into the polymeric matrix. However, their effect extended over the technological process itself. The data on their variation are given in application in Table 1. From this table it can be seen that the optimal parameters for the creation of PHB paclitaxel-loaded microparticles are the parameters used by us and listed in Table 1.

According to DSC data, the crystallinity of polymeric microparticles was 65.0% at a melting temperature T_m^{peak} from 133.9 to 155.2°C.

The polymeric nanoparticles created by direct membrane emulsification [28] were selected as a reference sample for the microparticles synthesized by the above-described high-tech semi-industrial method. This method is widely recognized and was earlier applied in our laboratory for the synthesis of submicro- and nanoparticles. In this case, the inclusion of paclitaxel was 10.5 ± 1.2%. The morphology of nanoparticles was determined by scanning electron microscopy (Fig. 4). Round nanoparticles had a diameter from 50 to 700 nm with an average diameter of 216 nm. The hydrodynamic diameter of particles was 351 ± 74 nm, and the ζ potential was –9.43 mV. The crystallinity of polymeric nanoparticles was 65.0% at a melting temperature T_m^{peak} from 132.0 to 157.3°C.

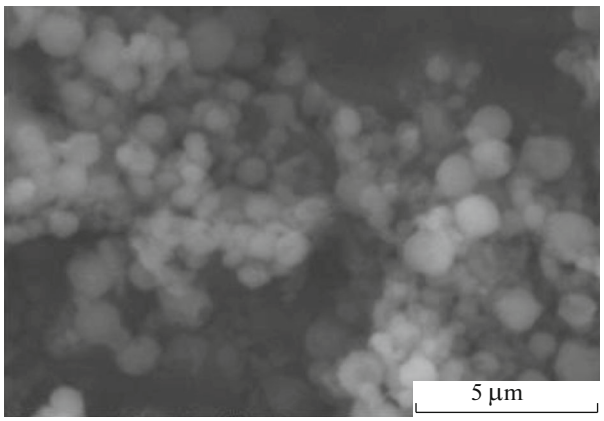


Fig. 4. SEM photo of nanoparticles prepared from PHB (6000 Da) with encapsulated paclitaxel.

Hence, it has been shown that paclitaxel-loaded microparticles, though being greater than nanoparticles in size, do not strongly differ from them in morphology and surface charge. The comparative characteristics of particles are given in summary Table 2. This allows us to hypothesize that the method of piezoelectric spraying in an inert atmosphere is a good approach to the scaling of the laboratory method for the preparation of a new paclitaxel medicinal form. However, in the future it will undoubtedly be required to compare micro- and nanoparticles by the method of delivering the medicinal substance into cells in vitro and in vivo, because despite the fact that the average diameter of microparticles is below the threshold value for cellular endocytosis [29], they have a dense hydrate shell, as is shown by the difference of more than two times between the diameter and hydrodynamic diameter of microparticles. Paclitaxel-loaded microparticles did not differ from the corresponding nanoparticles by their thermophysical parameters.

Studying the Sustained Release of Paclitaxel In Vitro

At the following stage of our work, the kinetic of medicinal substance release from the earlier created paclitaxel-loaded micro- and nanoparticles was studied in vitro. The particles were incubated in a phosphate buffer solution with the addition of an isotonic sodium chloride solution and Tween 80 under stirring

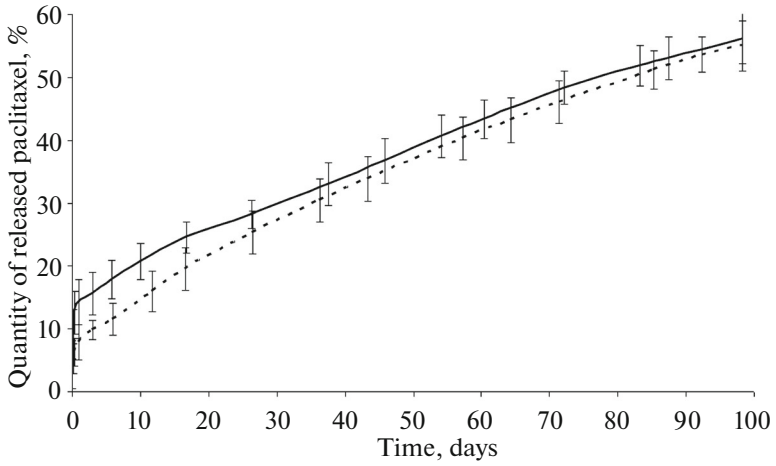


Fig. 5. Kinetic profile of paclitaxel release in vitro from microparticles prepared by electrodynamic drying (solid line) and nanoparticles prepared by one-stage emulsification (dashed line).

in a shaker. Due to the long time of experiment, sodium azide was added to the medium as an antibacterial agent.

The data (Fig. 5) demonstrate a rather low level of the initial "burst" effect (the fast release of the medicinal substance in the first hours), and the linear substance release kinetic component is dominant. This is a positive characteristic for the sustained release of paclitaxel, as this means that microparticles can provide long-term anticancer therapy at a specified rate. It should also be noted that the kinetic profile of paclitaxel release from polymeric nanoparticles (Fig. 5) is the same as for the kinetic of medicinal preparation release from microparticles created by piezoelectric spray drying, being in good agreement with the data, which were obtained earlier in our laboratory and show that the crucial factor influencing the kinetics of paclitaxel release from particles is their composition, and their size has only a slight effect on the kinetic profiles [30, 31].

CONCLUSIONS

Hence, paclitaxel-loaded microparticles have been prepared by the high-tech spray drying method. Microparticles have a regular spherical shape and are well-dispersible in an aqueous medium. The release of substance from them is characterized by a small initial burst release with further sustained release of paclitaxel, and this fact may be used for the long-term maintenance of a certain active substance concentration in dynamic aqueous media of living tissues and cultural media. These data allow us to speak about the development of a new paclitaxel anticancer medicinal preparation form ready for further tests in vitro and in vivo.

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