

Original scientific paper

5-fluorouracil-loaded polyhydroxyalkanoate micro- and nano-particles: preparation, characterisation and *in vitro* evaluation

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Abstract

Background and purpose: 5-fluorouracil (5-FU) is a widely used antimetabolite chemotherapeutic agent. However, it has substantial cytotoxicity and may cause serious adverse effects, including gastrointestinal toxicity, myelosuppression, and, less commonly, cardiac and neurologic toxicity. To achieve sustained release and reduce systemic exposure and administration frequency, 5-FU can be microencapsulated in biodegradable, non-toxic polyhydroxyalkanoate matrices. **Experimental approach:** In this research, 5-FU-loaded poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P3HBV) micro- and nanoparticles were prepared via spray drying and single emulsion solvent evaporation methods. The obtained particles were characterized in terms of their average hydrodynamic size, zeta potential, surface morphology, drug loading, encapsulation efficiency, release patterns, hemolytic activity, and cytotoxicity to HeLa cervical cancer cells. **Key results:** The obtained 5-FU-loaded micro- and nanoparticles had a regular spherical shape without surface defects. The nanoparticles were nearly identical in size regardless of the presence of 5-FU. In contrast, the encapsulation of 5-FU into microparticles by spray drying caused measurable increases in particle size, with the average hydrodynamic diameter increasing by 21.0 % for P3HB and by 28.6 % for P3HBV. Although the single emulsion solvent evaporation technique did not achieve therapeutically relevant 5-FU loading, spray-dried microparticles showed satisfactory drug loading and sustained release of 5-FU over 48 h. The microparticles exhibited pronounced cytotoxicity against HeLa cervical cancer cells while demonstrating lower hemolytic activity than free 5-FU. **Conclusion:** In this study, the promising drug-loaded systems based on polyhydroxyalkanoate microparticles were prepared and characterized for sustained 5-FU delivery in the therapy of cervical cancer.

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Keywords

Cervical cancer; polymeric nanoparticles; polymeric microparticles; poly(3-hydroxybutyrate); poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

Introduction

Cervical cancer remains a major global health threat, ranking among the most frequently diagnosed cancers in women and causing a substantial, yet largely preventable, burden of mortality. According to the World Health Organization, approximately 604,000 new cases and 350,000 deaths were recorded worldwide in 2024 [1]. A

commonly used chemotherapeutic agent in the management of cervical cancer is 5-fluorouracil (5-FU), an antimetabolite that primarily inhibits thymidylate synthase. Although 5-FU is employed in chemotherapy, its clinical utility is severely constrained by an extremely short plasma half-life, rapid catabolism by hepatic dihydropyrimidine dehydrogenase, and dose-limiting systemic toxicities including myelosuppression, mucositis, dermatitis and cardiotoxicity [2,3]. Encapsulation of 5-FU into biodegradable polymeric matrices offers a rational strategy to circumvent these limitations: it reduces the rate of metabolic inactivation of the drug, sustains local therapeutic concentrations over hours to days, attenuates peak plasma levels, and enables the formation of an injectable or implantable depot directly at the tumour site, a concept particularly attractive for locally accessible malignancies such as carcinoma of the cervix uteri [4].

A suitable matrix for drug encapsulation may be based on polyhydroxyalkanoates (PHAs), a family of microbially produced biodegradable polyesters that function as intracellular carbon and energy storage materials. Among the diverse range of PHAs, the homopolymer poly(3-hydroxybutyrate) (P3HB) and its copolymer with 3-hydroxyvalerate (P3HBV) attract the greatest interest. P3HB is the most extensively studied member of the PHA family and is characterized by high crystallinity, biodegradability, and biocompatibility; however, in its native form, it is notably brittle. Incorporation of 3-hydroxyvalerate (3HV) units into the P3HB main chain reduces crystallinity and improves processability. Owing to these properties, P3HB and P3HBV are considered promising materials for drug delivery systems. Notable drugs that have been successfully encapsulated into PHA matrices include doxorubicin [5], paclitaxel [6], doxycycline [7] and numerous other therapeutic agents [8-10]. These systems have demonstrated sustained release profiles, with the gradual release of active compounds maintained over periods ranging from several days to several weeks.

To date, most PHA-based drug-encapsulation approaches have targeted hydrophobic or amphiphilic molecules, which readily partition into the polymer matrix during particle preparation. However, encapsulating hydrophilic compounds remains a complex challenge. Thus, this study aimed to obtain and comprehensively characterize P3HB- and P3HBV-based microparticles (MPs) and nanoparticles (NPs) loaded with the hydrophilic drug 5-fluorouracil for potential treatment of cervical cancer.

Experimental

Materials

Microbial P3HB (363 kDa) and P3HBV (10.2 % of 3HV, 475 kDa) were produced at the Laboratory of Biotechnology of New Biomaterials of Siberian Federal University in Krasnoyarsk, RF [11,12]. 5-FU, poly(vinyl alcohol) (PVA) (31-50 kDa, degree of hydrolysis 98-99 %), dimethyl sulfoxide (DMSO) and standard antibiotic-antimycotic supplement for cell culture were purchased from Sigma-Aldrich (USA). Dichloromethane (DCM), chloroform, and CH₃OH were purchased from Merck (Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific (USA). Fetal bovine serum was purchased from HyClone (USA).

All reagents and solvents were of analytical grade and used as received without further purification. The water used was purified by an Arium® Pro Ultrapure water system (Sartorius AG, Germany).

Preparation of nanoparticles by emulsion solvent evaporation

P3HB and P3HBV NPs loaded with 5-FU (P3HB-5-FU-NPs and P3HBV-5-FU-NPs) were prepared by the single-emulsion solvent evaporation technique. In brief, 10 mg of P3HB or P3HBV was dissolved in 4 mL of CHCl₃ at 50 °C and mixed with 500 µL of 5-FU solution in DMSO (2 mg mL⁻¹). The resulting solution was added dropwise to 20 mL of PVA solution in deionized water (1.0 % w/v) and sonicated for 15 min in an ice bath using a Scientz JY92-IIN probe ultrasonic homogenizer (Scientz Biotechnology, China). The sonication

parameters were as follows: 20 % power, 6 mm probe, 20-25 kHz frequency, 2 s sonication cycle, 2 s pause. Then, the resulting emulsion was magnetically stirred at 875 rpm for 8 h until complete CHCl_3 and DMSO evaporation. The obtained NPs were collected by centrifugation, washed 5 times with deionized water, freeze-dried, and kept at $-20\text{ }^\circ\text{C}$.

Blank P3HB and P3HBV NPs (P3HB-NPs and P3HBV-NPs) were prepared according to the same method, with the exception that the initial PHA/ CHCl_3 solutions did not contain 5-FU.

Preparation of microparticles by spray drying

Spray-dried 5-FU-loaded P3HB and P3HBV MPs (P3HB-5-FU-MPs and P3HBV-5-FU-MPs) were prepared using Büchi Mini Spray Dryer B-290 (BUCHI Laboratory Equipment, Switzerland) according to the procedure described in [13]. Briefly, 150 mg of P3HB or P3HBV were dissolved in 150 mL of CH_2Cl_2 at $50\text{ }^\circ\text{C}$ and mixed with 10 mL of 5-FU solution in CH_3OH . The resulting solutions (5-FU to PHA ratio 1:10) were spray-dried using argon as a drying gas (solution feed rate 7.5 mL min^{-1} , flow rate $35\text{ m}^3\text{ h}^{-1}$; inlet temperature $100\text{ }^\circ\text{C}$; outlet temperature $67\text{ to }71\text{ }^\circ\text{C}$). The obtained MPs were collected, weighed and kept at $-20\text{ }^\circ\text{C}$.

Blank P3HB and P3HBV MPs (P3HB-MPs and P3HBV-MPs) were produced under the same conditions, except that the PHA/DCM solutions did not contain 5-FU.

Yield of the microparticles and nanoparticles

The yield of the obtained MPs and NPs ($Y / \%$) was determined according to Equation (1).

$$Y = \frac{M_{\text{ps}}}{M_{\text{polymer}}} 100 \quad (1)$$

where M_{ps} is the mass of the obtained MPs or NPs and M_{polymer} is the mass of polymer used.

Hydrodynamic particle size and zeta potential

The average hydrodynamic diameter and polydispersity index (PDI) of the obtained MPs and NPs were determined by dynamic light scattering (DLS), whereas zeta potential (ZP) was measured by electrophoretic light scattering (ELS) (Zetasizer Nano ZS, Malvern, UK). Aqueous suspensions of each sample containing 1 mg of particles in 2 mL of deionized water were sonicated for 1 min before the measurements (Scientz JY92-IIN, Scientz Biotechnology, China).

Scanning electron microscopy

To assess the shape and surface morphology of the obtained MPs and NPs, scanning electron microscopy (SEM) was used (Hitachi SU3500, Japan). The samples were positioned on a specimen stub and sputter-coated with platinum (Leica EM ACE200, Germany) prior to observation to increase conductivity and promote heat dissipation from the polymeric matrix.

Determination of 5-fluorouracil encapsulation efficiency and drug-loading

The encapsulation efficiency (EE, %) and drug loading (DL) of 5-FU were determined by UV-Vis spectroscopy using Genesys 10S UV-Vis (Thermo Scientific, USA). Briefly, the amount of 5-FU in the MPs and NPs was determined after dissolution of the corresponding samples in a mixture of DCM and CH_3OH (15:1 volume ratio). The absorbance was measured at 266.8 nm against the blank solution (containing unloaded particles). The amount of 5-FU in the obtained MPs and NPs was determined using the calibration curve. EE was calculated according to Equation (2).

$$EE = \frac{M_1}{M_2} 100 \quad (2)$$

where M_1 is the mass of 5-FU in MPs or NPs and M_2 is the initial mass of 5-FU.

Drug loading (DL, %) was calculated according to Equation (3).

$$DL = \frac{M_1}{M_{ps}} 100 \quad (3)$$

In vitro 5-fluorouracil release

In vitro 5-FU release was investigated spectrophotometrically in phosphate-buffered saline (10 mM, pH 7.4). 2 mg of the obtained particles were suspended in 2 mL of freshly prepared media and incubated at 37 °C. The samples were kept under continuous gentle agitation to simulate the *in vivo* movement of body fluids. At predetermined time intervals, the suspension was centrifuged, aliquots were withdrawn, and an equivalent volume of buffer was added to maintain a constant volume through the course of the experiment. Finally, the absorbance of the aliquots at 266.8 nm was recorded.

Drug release, % was calculated by Equation (4)

$$\text{Drug release} = \frac{M_t}{M_x} 100 \quad (4)$$

where M_t is the mass of 5-FU released at each time point and M_x is the mass of 5-FU contained in the weighted mass of particles.

Cell culture

HeLa human cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. The cells were maintained at 37 °C in a 5 % CO₂ humidified atmosphere and subcultured using 0.25 % trypsin-ethylenediaminetetraacetate (trypsin-EDTA) after reaching 70 to 80 % confluence.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

HeLa cells were seeded at a density of $1.5 \cdot 10^4$ cells *per* well in 96-well plates. After seeding and 24 h of incubation, the medium was replaced with fresh culture medium containing 5-FU aqueous solutions or particle suspensions with various concentrations (to obtain 5-FU concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, and 15 µg mL⁻¹ in wells) and the cells were incubated with NPs for 72 h. Then, the cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of the resulting formazan dissolved in DMSO was recorded using an iMark Microplate Reader (Bio-Rad Laboratories, USA) after 4 h of incubation with 200 µL of MTT solution at 37 °C ($\lambda = 550$ nm). Cell viability, % was calculated relative to untreated cells according to Equation (5).

$$\text{Cell viability} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} 100 \quad (5)$$

where A_{test} is the absorbance of samples incubated with the tested formulations, A_{control} is the absorbance of the control sample, and A_{blank} is the absorbance of the blank solution.

Following the same protocol, cells were exposed to aqueous suspensions of blank P3HB and P3HBV particles diluted in DMEM at concentrations corresponding to the amount of 5-FU encapsulated in the studied particles (5.4-323 and 4.5-271 µg mL⁻¹ for P3HB and P3HBV, respectively).

Live/dead staining

To confirm the results of the MTT assay, live/dead double staining of the cells exposed to the studied particles was performed using ReadyProbes™ Cell Viability Imaging Kit (Thermo Fisher, USA). Cells were seeded and treated with 5-FU and drug-loaded particles as described in the previous section. Then, the medium was removed, and the cells were incubated with NucBlue® Live reagent (Hoechst 33342, stains nuclei of all cells, blue) and NucGreen® Dead reagent (stains dead cell nuclei only, green) for 15 min at 37 °C. The stained cells were imaged using a Leica DM6000 B digital microscope (Leica Microsystems, Germany).

Hemolytic activity

The hemolytic activity of 5-FU and 5-FU-loaded P3HB and P3HBV particles was determined according to the procedure described in [13] with minor modifications. In brief, the whole human blood containing EDTA as an anticoagulant was centrifuged at 3000 rpm for 10 min. Plasma and buffy coat were discarded, while the red blood cell (RBC) pellet was collected, washed with normal saline and resuspended in normal saline to a concentration of $1 \cdot 10^8$ cell mL⁻¹. Then, 20 µL of 5-FU aqueous solutions or particle suspensions with various concentrations (to obtain the 5-FU concentrations of 0.25, 0.5, 1.0, 2.5, 5.0 and 15 µg mL⁻¹ in wells) were added to 130 µL of the obtained RBC suspension in a 96-well plate and incubated for 1 h at 37 °C with gentle agitation using a ST-3M thermostatic shaker (Elmi, Latvia). After incubation, RBCs were separated by centrifugation, and the absorbance of haemoglobin in the supernatant at 550 nm was determined using an iMark Microplate Reader (Bio-Rad Laboratories, USA). RBCs in the positive control (PC) were treated with aqueous Triton X-100 solution (1 %), which causes the complete lysis of the RBC membranes, whereas RBCs in the negative control (NC) were incubated with normal saline.

Hemolytic activity was calculated according to Equation (6):

$$\text{Hemolysis} = \frac{A_{\text{test}} - A_{\text{nc}}}{A_{\text{pc}} - A_{\text{nc}}} 100 \quad (6)$$

where A_{test} is the absorbance of samples incubated with 5-FU or 5-FU-loaded P3HB and P3HBV particles, A_{pc} is the absorbance of the positive control sample (RBCs incubated with Triton X-100 solution) and A_{nc} is the absorbance of the negative control sample (RBCs incubated with normal saline).

According to the same protocol, the hemolytic activity of the corresponding blank particles was determined.

Statistical analysis

Experimental data were presented as the mean $\pm$ standard deviation ($n = 3$ for all experiments). The statistical analysis of the results was performed using Microsoft Excel 2021. Student's t-test was used to determine the statistical significance of the experimental results. The significance threshold was set at $p < 0.05$.

Results and discussion

In this research, 5-FU-loaded P3HB and P3HBV micro- and nanoparticles were prepared by the spray-drying method and emulsion solvent evaporation technique. The obtained MPs and NPs were characterized in terms of their average hydrodynamic size, zeta potential, surface morphology, drug loading, encapsulation efficiency (Table 1), release patterns, hemolytic activity, and cytotoxicity in HeLa cervical cancer cells.

Table 1. Characteristics of the obtained NPs and MPs.

Formulation	Y / %	d / nm	PDI	ZP, mV	EE, %	DL, %
P3HB-NPs	93±4	149±15	0.18±0.04	-25.0±5.2	-	-
P3HBV-NPs	93±3	155±17	0.22±0.04	-24.9±4.6	-	-
P3HB-5-FU-NPs	92±5	154±12	0.19±0.03	-27.6±4.9	not detected	not detected
P3HBV-5-FU-NPs	92±4	159±12	0.10±0.03	-25.3±5.7	not detected	not detected
P3HB-MPs	20±5	2391±110	0.47±0.08	-42.9±5.4	-	-
P3HBV-MPs	20±3	2336±80	0.49±0.08	-42.0±5.1	-	-
P3HB-5-FU-MPs	18±2	2892±120	0.48±0.05	-45.4±4.5	9.6±0.5	4.6±0.3
P3HBV-5-FU-MPs	22±4	3004±114	0.46±0.07	-47.9±5.1	10.2±0.3	5.5±0.2

Characteristics of the obtained 5-fluorouracil-loaded microparticles and nanoparticles

To determine the average hydrodynamic diameter and zeta potential of the particles, we used DLS and ELS, respectively. The average hydrodynamic diameters of the nanoparticles were 149±15, 155±17, 154±12, and 159±12 nm for P3HB-NPs, P3HBV-NPs, P3HB-5-FU-NPs and P3HBV-5-FU-NPs, respectively. The obtained nanoparticles were nearly identical in size, which is primarily attributed to the absence of any significant influence of the encapsulated drug on polymer chain packing and folding during nanoparticle formation (due to the extremely low DL of 5-FU; see the *5-fluorouracil encapsulation efficiency and drug loading* section). In contrast, encapsulating 5-FU in PHA microparticles *via* spray drying leads to measurable changes in particle size. The average hydrodynamic diameters of the microparticles were 2391±110, 2336±80, 2892±120 and 3004±114 nm for P3HB-MPs, P3HBV-MPs, P3HB-5-FU-MPs, and P3HBV-5-FU-MPs, respectively. The encapsulation of 5-FU resulted in an increase in the average hydrodynamic diameter of the microparticles by 21 % (P3HB) and 28.6 % (P3HBV). This phenomenon has also been observed in several studies. For instance, Shershneva *et al.* [14] noted that encapsulating 5-FU in the P3HB matrix increased the MPs' size by 24 % compared with unloaded MPs. Pozdniakova *et al.* [15] also reported that encapsulation of etamsylate into a P3HB matrix resulted in an increase in microparticles' size from 15.0±0.48 µm (for unloaded microparticles) to 18.0±0.63 µm (for drug-loaded microparticles).

Scanning electron microscopy revealed that all the prepared particles were spherical in shape and exhibited no obvious surface defects (Figure 1). Notably, the particle sizes determined by SEM were in good agreement with those obtained by dynamic light scattering.

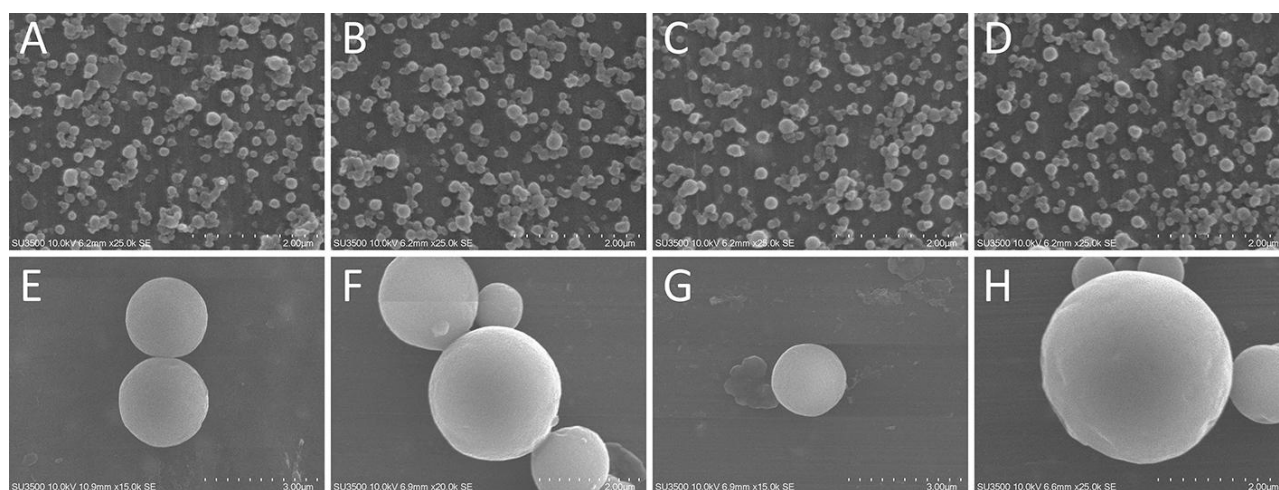


Figure 1. SEM images of the obtained NPs and MPs. (A) P3HB-NPs. (B) P3HB-5-FU-NPs. (C) P3HBV-NPs. (D) P3HBV-5-FU-NPs. (E) P3HB-MPs. (F) P3HB-5-FU-MPs. (G) P3HBV-MPs. (H) P3HBV-5-FU-MPs

The obtained micro- and nanoparticles showed ZP values in the range of -47.1 mV to -24.0 mV, suggesting that the resulting colloidal dispersions possessed electrostatic stability (moderately stable for nanoparticles and highly stable for microparticles [16]). Such ZP values are typical of micro- and nanoparticles prepared

from polyhydroxyalkanoates as well as from polymeric materials of different origins [9]. Ali *et al.* [17] reported that the ZP of 5-FU-loaded solid lipid nanoparticles (obtained from glyceryl palmitostearate, Geleol® and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[ethoxy(polyethylene glycol)-2000]) was measured to be -26.2 ± 0.36 mV. Kole *et al.* [18] noted that the ZP of isoniazid-loaded poly(caprolactone) nanoparticles was equal to -24.8 ± 0.30 mV.

The encapsulation of 5-FU into PHA-based micro- and nanoparticles did not result in significant changes in ZP across all formulations. The measured ZP values remained nearly constant within the experimental variability, with overlapping ranges observed for unloaded and 5-FU-loaded particles, indicating no substantial shift in surface charge. This suggests that 5-FU encapsulation does not markedly alter the interfacial properties of the particles, although partial localization of the drug at or near the particle surface cannot be excluded.

5-fluorouracil encapsulation efficiency and drug loading

Spray drying technique proved an effective method for loading 5-FU into PHA microparticles. EE and DL for P3HB-5-FU-MPs were 9.6 ± 0.5 and $4.6 \pm 0.3\%$, respectively, whereas for P3HBV-5-FU-MPs they were higher, reaching 10.2 ± 0.3 and $5.5 \pm 0.2\%$. In contrast, 5-FU was not detected in P3HB-5-FU-NPs and P3HBV-5-FU-NPs because the optical density of these samples was below the limit of detection. Thus, the amount of the encapsulated 5-FU in P3HB-5-FU-NPs and P3HBV-5-FU-NPs was considered therapeutically irrelevant. The results obtained are in accordance with McCarron *et al.* [19]. Despite adequate initial drug loading into poly(isobutylcyanoacrylate), poly(lactide-co-glycolide), poly(caprolactone), and Eudragit matrices (up to 5.49 %), a sharp decrease in encapsulated 5-FU was observed after one washing step: DL dropped to below 1 %. In addition, all obtained 5-FU-loaded samples showed pronounced burst release in aqueous solution, indicating that 5-FU was loosely associated with the hydrophobic polymeric surfaces. Similar data were obtained by Öcal *et al.* [20] and Tummala *et al.* [21] for poly(DL-lactic acid)/poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) copolymer and chitosan, respectively. The authors reported relatively high 5-FU loadings in the corresponding polymeric NPs and noticeable burst release (more than 60 and 90 % of the encapsulated 5-FU after 10 h of exposure to aqueous media), indicating surface localization of the drug.

Encapsulating highly hydrophilic drugs such as 5-FU into hydrophobic polymeric matrices using emulsion solvent evaporation techniques remains challenging. This phenomenon is attributed to a limited affinity of these molecules for hydrophobic polymers, which leads to their partitioning into the continuous water phase during encapsulation [22, 23]. In our preliminary studies, we attempted to increase DL and EE values by varying key formulation parameters, including 5-FU amount, surfactant concentration, and the nature and proportion of the co-solvent (DMSO, CH₃OH) in the organic phase. These modifications did not significantly improve EE or DL, which remained very low. Moreover, the resulting nanoformulations showed increased size and pronounced aggregation, which is unfavorable for NP-based drug delivery systems. Taken together, these results indicate that conventional emulsion-based encapsulation of 5-FU into hydrophobic PHAs requires alternative strategies to achieve therapeutically relevant drug loading.

Spray drying enables higher drug loading than the emulsion solvent evaporation technique because it avoids competing drug dissolution in the aqueous phase during encapsulation [24]. Relatively low DL (theoretical DL is equal to 9.1 %) in the case of P3HB-5-FU-MPs ($4.6 \pm 0.3\%$) and P3HBV-5-FU-MPs ($5.5 \pm 0.2\%$) may be attributed to the rapid solvent evaporation during spray drying (dichloromethane boils at 40 °C, whereas the inlet temperature of the drying chamber is 100 °C), which does not allow the polymer droplet to fully form a solid microcapsule around the encapsulated substance; as a result, the latter remains on the particle surface and subsequently adheres to the inner walls of the drying chamber. At lower inlet

temperatures, as shown in our previous study [25], particle size increased substantially, limiting their potential for further *in vivo* application.

Because the amount of encapsulated 5-FU in P3HB-5-FU-NPs and P3HBV-5-FU-NPs was considered unacceptable for biomedical applications, we selected only P3HB-5-FU-MPs and P3HBV-5-FU-MPs for further analysis.

In vitro 5-fluorouracil release

The release of 5-FU from P3HB-5-FU-MPs and P3HBV-5-FU-MPs was investigated in phosphate-buffered saline (10 mM, pH 7.4, 37 °C). *In vitro* 5-FU release profiles are presented in Figure 2.

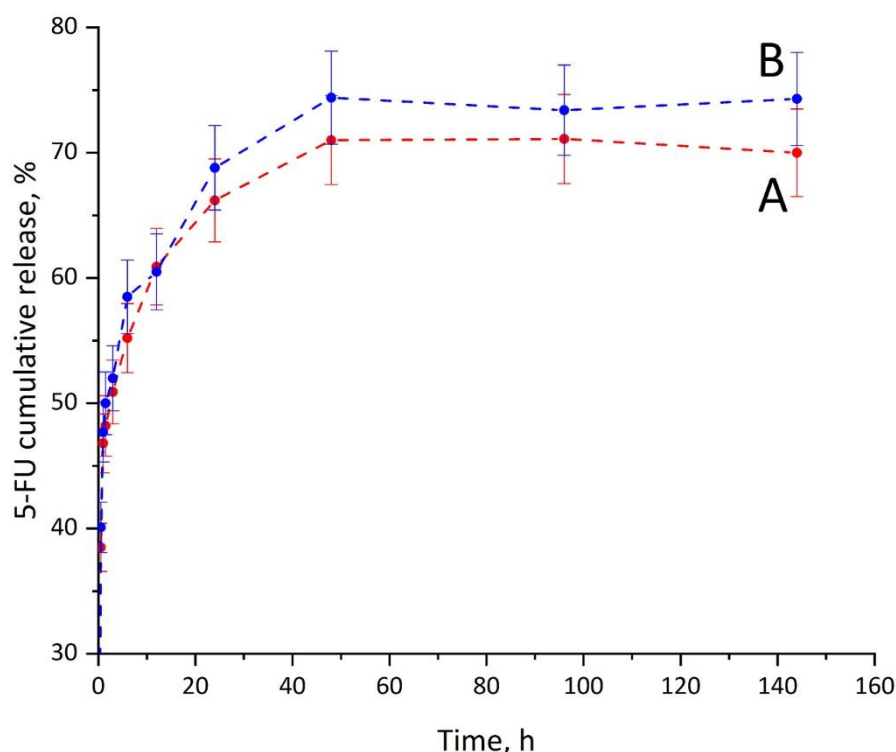


Figure 2. *In vitro* 5-FU release profiles. (A) P3HB-5-FU-MPs. (B) P3HBV-5-FU-MPs.

The kinetic curves exhibit two characteristic regions typical of PHA particles [26]: an initial burst release from the surface of microparticles and a gradual release from the inner layers of the microparticles. During the 1-hour burst-release stage, 46.8 % (P3HB-5-FU-MPs) and 47.7 % (P3HBV-5-FU-MPs) of 5-FU were released. The pronounced burst release of the drug from the investigated microparticles may be attributed to the low affinity of hydrophilic 5-FU for the hydrophobic PHA matrix, which promotes redistribution of the drug toward the particle surface and rapid initial desorption (see the *5-FU encapsulation efficiency and drug loading* section). The relatively higher DL values of P3HB-5-FU-MPs and P3HBV-5-FU-MPs led to a correspondingly higher drug release. Thus, both types of MPs achieved maximum 5-FU release after 48 hours of exposure: 71.0 % for P3HB-5-FU-MPs and 74.4 % for P3HBV-5-FU-MPs. In general, because 5-FU release from both microparticle types follows a similar pattern and shows the same burst-phase duration and total release, differing only in the total amount of drug released, the release behavior of 5-FU from P3HB and P3HBV may be considered identical.

From a therapeutic perspective, the two-stage profile may be advantageous for applications in which a rapid initial increase in local drug concentration is desirable, followed by sustained drug exposure over the subsequent release period. For instance, a locally administered depot designed to eliminate residual tumor cells may benefit from an initial burst release followed by a more sustained release phase.

To determine the mechanism of 5-FU release from the PHA microparticles, zero-order, first-order, Higuchi, Hixson-Crowell, and Baker-Lonsdale mathematical models were fitted to *in vitro* release profiles of 5-FU [27]. The obtained coefficients of determination (R^2) are presented in Table 2.

Table 2. R^2 values of drug release kinetic models

Sample	R^2				
	Zero-order	First-order	Higuchi	Hixson-Crowell	Baker-Lonsdale
P3HB-5-FU-MPs	0.859	0.908	0.966	0.888	0.963
P3HBV-5-FU-MPs	0.889	0.885	0.972	0.858	0.945

Because polyhydroxyalkanoates are hydrophobic polymers, encapsulated drug release from PHA matrices is mainly governed by Fickian diffusion [28], driven by concentration gradients of the encapsulated drug, buffer components, solvent, *etc.* [29]. This statement is supported by the results presented in Table 2. The highest R^2 values were obtained for the Higuchi (0.966 and 0.972 for P3HB-5-FU-MPs and P3HBV-5-FU-MPs, respectively) and Baker-Lonsdale (0.963 and 0.945 for P3HB-5-FU-MPs and P3HBV-5-FU-MPs, respectively) diffusion models, indicating that these models best fit the sustained release kinetics of 5-FU from PHA microparticles. The release is least well described by the zero-order and first-order models, as well as by the Hixson-Crowell (erosion-based) model.

Cytotoxicity to HeLa cells

The influence of the non-encapsulated 5-FU, as well as blank and 5-FU-loaded P3HB and P3HBV microparticles on the viability of HeLa cells *in vitro* was evaluated via the standard MTT assay at six 5-FU concentrations ranging from 0.25 to 15 $\mu\text{g mL}^{-1}$ (Figure 3A). The free drug showed potent cytotoxicity against the studied cells, with a calculated half-maximal inhibitory concentration (IC_{50}) of $1.42 \pm 0.36 \mu\text{g mL}^{-1}$. The obtained results are consistent with [30,31]. Notably, broad variations in IC_{50} values of 5-FU in HeLa cell culture reported in the literature over the past decades may be attributed to the strong time dependence of the drug's *in vitro* cytotoxic effects [32], since 5-FU is phase-specific and exerts its toxicity only during the S-phase of the cell cycle [33]. For P3HB-5-FU-MPs and P3HBV-5-FU-MPs, the IC_{50} in HeLa cells increased compared with the pure drug (to 2.63 ± 0.32 and $2.43 \pm 0.44 \mu\text{g mL}^{-1}$, respectively). This drop in acute cytotoxicity is expected for sustained-release formulations in which the total amount of encapsulated drug is not immediately bioavailable, as previously shown by Halevas *et al.* [34], Kang *et al.* [35], Wang *et al.* [36] and others. The similar IC_{50} values observed for P3HB and P3HBV indicate that including 3HV units in the polymer chain had little effect on the short-term cytotoxicity profile under the studied conditions, although further investigation is required to ensure the absence of 3HV influence over longer timeframes. In addition, exposure to blank P3HB and P3HBV MPs did not reduce HeLa cell viability even at the highest concentrations tested, so the polymer matrices themselves did not contribute to the cytotoxic effects of the studied systems. These data align with many reports of negligible cytotoxicity in PHA-based micro- and nanoparticulate formulations [37-39], confirming the high biocompatibility of the studied polymers and their applicability for designing sustained-release drug delivery systems.

Live/dead double staining corroborated the results of the MTT assay (Figure 3B). At the highest concentration of the free drug (15 $\mu\text{g mL}^{-1}$), few to no live cells were observed under fluorescence microscopy. In contrast, 5-FU-loaded P3HB and P3HBV MPs maintained a low but significant number of viable cells, which is consistent with the MTT assay data. Meanwhile, images of blank MPs of both polymers did not differ from the control.

Hemocompatibility

To evaluate the toxic effects of the obtained MPs and pure 5-FU on RBCs, an *in vitro* hemolysis assay was carried out. It has been shown that P3HB-MPs and P3HBV-MPs exhibited negligible hemolytic activity across the entire tested concentration range (Figure 3C). The results obtained are consistent with the studies of our research team [13,40] and others [41]. For free 5-FU, only slight dose-dependent hemolysis was observed even at the highest concentration tested (1.31% at $15\ \mu\text{g mL}^{-1}$), in accordance with previously reported data [42,43]. Encapsulating the drug in P3HB or P3HBV MPs further reduced its hemolytic potential, which may result from the shielding effect of the PHA matrices, ensuring sustained release of the loaded drug into the surrounding medium. According to established criteria, formulations inducing less than 5% hemolysis may be regarded as non-hemolytic [44]. Thus, at the initial evaluation, both blank and 5-FU-loaded P3HB and P3HBV MPs obtained in the present study were considered safe and hemocompatible for further biomedical investigations.

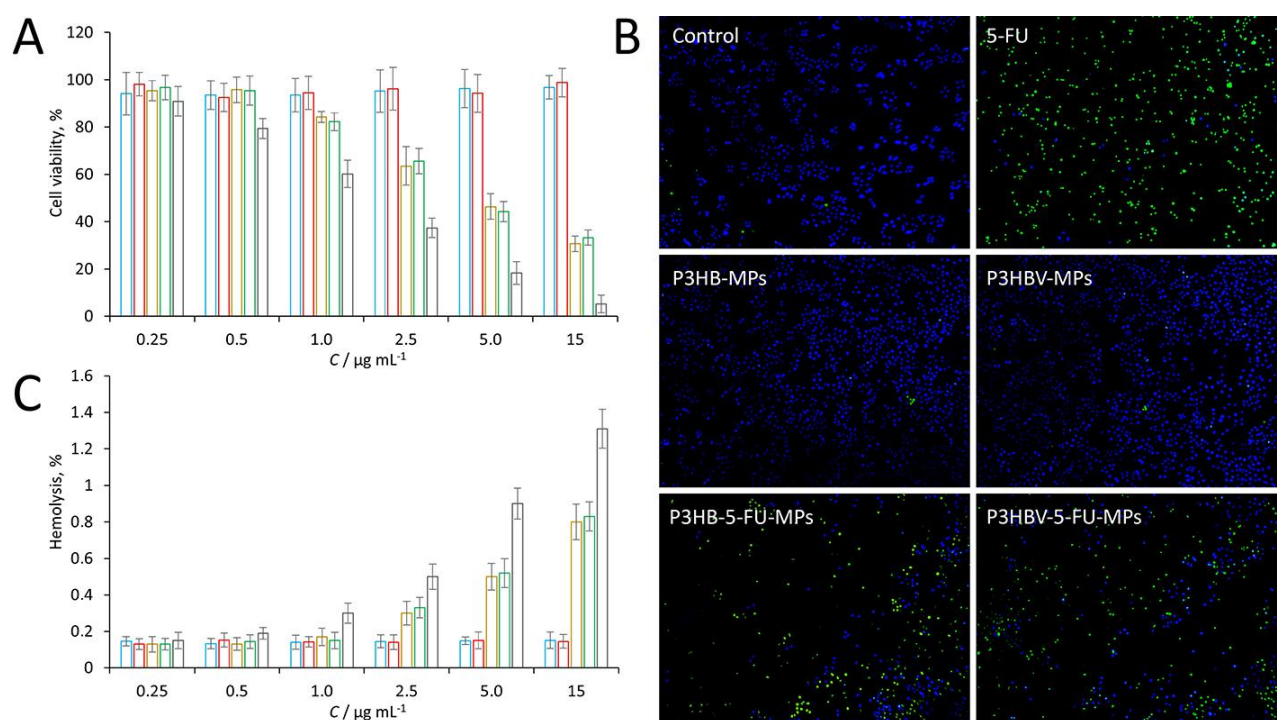


Figure 3. (A) Viability of HeLa cells exposed to P3HB-MPs (blue), P3HBV-MPs (red), P3HB-5-FU-MPs (yellow), P3HBV-5-FU-MPs (green) and 5-FU (black) for 72 h, determined via MTT assay. The x-axis is the total 5-FU concentration in wells. For MPs, values represent the equivalent 5-FU concentrations achieved by the corresponding doses of MPs (5.4 to 323 and 4.5 to $271\ \mu\text{g mL}^{-1}$ for P3HB and P3HBV, respectively). (B) Live/dead double staining images of HeLa cells exposed to $15\ \mu\text{g mL}^{-1}$ 5-FU and the corresponding concentrations of PHA microparticles. Live and dead cells had blue and green fluorescence, respectively. (C) Hemolytic activity of the free 5-FU, as well as blank and 5-FU-loaded PHA MPs. Colors represent the same formulations as in (A)

Conclusions

In this research, 5-FU-loaded P3HB and P3HBV micro- and nanoparticles were prepared via spray drying and single emulsion solvent evaporation methods, respectively. All particles had a regular spherical morphology, smooth surfaces, and suitable zeta potential. The spray-dried microparticles showed satisfactory drug loading and released encapsulated 5-FU in a sustained manner over 48 h. The microparticles showed potent cytotoxicity against HeLa cervical cancer cells and reduced hemolytic activity compared with free 5-FU. Thus, these microparticulate formulations may represent a promising biodegradable sustained-release platform for 5-FU delivery in cervical cancer therapy. Future research will focus on optimizing particle composition and preparation conditions to increase 5-FU loading, reduce initial burst release and extend release duration, followed by comprehensive *in vivo* evaluations of pharmacokinetics, biodistribution, antitumor efficacy and systemic toxicity.

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