A.Shershneva, A.Murueva, E. Nikolaeva, E.Shishatskaya & T.Volova.

Novel spray-dried PHA microparticles for antitumor drug release

Abstract

The production of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate/polyethylene glycol – based microparticles, loaded with antitumor drugs Paclitaxel (PTX) and 5-Fluorouracil (5-FU) by spray-drying technique was investigated. The average diameter of microparticles was found to be 3.4 ± 0.5 μm and zeta potential was about -44 mV. The addition of surfactant (PEG) did not show any effect on the morphological characteristics of the particles. But the chemical structure of drug influenced on the properties. Microparticles had heterogeneous pores on the surface when the hydrophobic PTX was encapsulated. For 5-FU loading microparticles, were established that the addition of surfactant positively influenced on the properties of particles and led to the loading of drug directly into the matrix. This is confirmed by the results of electron microscopy and dynamics of drug release *in vitro*. As a whole, the release profiles of PTX and 5-FU from composite P3HB/PEG-microparticles were less than from P3HB-microparticles. The results of the morphological evaluation of Hela cells demonstrated that the use of cytostatic drugs loaded in P3HB microparticles induces morphological changes associated with apoptosis (chromatin condensation, core fragmentation, margination of nucleus). Thus, the obtained results can serve as the basis for the development of new antitumor drugs of prolonged action, intended for various modes of administration.

Keywords: polyhydroxyalkanoates, spray-drying, microparticles, paclitaxel, 5-fluorouracil, efficiency

Introduction

Biopharmacology perceives the development of the antitumor drug as one of the top priority research. The drugs such as taxane derivatives and pyrimidine antagonists are well documented against different types of cancer such as lung cancer, ovarian cancer, prostate cancer, etc. The ongoing efforts to evaluate antitumor drugs are often bound with myriad limitations such as low selectivity and high systemic toxicity which leads to the adverse effects on the individual health. The situation becomes deleterious with immuno-compromised patients. In most cases, the efficacy and dosage of anticancer drugs do not exhibit the complete activity. In the current scenario, minimizing the toxicity of chemotherapy and enhancing the drug solubility with a specific mode of action on tumor cells has become a subject of interest for various scientific studies. Based on these facts, different strategies are implemented to enhance the therapeutic index of the drug for instance, encapsulation of antitumor drugs. One such method includes the development of new dosage of chemotherapeutic agents in the form of nano/microparticles from biodegradable and biocompatible polymers.[1-3] There are different polymers which are widely in practice but they usher their own toxicity and few are reported to cause severe allergic reactions. Hence, care must be taken during the selection of polymers to develop nano/microparticles.

The polyesters of monocarboxylic acids such as polylactide and polyglycolides are popular choices to produce nanoparticles/microparticle. But in recent years, use of polyhydroxylalkanoate (PHA) polymer has grabbed scientific attention owing to its unique properties such as biodegradability, biocompatibility and easy production via microbial fermentation. In comparison with popular polylactides, PHAs are thermoplastic, have less effect on pH of tissues and have a longer *in vivo* degradation period, which makes them one of ideal polymer, for targeted drug delivery systems.

The most common technique for obtaining microparticles from PHA is emulsion method, which includes evaporation/solvent extraction.[4-7] These methods are based on packing polymeric chains in an aqueous medium to obtain spherical particles onto which the drug can be incorporated within the internal cavity of these particles or adsorbed on their surface.

Among the different techniques used, spray-drying method is one of the simplest, inexpensive and efficient routes to obtain particles.[8 – 10] Spray-drying is a fast, continuous and cost-effective process for the production of dry powders by spraying solutions in a hot gas environment. This method is widely used in different sectors like pharmaceutical, chemical, cosmetic and food industries.[11-14]

One of the main advantages of obtaining particles from hydrophobic polymers with spray-drying is the use of solvents with a low boiling point. For example, the use of dichloromethane (40 °C) or acetone (56 °C) prevents sticking and agglomeration of particles. The reason is that these solvents evaporate at a temperature lower than the melting point and the glass transition temperature of the most of the other hydrophobic polymers.[13, 15-16]

Well-known, spray-drying was considered as dewatering process, however, it is also possible to encapsulate hydrophilic and hydrophobic active compounds in various formulations.[17-19] One of the interesting facts of spray-drying is that the droplets are exposed to temperatures for milliseconds or seconds[15, 20], which protects active compounds from thermal degradation. [21] In addition, the solid particles obtained by this method have higher chemical and physical stability. Moreover this method allows obtaining particles with the higher values of encapsulation efficiency, in contrast to the emulsion method, in which the drug separates between phases in the emulsion and, as a consequence, leads to decrease encapsulation efficiency.[22-23]

The perusal of scientific studies report that the use of biopolymeric microparticles containing antitumor drugs 5-fluorouracil and paclitaxel, non-steroidal anti-inflammatory drug celecoxib, as well as antibiotics, antiviral drugs can be achieved by the spray-drying method[16, 24-29]. Recently, dosage forms of poorly soluble, toxic, short half-life drugs were obtained using the spray-drying method for treatment of lung cancer[30-31], bronchial asthma[32], hypertension[18], etc.

The preparation of microparticles from poly-3-hydroxybutyrate (P3HB) by spray-drying process was described by our scientific group in earlier studies. In these studies the different parameters and characteristics in the development of microparticles are demonstrated, e.g. production yield, average diameter, zeta potential and production processes, like inlet temperature, polymer solution feeding rate and polymer solution concentration are established.[33-34] However, in the modern literature single example of using spray drying for the preparation of PHA microparticles is presented.[35] Thus, paracetamol loaded microspheres from aqueous dispersions of extracted poly-3-hydroxybutyrate / poly-3-hydroxy-4-pentenoate granules were synthesized and briefly characterized.[35] Based on these facts and consideration, the present study was designed and executed for developing microparticles from organic solutions of P3HB, as well as in a blend with polyethylene glycol containing antitumor drugs paclitaxel and 5-fluorourocyl, by spray-drying technique. The activity of microparticles, controlled drug release and the cytotoxic effect were studied in culture of human cervical carcinoma cells in (HeLa). The results were validated and compared with commercial antitumor drugs.

Materials and methods

Materials

Poly-(3-hydroxybutyrate) (P3HB) with different molecular weight was produced at Institute of Biophysics of the SB RAS by the microbial fermentation process. Registered mark of material – "Bioplastotan™". Polyethelenglicol (PEG, 4600 Da), 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (USA), paclitaxil (PTX) from Veropharm LENS (Russia).

Methods

Preparation of microparticles by spray-drying

Microparticles were prepared by spray-drying process of PHA solutions using Büchi B-290 Spray dryer (BÜCHI Laboratory Equipment, Switzerland, Flawil). P3HB or blend of P3HB and PEG (75:25) were dissolved in dichloromethane (1wt %) for obtaining microparticles. The parameters selected for preparation of the microparticles were inlet temperature 75 °C, aspirator speed 100%, and feed pump speed 3.2 mL·min^{-1} with a nozzle orifice (diameter of 0.7 mm).

Preparation of drug loaded microparticles by spray-drying

Microparticles with 5-FU were obtained from 1wt % P3HB dissolved in dichloromethane and 5-FU dissolved in methanol (based on 1% of the weight of the polymer carrier). Microparticles with PTX were prepared by mixing commercial PTX (1 wt % of the weight of the polymer carrier) with 1 wt % solution of the polymer in dichloromethane by spray-drying process. Assay conditions were similar to those described earlier: inlet temperature 75 °C, feed pump speed 3.2 mL·min^{-1}. The obtained microparticles were stored under vacuum.

Characterization of microparticles

Production yield

The production yield (P) was defined as the percentage of the weight of spray-dried microparticles (W_m) compared to the weight of polymer (W_p) in the initial feed solution as shown in Eq. (1):

$$P\ (\%) = \frac{W_m}{W_p} \times 100 \qquad (1)$$

Scanning electron microscopy

The morphological characteristics of the microparticles were studied using scanning electron microscopy S-5500 and TM-3000 electron microscopes (Hitachi, Tokyo, Japan). The samples were sputter-coated with platinum under conditions 3 cycles of 20 seconds at 10 mA by a sputter coater K550X (Emitech, Quorum Technologies Ltd., East Sussex, UK).

Particle size and zeta potential measurements

The size distribution and polydispersity index (PdI) of microparticles were determined on a particle size analyzer Zetasizer Nano ZS (Malvern, Worcestershire, UK) using the dynamic light scattering method. Each sample was measured in triplicates. The surface charge of microparticles was characterized by value of the electrokinetic potential (zeta potential), which was determined by the electrophoretic mobility of the particles in the suspension on particle size analyzer.

Antitumor drug loading and encapsulation efficiency

The amount of drug inclusion in the polymeric microparticles was determined by spectrophotometric analysis. The 5-FU- loaded and PTX-loaded microparticles were dissolved in a mixed solution of dichloromethane and methanol (v/v 3:1) and dichloromethane, respectively. The quantity of PTX and 5-FU loaded in the microparticles was determined on a UV-Vis spectrophotometer Cary 60 (Agilent Technologies, Selangor, Malaysia) by measuring the UV-Vis absorbance at 227 nm (PTX) and 265 nm (5-FU) using pre-built calibration graphs. The experiment was carried out in triplicates.

The encapsulation efficiency (E) was defined as the percentage of the weight of drug in microparticles (W_m) compared to the initial weight of drug (W_i) as shown in Eq. (2):

$$E\ (\%) = \frac{W_m}{W_i} \times 100 \qquad (2)$$

In vitro drug release studies

The controlled drug release studies of 5-FU- loaded and PTX-loaded microparticles werecarried out *in vitro*. The microparticles were initially sterilized by UV radiation for 20 minutes and placed in a sterile 12-well culture plate, containing 2 ml of phosphate-buffered saline (PBS, pH 7.4). The plate was exposed to shaker-thermostat ST-3M (ELMI, Riga, Latvia) at 37 °C (n=3). Microparticles were settled by centrifugation (4000 rpm, 20 min), and in the samples the amount of the drug, released at different intervals, was determined. 1 ml samples were withdrawn from the solution to observe the change in 5-FU and PTX concentration by UV-Vis spectroscopy (Agilent Technologies, Selangor, Malaysia). The amount of volume used from culture plate was replaced with phosphate buffer. The amount of 5-FU and PTX in the supernatant was determined at 227 nm and 265 nm, respectively. Triplicate measurements were performed during every analysis.

Cell culture

The efficacy of the drug loaded microparticles was investigated with HeLa tumor cells, at the concentration of 5×10^3 cells / ml placed in 24-well plate. The suspension of drug loaded microparticles was introduced into the cell culture; the concentrations of the encapsulated drugs were 0.6 µg / ml. The cell culturing was carried out according to the protocol described by Freshney.[36] In brief, the DMEM suspension contained 10% solution of embryonic bovine serum and solution of antibiotics (streptomycin 100µg /ml, penicillin 100 U/ml) was incubated in a CO_2 incubator (New Brunswick Scientific, St. Albans, UK) with 5 % atmospheric CO_2 at 37 °C. The medium was replaced every three days.

In vitro cytotoxicity assay

The level of cellular metabolism was studied with MTT assay with respect to the positive control. The commercial drugs were introduced into cell cultures at similar concentration.

The viability of HeLa cells was evaluated in a reaction with MTT based on the ability of cell dehydrogenase to restore MTT to formazan, which characterizes the activity of mitochondria and the number of living cells and indirectly reflects the ability of cells to proliferate on matrices. To HeLa cells, 50 µl of 5% MTT solution and 950 µl of the total nutrient medium were added to the well, containing each polymer sample. The medium with the MTT solution was replaced with DMSO to dissolve the resulting MTT formazan crystals. After 30 minutes, the supernatant was transferred to a 96-well plate (Corning, USA), and the optical density was measured at a wavelength of 540 nm on a Bio-Rad 680 microplate reader (Bio-Rad Laboratories Inc., Osaka, Japan). The number of cells was estimated from the calibration curve.

Fluorescent staining of Hela cells

The cell viability in HeLa culture was investigated by the differential staining techniques using DNA-intercalate fluorescent dyes such as acridine orange (AO) and ethidium bromide (EB). Cell staining was performed according to procedure, the previously described by Liu et al.[37] The cells were initially fixed with a 1% formalin solution at room temperature. The culture was washed with DPBS solution, 70% alcohol. The degradation of RNA was achieved using 0.2 ml of RNAse which was added to the cells culture and incubated at 37° C for 30 minutes. Further, solution mixture containing AO (100 µg / ml) and EB (100 µg / ml) was added in a ratio of 1:1. The cell morphological analysis and counting were performed on the first and third day using a fluorescence microscope Leica DM6000B (Leica Camera AG, Wetzlar, Germany) at a lens of 20× in 10 fields of vision. The staining with DAPI and FITC (Sigma-Aldrich, USA) was also performed to study cell morphology and the results of MTT. Visualization and counting of cells were carried out in a similar way.

Statistical analysis

The results obtained are expressed as a mean ± standard deviation. The statistical processing of the results was carried out using the standard Microsoft Excel, STATISTICA 8. Arithmetic means, mean square error, and arithmetic means error were calculated. Significant differences in average values were assessed using Student's t-test. P values < 0.05 are considered as statistically significant.

Results and discussion

Properties of spray-dried P3HB microparticles

Effect of molecular weight on the characteristics of microparticles

The present study presents the results of the preparation of P3HB microparticles by spray drying. During the process of development, the micronization of solutions containing high molecular weight poly (3-hydroxybutyrate) (over 1000 kDa) resulted in the formation of "polymer threads" (Figure 1a). The part of high-molecular polymer chains are not completely dried due to high pressure and fast gas flow in the system. Therefore, these were resulted in a thread-like structures. In addition, production yield product was not more than 10% (Table 1). The formation of such structures may be due to the high viscosity of P3HB solution. According to literature

sources, the viscosity of the P3HB solution varies from 4 to 60 Pa·s[38], which makes it difficult to obtain microparticles with a high production yield. However, the low molecular weight polymer (100 kDa) was ideal to obtain a good quality of microparticles, and the production yield was also increased up to 85% (Table 1). This is due to the decrease in the viscosity of the spray solution.

Microparticles, obtained from low molecular weight P3HB, had the spherical shape as shown in Figure 1b. It is noable, that there was a formation of agglomerates between microparticles with the smaller diameter. All in all a broader particle size distribution was shown (Figure 1b), that was investigated by dynamic light scattering method (Table 1). The average diameter of microparticles was to be 3.4 ± 0.5 μm.

In addition, it was also found that the P3HB microparticles obtained by the spray-drying method displayed a significantly higher zeta potential (-45 mV) compared to the microparticles obtained by the popular emulsion method (about -15 mV).[39] This result indicated that the microparticles obtained via low molecular weight P3HB, by spray-drying technique, have negative zeta potentials with enhanced physical stability. The result justifies the previous findings, which suggest that absolute value of zeta potential over | 30 | mV is optimal and satisfactory. Absolute value of zeta potential of more than | 60 | mV is said to have high physical stability.[40]

Influence of PEG surfactant on the characteristics of microparticles

The addition of PEG to the P3HB solution (25:75) did not show any effect on the morphological characteristics of the particles. Further, the electrophoretic activity of the microparticles was found to have zeta potential -43 mV, and the average diameter did not exceed 3.6 μm. However, the addition of PEG led to decrease agglomeration of the particles, and in consequence to decrease PdI (Table 1).

It is reported that the addition of surfactants to polymers like polyhydroxybutyrate, affect physicochemical properties of the particles.[41] Studies also highlight the role of surfactants in increasing the elasticity, reducing the degree of crystallinity of P3HB, facilitates the penetration of water into the polymer matrix.[42] PEG is the most commonly used non-ionic surfactant, which is said to be a hydrophilic non-toxic segment in combination with hydrophobic biodegradable aliphatic polyethers.[43-44] PEG escapes the initial defense process from the immune system due to its biocompatible properties, which result in higher circulation period in the blood stream by creating a spatial barrier that prevents the opsonization process.[45]

When studying the morphology of the obtained samples, it was established, that microparticles of P3HB, as well as the particles with PEG, had a smooth surface (Figure 2). The only characteristic feature of composite microparticles was the presence of small pores on the surface, indicated by the arrows in Figure 2b. The results obtained from the smooth surface of the composite microparticles with PEG are consistent with a study conducted by Monnier[46], who showed that the addition of low molecular weight PEG led to the formation of a smoother surface of the poly(hydroxybutyrate-co-valerate) microparticles obtained by emulsion method.

Characterization of P3HB microparticles and P3HB/PEG composite microparticles loaded with antitumor drugs

The composite characterization of P3HB microparticles and P3HB/PEG microparticles loaded with antitumor drugs was studied. Drug loading did not affect the dimensional characteristics of the zeta potential of microparticles (Table 1). Probably, such an effect was associated with a low concentration of drugs - 0.6 mg/ml. In the present study, PTX and 5-FU were used as model hydrophobic and hydrophilic drugs, respectively. The obtained results are presented in Figure 3. The SEM analysis revealed that the accumulation of 5-FU was mainly localized on the surface of P3HB-microparticles, whereas the addition of PEG to P3HB led to the loading of the drug directly into the matrix of carriers. This was confirmed by the results of drug encapsulation efficiency (Table 1). Thus, the addition of a surfactant positively influenced the properties of hydrophilic drug-loaded P3HB microparticles, which correlates with the results presented in Paudel.[30]

It is known that partial amorphization of substances occurs in spray-drying process[30], and the addition of surfactant reduces amorphization. This surfactant leads to the formation of a smooth and spherical surface of carriers due to dense packing of molecules of substances and decrease of their mobility. Such effect provides high encapsulation efficiency and sustained release of drugs from carriers.[47-48] For example, Hamzehloo et al. 2017 showed that the addition of PEG to Eudragit E100 provided a sustained release of the drug by swelling the carriers and diffusing the drug.[19]

Interestingly, with respect to PTX, a marked change in the surface structure was observed, most likely due to the presence of polyoxyethylated castor oil (Cremophor® EL *) in the commercial preparation. Due to the fact that the oil component of PTX has a boiling point much higher than the originally set inlet temperature 75 °C it could not dried during the spraying process, which led to the formation of an uneven and porous surface of microparticles (Figure 3a, b). These results highlight that addition of surfactant does not change the porosity of the surface of the microparticles, while the presence of the oil component in the solution during sputtering produces large and heterogeneous pore sizes on the surface.

Study of the drugs release from P3HB and composite microparticles in vitro

The controlled release of the antitumor drugs such as PTX and 5-FU from P3HB and composite P3HB/PEG microparticles in the PBS are shown in Figure 4. The results showed that during the first phase, the release of PTX and 5-FU from polymeric microparticles was characterized by a high yield of the drugs (72-120 h), which was also

justified by the presence of a large amount of drug on the surface of microparticles and weak hydrophobic interaction between the drug and the polymer. The next phase of antitumor drug release (144-168 h) was dominated by the diffusion mechanisms associated with the release of the drug from the internal structures of microparticles, that is why drug release slowed down and the concentration of preparations in the PBS was constant. Earlier scientific literature reports that the drug release from polymer systems with prolonged action can be implemently by diffusion, in which the preparations move to the edge of the polymer product and then pass into the external environment.[49] It is known that P3HB in the absence of biological factors (enzymes, cells) does not hydrolyze into carbon chains[50], so the release of preparations from polymer carriers occurs according to the laws of chemical reactions and does not depend on the state of the carrier material.

In the first 48 hours, the drug release of PTX from the P3HB microparticles was 0.024 mg/ml and 0.012 mg/ml from the P3HB/PEG composite microparticles, indicating the 40% and 20% of the drug loading, respectively. The drug release of 5-FU from P3HB and composite P3HB/PEG microparticles was less than that of PTX and was 16% and 3.5%, respectively. After 140 h the outline of the profile curve on the plateau was determined in both variants of microparticles and the rate of drug release gradually decreased. After the completion of the experimental process the PTX release in the medium was 67% and 50%, and 5-FU release was respectively 49.7% and 22% from microparticles obtained from P3HB and composite P3HB/PEG respectively. On average, the total drug release of antitumor drugs from composite microparticles was 1.5 times lower compared with P3HB microparticles. The drug release from biodegradable microparticles depends on a number of factors and some of the important factors include the size, microparticle surface structure, and the chemical nature of the drug[51]. The decrease in the total release of antitumor drug from composite microparticles is most likely due to the fact that the surface of the microparticles obtained from P3HB containing 5-FU was coated with the drug, while the surface of the composite P3HB/PEG microparticles was heterogeneous with small pores and the drug was not detected (Figure 3c, d). High porosity and heterogeneity of P3HB microparticles and P3HB/PEG composite microparticles loaded with PTX contributed to increase in drug release. The obtained results are in accordance with the study carried out by Monnier[46]. The release rate of heparin from microparticles obtained on the dasis of P3HB/P3HV with the addition of low molecular weight PEG, was in 2-3 times lower if compared with the release from P3HB-microparticles. In general, the addition of PEG to P3HB contributed to a reduction of the so-called "burst release effect" of drugs at the initial stages of kinetic curves (1-2 days). Reduction of this effect is essential in the development of prolonged drug delivery systems, which will be highly important for minimizing the toxicity of highly toxic antitumor drugs. It is noteworthy to mention that the rate of drug release may depend on the degree of binding drug molecules to the polymer, which, in turn, is determined by the molecular weight of the drug, its solubility, the presence of polar or functional groups. In the present study, a slight decrease in the release rate of 5-FU in comparison with PTX might be due to the presence of functional groups in the structure of 5-FU that are capable of forming hydrogen bonds with the carboxyl and hydroxyl groups of the polymer. In general, the addition of a low molecular weight and non-ionic surfactant can regulate the efficiency of drug loading into microparticles, and with respect to the chemical structure of the drug. Hence it is possible to obtain prolonged release systems with the optimal release rate of the drugs to maintain its necessary concentration in the body.

Cell culture

Efficacy of antitumor drugs loaded in P3HB and composite P3HB/PEG microparticles

The functional properties of PTX and 5-FU loaded in microparticles were analyzed in comparison with commercial drugs in the culture of the tumor cell. Previously, it has been shown that microparticles based on PHA such as P3HB, P3HB/3HV, P3HB/3HH, P3HB/4HB, P3HB/3HO are reported to have no toxic effect on the NIH 3T3 fibroblastic cell line, L929 fibroblastic cell line, prenatal rat neuronal hippocampal cells and HeLa tumor cells.[39, 52-54] In the present study, HeLa tumor cells were selected as a subject of investigation. The proliferative potential of Hela was evaluated by the MMT test (Figure 5). After the application of free PTX and PTX-loaded in microparticles, the number of cells in the intact control was significantly higher than in the experimental groups. After 3 days, the number of cells with the addition of P3HB and composite P3HB/PEG microparticles containing PTX, was almost the same, respectively, 7.4×10^3, 4.9×10^3, that on the average 12 times lower, when compared to intact control.

The maximum antiproliferative effect of 5-FU was observed on the first day with P3HB / 5-FU, for which the number of viable cells was 9.6×10^3. By the third day, the antiproliferative effect of this sample was similar to free drug, with a number of cells not exceeding 4×10^3. As mentioned earlier, the addition of surfactant to P3HB resulted in the decreased release of 5-FU from the particles and, as a consequence, less effect was observed during initial days (the number of viable cells was 26.3×10^3).

The delayed effect of free 5-FU on the first day is most likely due to the functional peculiarity of drug, as to prevent DNA synthesis and to form structurally deficient RNA it is necessary to convert 5-FU into active metabolites, including 5-fluoro-2'-deoxyuridine-5-monophosphate and 5-fluorouridine tri-phosphate.

It can be estimated that the efficacy of products during production of micro- and nano-carriers is an important parameter especially in the case of sensitive substances such as drugs, proteins, genes, vitamins, etc. Earlier studies reported that the encapsulation of substances retained their activity after spray-drying.[55-58] For example, Estevinho

et al. loaded vitamins B_{12} and C in chitosan-microparticles by spray-drying and confirmed the biological activity and the integrity of vitamins.[59]

It can be predicted that the maximum activity of 5-FU-loaded in P3HB-microparticle might be due to the emergence of polymorphic changes or amorphization of 5-FU during the drying process. According to the literature, the spray-drying process can lead to increasing the solubility and the rate of absorption of the loaded substances.[60-62] A similar effect was noted in the work carried out by Tran.[61] Deposition of raloxifene in the microparticles from polyvinylpyrrolidone K30 resulted in transition of the drug from crystalline to amorphous state.[61] Similar observation was seen with flurbiprofen-loaded sucrose nanoparticles.[63] Flurbiprofen was characterized by amorphization of the drug during the drying process. Concentration of drug in the blood plasma was 9 times higher than in the group with the administration of the commercial preparation, when the deposited flurbiprofen was administered orally to rats.[63] Nevertheless, a possible transformation of 5-FU was observed in the study, which can serve as the basis for future investigations.

Morphological evaluation of Hela tumor cells upon culturing with antitumor drugs

It is known that antitumor drugs are capable of inducing both apoptotic and necrotic cell death in cell culture. For potential antineoplastic agents, the key point is to initiate cell death by apoptosis without the development of an inflammatory process. In the present study, the morphological evaluation of Hela cell death was carried out using the double staining method (AO/ EB) which was examined under a fluorescent microscope. AO is one of the most important fluorescent dyes that can stain the DNA nucleus in an intact cell membrane, while EB stains the cells that have lost the integrity of the cell membrane.[64] Consequently, living cells have a green-colored core, and early apoptotic cells display the bright green condensed core with chromatin fragmentation. Further, the late apoptotic cells condense with a fragmentary chromatin displaying yellow-orange or red color, while dead cells show bright orange or red homogeneous nucleus.

The morphological studies of Hela cells with the addition of drugs in free form and loaded in microparticles showed visual changes compared to intact cells (control). During the observation, the control cells had an oval shape and were evenly colored green. At the same time, the number of cells in the control group with signs of early apoptosis did not exceed 4% of the total number of cells in the field of vision. This may be due to the high proliferative potential of Hela tumor cells and the limited space for cell growth (Figure 6, 1-3 day).

The Hela cells showed typical signs of apoptosis, like the decrease in volume, fragmentation and condensation of chromatin and formation of apoptotic bodies (Figure 6, shown by arrows).[65-67] Chromotin condensed and fragmented by the apoptotic type was detected after one day both in dead cells and in alive cells, preserving the integrity of the plasma membrane (Figure 6). A significant increase in the proportion of apoptotic cells was noted due to the action of loaded PTX. Thus, the proportion of cells with condensed chromatin when P3HB and P3HB / PEG were applied to microparticles loaded with PTX was 1.5-2 times the higher than proportion of cells with fragmented chromatin, into which 5-FU loaded microparticles were added. With the use of free PTX, an increase in the number of necrotic cells was observed after 24 hours, which was 80-90 %.

The enhanced cytotoxic effect of PTX loaded in microparticles and free PTX obtained in the present investigation is consistent with the data on the kinetics of PTX release from microparticles. The results showed higher PTX yields, which inhibited the growth that, in its turn, induced apoptosis in Hela culture, compared to 5-FU loaded microparticles. On the first day, the yield of 5-FU in the PBS from the microparticles was at the level of 0.0005 mg/ml, and this concentration was not sufficient to suppress cell growth (Figure 4).

After 3 days, the experimental groups showed an increase in the number of necrotic cells whereas, in the control group, single cells with fragmented chromatin were observed (Figure 6).

The FITS/DAPI staining was performed to evaluate the morphological changes of Hela cells when cultured with the free form and loaded in microparticles forms of PTX and 5-FU. Morphological changes associated with apoptosis (chromatin condensation, core fragmentation, margination of nucleus) are indicated by the arrows in Figure 7. In general, the results obtained confirmed the effectiveness of PTX and 5-FU loaded in P3HB and P3HB/PEG microparticles and their possible role in initiating cell death by apoptosis.

Conclusion

The present investigation are promising enough to reveal the development of microparticles from P3HB and P3HB/PEG composite microparticles loaded with PTX and 5-FU using the spray-drying method. The study attributes towards reporting the spray-drying technique as one of the most simple and feasible processes for obtaining microparticles. The technique showed an increase in the yield of the product and displayed high efficiency of incorporating the preparations into microparticles. One of the interesting facets of the rapid drying process is to prevent the destruction of encapsulated drugs and maintain its activity. Further study also highlights the importance of using low-molecular surfactant to P3HB microparticles, which allows to regulate the effectiveness of drug loading and influence the morphology of the microparticle surface. The kinetics and drug release of PTX and 5-FU from P3HB and composite P3HB/PEG microparticles was studied and their efficiency with the mode of action against Hela tumor cells was evaluated. The prolonged release of antitumor preparations from microparticles led to inhibition tumor cell proliferation. Overall the obtained results can envision and serve as the basis for the development of new antitumor drugs of prolonged action, intended for various modes of administration.

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Figure caption

Figure 1. SEM images of microparticles obtained by spray drying from poly-3-hydroxybutyrate with different molecular weights: 1479 kDa (a); 50 kDa (b). The bar is 100 μm and 30 μm

Figure 2. SEM images of microparticles obtained by spray drying from PHA: poly-3-hydroxybutyrate (a); poly-3-hydroxybutyrate/polyethylene glycol (b). The bar is 2 μm

Figure 3. SEM images of microparticles obtained by spray drying from polymer solutions: poly-3-hydroxybutyrate/Paclitaxel (a), poly-3-hydroxybutyrate/polyethylene glycol/ Paclitaxel (b), poly-3-hydroxybutyrate/5-Fluorouracil (c), poly-3-hydroxybutyrate/polyethylene glycol/5-Fluorouracil (d)

Figure 4. Dynamics of Paclitaxel (a) and 5-Fluorouracil (b) release from poly-3-hydroxybutyrate and composite poly-3-hydroxybutyrate/polyethylene glycol microparticles

Figure 5. MTT assay: the effect of Paclitaxel (a) and 5-Fluorouracil (b) encapsulated in polymer microparticles on the number of viable cells in HeLa: concentration of drugs is – 0,6 mg /ml

Figure 6. Apoptotic morphology detection by acridine orange-ethidium bromide fluorescent staining of HeLa treated with Paclitaxel, poly-3-hydroxybutyrate/Paclitaxel, poly-3-hydroxybutyrate/polyethylene glycol/Paclitaxel, 5-Fluorouracil, poly-3-hydroxybutyrate/5-Fluorouracil, poly-3-hydroxybutyrate/polyethylene glycol/5-Fluorouracil

Figure 7. DAPI and FITC staining of HeLa cells treated with Paclitaxel, poly-3-hydroxybutyrate/Paclitaxel, poly-3-hydroxybutyrate/polyethylene glycol/Paclitaxel, 5-Fluorouracil, poly-3-hydroxybutyrate/5-Fluorouracil, poly-3-hydroxybutyrate/polyethylene glycol/5-Fluorouracil