Development of Biodegradable Delivery Systems Containing Novel 1,2,4-Trioxolane Based on Bacterial Polyhydroxyalkanoates

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In this work, delivery systems in the form of microparticles and films containing 1,2,4-trioxolane (ozonide, OZ) based on polyhydroxyalkanoates (PHAs) were developed. Main systems’ characteristics were investigated: the particle yield, average diameter, zeta potential, surface morphology, loading capacity, and drug release profile of microparticles, as well as surface morphology and release profiles of OZ-containing films. PHA-based OZ-loaded microparticles have been found to have satisfactory size, zeta potential, and ozonide loading-release behavior. It was noted that OZ content in influenced the surface morphology of obtained systems.

1. Introduction

Over the past decade, cyclic organic peroxides have been intensively researched by chemists and pharmacologists. The discovery of the natural peroxide artemisinin [1–3], which has antimalarial properties, was the beginning of the development of biologically active peroxides (Figure 1). It has been found that cyclic peroxides have antimalarial [4–12], antiparasitic [13–19], anticancer [20–31], antiviral [32–34], and fungicidal [18, 35, 36] activities. On the basis of the fully synthetic peroxide artevorolane, which contains a 1,2,4-trioxolane (ozonide) fragment, has been created an antimalarial drug of the latest generation—Synriam [37]. It was recently found that artemesate is able to inhibit NL63 α-coronavirus and OC43 β-coronavirus, while ozonides OZ277 and OZ418 are able to inhibit both α-coronavirus NL63, β-coronavirus OC43, and SARS-CoV-2 [38].

Controlled release drug delivery systems based on biodegradable polymeric films and microparticles can reduce detrimental side effects of biologically active substances. The matrix of such systems must be biocompatible, in the case of medical use, and environmentally safe and friendly, for use in agriculture.

Poly(lactic-co-glycolic acid) (PLGA) [39–41], polycaprolactone (PCL) [42, 43], and polyhydroxyalkanoates (PHAs) [44, 45] are widely used as a polymer matrix for depot systems. One of the most perspective matrices of such systems is PHAs, bacterial polyesters that possess biodegradability in biological media, biocompatibility, and nontoxicity. PHAs are water-insoluble semicrystalline substances suitable for medical, agricultural, and industrial applications [46–48].

Pol-3-hydroxybutyrate (P3HB) is the first well-described and studied microbial short-chain length polyhydroxyalkanoate (Figure 2(a)). The P3HB degree of crystallinity is about 72%, the melting point is between 174 and 179°C, and it also has a wide range of molecular weight distribution, depending on the cultivation procedure [49]. P3HB copolymers with poly-3-hydroxyvalerate, P3HV (PHBV, the molar fraction of P3HV varies from 10% to 80%), are recognized as more elastic and plastic material, surpassing P3HB in absolute strength and Young’s modulus. These properties make PHBV more suitable in bioengineering, for obtaining particles, films, scaffolds,
and surgical suture (maintaining high biocompatibility for different biological systems and complete biodegradation) [47]. Anti-inflammatory [50], bactericidal [51], antitumor [52], herbicidal [53], and other drugs have been successfully encapsulated in the PHA matrix.

The natural antimalarial drug artemisinin (ART) is the most commonly used substance in the development of peroxide-containing controlled release systems. Polymer-based systems for ART delivery are usually based on PLGA [54], PCL [55], and cyclodextrin [56].

As far as we know, there are no data in the literature about the encapsulation of synthetic peroxides into the PHA matrix. In this work, we have chosen the synthetic ozonide (OZ) as the model object. OZ (Figure 2(b)) is a hydrophobic white powder that dissolves in DMSO, chloroform, and acetonitrile. The absorption maxima of the substance are in the UV region (268 and 276.5 nm). Since ozonides related to OZ exhibit anti-cancer [25–27], antiparasitic [57], and fungicidal activity [36], PHA-microparticles containing OZ might be potentially used for cancer treatment along with other known drug delivery systems based, for example, on lipid nanovesicles [58], leukocyte-mimicking nanovesicles [59], and immunoliposomes [60].

The aim of this research is to obtain OZ-containing depot systems based on P3HB and PHBV in the form of microparticles and casting films and to study their basic physical, chemical, and cytotoxic properties against HeLa cells.

2. Material and Methods

2.1. Material. Microbial P3HB was obtained by the fermentation process: \( M_w = 1480 \text{kDa} \) (for emulsification method), \( M_w = 96\text{kDa} \) (for spray drying method), and PHBV polymer with molar ratio 90:10 (P3HB: P3HV) and \( M_w = 260\text{kDa} \). Studied polymers and copolymer were produced at the laboratory of Biotechnology of new biomaterials of Siberian Federal University in Krasnoyarsk, RF. OZ was synthesized according to the following equation:

\[
\gamma_{SL} = \gamma_L(1 + \cos \theta)^2/4, \tag{1}
\]

where \( \gamma_L \) is free water surface tension, 72.8 erg/cm².

Free surface energy of PHBV films was calculated according to the following equation:

\[
\gamma_S = \gamma_L + \gamma_W - W_{SL}, \tag{2}
\]

The cohesive force was calculated from the formula

\[
W_{SL} = 2\sqrt{\gamma_S \gamma_L}. \tag{3}
\]

2.2. Preparation of OZ-Loaded PHBV Films (OZ-F). PHBV films containing OZ were prepared by the solution casting method. 250 mg of PHBV was dissolved in chloroform under heating. After that, the solution of OZ in chloroform with a concentration of 1 mg ml⁻¹ was added to obtain films containing 0.008 wt.%, 0.016 wt.%, and 0.024 wt.% of OZ (23.5, 47, and 70.5 μmol g⁻¹, respectively). The resulting solution was transferred to a sterile Petri dish and left until complete solvent evaporation (at room temperature for 48 h). As a control, PHBV-based films without OZ were obtained under similar conditions.

2.3. Preparation of OZ-Loaded P3HB Microparticles by Emulsification Method (OZ-MP_EM). OZ-MP_EM were prepared using water in oil emulsion containing 0.4 g of P3HB, 0.04 g of OZ in 40 ml of dichloromethane (DCM), and 100 ml of 0.5% aqueous PVA solution. The resulting emulsion was mechanically stirred at 5000 rpm for 3 min (Heidolph SilentCrusher M, Germany). After complete evaporation of DCM, OZ-MP_EM were collected by centrifugation at 4000 rpm for 3 min, washed multiple times with deionized water, and then lyophilized.

Unloaded microparticles (MP_EM) were obtained under similar conditions.

2.4. Preparation of OZ-Loaded P3HB Microparticles by Spray Drying Method (OZ-MPSD). The Büchi spray dryer (BUCHI Laboratory Equipment, Switzerland) OZ-MP_SD was used for the spray drying of P3HB/OZ solution (80 mg of P3HB, 40 mg of OZ dissolved in 80 ml of DCM), with nitrogen as a drying gas, at a flow rate of 4.5 ml min⁻¹.

Microparticles (MP_SD) without OZ were obtained under similar conditions.

2.5. Characterization of Films

2.5.1. Morphological Analysis. The surface morphology analysis of films was carried out by scanning electron microscopy (SEM) using SU3500 and TM4100 (Hitachi, Japan). In order to determine the average roughness (Sa) of the surface atomic force, microscopy (AFM) (Solver Nano, NT-MDT, Russia) was used.

2.5.2. Determination of Surface Characteristics. Film surface characteristics towards liquids were measured using the Drop Shape Analyzer (KRÜSS, Germany) with distilled water as a polar liquid.

By measuring contact angles (θ, degrees) (water/air), free surface energy (\( \gamma_S \)), free interface energy (\( \gamma_{SL} \)), and cohesive force (\( W_{SL} \)) (erg/cm²) were calculated.

Free surface energy of PHBV films was calculated according to the following equation:

\[
\gamma_S = \gamma_L(1 + \cos \theta)^2/4, \tag{1}
\]

Free interface energy (polymer/water) was found from the formula:

\[
\gamma_{SL} = \gamma_S - \gamma_L - W_{SL}. \tag{2}
\]

The cohesive force was calculated from the formula

\[
W_{SL} = 2\sqrt{\gamma_S \gamma_L}. \tag{3}
\]

2.6. Characterization of Microparticles

2.6.1. Morphological Analysis. The surface morphology analysis of polymer microparticles was carried out as above. A number of samples were sputter coated with platinum using Leica EM ACE200 (Leica Microsystems, Germany) to obtain a good-quality SEM image.
Artemisinin (ART) antimalarial

Artesunate antimalarial

Artesunate (OZ277)

Synriam™ antimalarial

Tricyclic monoperoxide Anthelmintic

Bridged tetraoxane Antifungal

Bridged ozonide Anticancer

OZ418 antischistosomal

**Figure 1:** Biologically active peroxides of natural, semisynthetic, and synthetic origin.

**Figure 2:** Structures of poly-3-hydroxybutyrate (P3HB) and OZ.
2.6.2. Total Yield of Microparticles. Total yields of microparticles (%) were determined according to

\[
\text{Yield} \% \left( \right) = \frac{M_m}{M_p} \cdot 100, \tag{4}
\]

where \( M_m \) is the mass of microparticles and \( M_p \) is the initial mass of P3HB, used in synthesis.

2.6.3. Particle Size and Zeta Potential. Zetasizer Nano ZS (Malvern, UK) was used to determine the average particle size, zeta potential, particle size distribution (PSD), and polydispersity index (PDI). 5 mg of each sample was suspended in deionized water and sonicated at 6 W for 1 min before the measurements.

2.6.4. DSC Analysis. Differential scanning calorimetry, DSC, analysis was carried out to determine the melting behavior of \( \text{MP}_{EM} \) and \( \text{OZ-MP}_{EM} \) using Mettler Toledo STAR DSC 1 (USA), samples were heated at a rate of 10°C min\(^{-1}\), and the data were recorded from 20 to 300°C.

2.6.5. Drug Encapsulation Efficiency (for Microparticles) and In Vitro OZ Efflux. The amount of OZ encapsulated in the P3HB microparticles was determined spectrophotometrically using Genesys 10S UV-Vis (Thermo Scientific, USA). Firstly, \( \text{MP}_{EM} \) and \( \text{MP}_{SD} \) containing OZ were dissolved in DCM. Then, the absorbance (at 268 nm) of each solution was measured, and a calibration curve (pure OZ in DCM) was plotted. The amount of OZ in MPs was determined using the standard curve.

The encapsulation efficiency (EE) was determined according to the formula

\[
\text{EE} \% \left( \right) = \frac{M_1}{M_2} \cdot 100, \tag{5}
\]

where \( M_1 \) is the mass of OZ in microparticles and \( M_2 \) is the initial mass of OZ.

The investigation of the release of OZ from OZ-MPs was carried out in vitro. Samples of the studied microparticles were placed in sterile centrifuge tubes containing DMSO (2 mg MPs in 1 ml DMSO). Tubes were thermostated at 37°C, with the microparticles precipitated by centrifugation, 11000 rpm, 5 min, at predetermined time intervals. The amount of OZ released was determined by the measurement of absorbance of the supernatant at 268 nm.

To assess the release profile of OZ from OZ-containing films, each film was cut in circles with a diameter of 6 mm. Then, the circles were placed in sterile tubes containing DMSO, and the amount of OZ released during 9 days was measured as mentioned above.

2.7. Cell Culture. HeLa cells, as a model adhesive culture for the estimation of cytotoxic effect, were cultured in Gibco Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific, USA) with the addition of fetal bovine serum (HyClone, USA) and standard antibiotic-antimycotic supplement (Sigma-Aldrich, USA). 3-(4,5-Dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique was used to evaluate the cytotoxic activity of the obtained depot systems.

For the MTT study, the cell suspension was incubated with the studied systems for 24 hours. Then, MTT (in PBS buffer) was added to all samples; the cell culture plate was then incubated at 37°C for 4 h. The resulting formazan was dissolved in DMSO, and the absorbance was determined at 550 nm using an iMark Microplate Reader (Bio-Rad Laboratories, USA).

Cell viability (%) was calculated according to the equation below:

\[
\text{Cell viability} = \frac{[A]\text{test}}{[A]\text{control}} \cdot 100, \quad (6)
\]

where \([A]\text{test}\) is the absorbance of the test sample and \([A]\text{control}\) is the absorbance of the control sample.

The Trypan blue assay was used to assess cell death as a result of interaction with depot systems. After co-incubation for 24 hours, cells were harvested using 0.25% Trypsin-EDTA (Gibco, USA) and centrifuged; the supernatant was removed and resuspended in DMEM. Trypan blue solution (Gibco, USA) was added to the cell suspension, which was layered on a microscope slide.

The number of living cells was determined in the hemocytometer using the following formula:

\[
X = N \cdot 0.05 \cdot 10^m \cdot m, \quad (7)
\]

where \(X\) is the number of cells in 1 ml, \(N\) is the number of cells in 5 large squares, and \(m\) is dilution.

Four replicates were performed for each sample.

To visualize cell viability during interaction with the studied systems, a LIVE/DEAD assay was performed using a ReadyProbes™ double staining kit (Thermo Fisher, USA) in accordance with the manufacturer’s protocol. Images were obtained using the Leica digital microscope (Leica Microsystems GmbH, Germany). Live and dead cells had blue and green fluorescence, respectively. Images were analyzed using NIH ImageJ software.

### 3. Results and Discussion

In this work, for the first time, two OZ-loaded depot systems based on PHA were developed. The systems obtained were sufficiently stable in model medium and had high drug loading and encapsulation efficiency. We were not absolutely sure about the successful encapsulation of organic peroxides into the PHA matrix, since the interaction of the carboxyl groups of PHAs with peroxides can lead to the decomposition of the peroxide or its rearrangements, such as the rearrangements of Baeyer-Villiger, Criegee, Hock, etc. [62].

According to the obtained absorption spectra of pure OZ and PHAs before and after the encapsulation procedure (not shown), there were no changes noted in the structure of the peaks and their position in the spectra, which was an indication of the absence of any rearrangements or decomposition of molecules.

PHAs have undeniable advantages as a matrix for depot systems, compared to PLGA and other hydrophilic polymers. PHAs are almost inert to hydrolysis and only biodegrade under enzymatic processes in aerobic conditions, without local shifts in the pH of the biological system to the acidic region, as in the case of PLGA. In this work, we studied the release of OZ from microparticles and films containing 7.1% (OZ-MP SD), 9.7% (OZ-MP EM), 0.008%, 0.016%, and 0.024% of OZ (wt.%).

#### 3.1. Characterization of Films

For the estimation of the applicability of the approach, we obtained casting films by combining chloroform solutions of two components of the depot system. Resulted films had relatively homogeneous...
structures. The surface of OZ-F was rough and porous, with pore sizes ranging from 0.5 to 6 μm (Figure 3).

As a result of the addition of OZ to the PHA, we detected the increasing of the porosity and average roughness of the sample surface, compared with the control, with both microscopy approaches—SEM and AFM. Increasing the ozonide concentration in the film leads to a linear increasing in the average surface roughness (Table 1).

The films' surface characteristics are presented in Table 1.

Since OZ is encapsulated into the PHBV matrix, all the studied parameters of the obtained films significantly change. When the amount of OZ in the film increases from 0% to 0.008%, the contact angle (water in air) increases by more than 30%: from 62.3 ± 1.56 to 92.5 ± 1.49 degrees, so the resulted film surface becomes more hydrophobic. The free surface energy reaches its maximum at 0.016% of OZ in PHBV film, 48.0 ± 0.71 erg/cm²; the presence of OZ reduces the free interface energy of the PHBV-OZ composite film. The cohesion forces are also increased, when OZ is added, by about 5-11%.

Thus, with an increase of the ozonide concentration in the PHBV film, both its morphological, average roughness, and physical characteristics: water contact angle and free surface energy, free interface energy, and cohesion forces change. These results make it possible to predict the properties of these depot systems related to biological objects—water-containing medium, tissues, and cells. An increasing of cohesive forces can be regarded as a positive factor contributing to the tight attachment of cellular elements.

3.2. Characterization of Microparticles. OZ-MP have a uniform spherical shape. OZ-MP SD were found larger and smoother than OZ-MP EM. It was also noted that OZ-MP EM (Figure 4(b)) have minor surface defects.

The encapsulation of OZ into microparticles leads to the insignificant increasing in particle size: the average diameter of OZ-MP EM and OZ-MP SD was 0.44 ± 0.03 μm and 2.6 ± 0.1 μm, respectively, while the average diameter of MP EM was 0.35 ± 0.02 μm and the average size of MP SD was 2.1 ± 0.1 μm.

The results also indicate that ζ-potential markedly decreases when OZ is encapsulated into both types of microparticles. The value of zeta potential of OZ-MP was −13.8 ± 0.3 for EM and −39.6 ± 0.4 mV for SD, respectively (Table 2). The zeta potential of MP EM was −23.8 ± 0.2 and −45.2 ± 0.2 mV for MP SD. In general, the morphology, size, and zeta potential of the microparticles obtained in this research are typical for PHA-based depot systems. Thus, encapsulation of ceftriaxone into the P3HB matrix (Murueva et al. [63]) also leads to increasing the microparticle size.

The size of microparticles was 0.89 ± 0.02 μm for EM and 4.2 ± 0.7 μm for SD, compared to 0.74 ± 0.02 μm (EM) and 6.51 ± 0.47 μm (SD) for empty particles obtained under
Figure 5: Release profiles (kinetic curves) of OZ-MP: (a) OZ-MP_{EM}; (b) OZ-MP_{SD}.

Figure 6: Initial region of the kinetic curve illustrating OZ release from MPs: (a) OZ-MP_{EM}, \( R^2 = 0.988 \); (b) OZ-MP_{SD}, \( R^2 = 0.998 \).

Figure 7: Final region of the kinetic curve illustrating OZ release from MPs: (a) OZ-MP_{EM}, \( R^2 = 0.960 \); (b) OZ-MP_{SD}, \( R^2 = -0.927 \).
reported that the average diameter of P3HB-MP SD contain-
respective (compared to
According to the DSC results,
3.2.1. Thermal Characteristics.
method [63].
explained by the peculiarities of the spray drying
3
parameters of blank P3HB-MP SD: the average diameter was
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mV for unloaded microspheres). Shershneva et al. [52]
reported that the average diameter of P3HB-MP SD contain-
ing paclitaxel was 3.08 ± 0.7 μm, and zeta potential was –
40.8 ± 0.9 mV, which is slightly lower than the same param-
eters of blank P3HB-MP SD; the average diameter was 3.36
± 0.46 μm, and zeta potential was –44.6 ± 0.4 mV.

The yield of microparticles was excellent for OZ-MP EM
92.4 ± 0.2%, and for OZ-MP SD 26.6 ± 0.4%.
The encapsulation efficiency of OZ was 89.4 ± 0.2% and
37.7 ± 0.3% in MP EM and MP SD, respectively. EE of MP SD is
expectedly lower compared to EE of MP EM. It can be
explained by the peculiarities of the spray drying method [63].

3.2.1. Thermal Characteristics. According to the DSC results,
OZ-MP EM have a lower melting point (T m = 161.3°C) and
crystallization temperature (117.4°C) compared to MP EM
(176°C and 123.6°C, respectively). This is consistent with
the results of Senhorini et al. [64], who noted a decrease in
T m of PHBV (8.7% of P3HB) MP after encapsulation of
andiroba oil, which has anti-inflammatory and insecticidal
properties. Bidone et al. [65] and Volova et al. [66] reported
that there is also a decrease in the melting point of polymer
compositions based on P3HB containing ibuprofen (by
10°C) and metribuzin (by 2°C), respectively.

As a result, OZ provides greater fusibility of the
microparticle-based depot system, which can potentially
lead to faster biodegradation.

3.2.2. OZ Release from MPs and Fs In Vitro. OZ release pro-
files from MP EM and MP SD are presented in Figure 5.
A significant drug release—60% for OZ-MP EM and 48%
for OZ-MP SD—is achieved in 24 hours, and the full release
of OZ occurs in 144 and 216 hours, respectively.
It was found that the OZ release from OZ-MP EM and
OZ-MP SD depends on the sizes of microparticles and their
surface structure having defects as pores and microcracks.
OZ-MP SD have a smoother surface and larger sizes com-
pared to OZ-MP EM, so the release rate from these micropar-
ticles is slower.

Each kinetic curve is characterized by two regions: the
linear (0–24 h) and nonlinear (48–312 h) stages of OZ
release from microparticles. To determine the drug release
mechanism according to the Higuchi model, the diffusion
coefficients were calculated at each of these regions.
In case of a short desorption time of OZ at the initial
stage, Fick’s second law (according to Higuchi model [67])
can be written as follows:
\[
\frac{m_t}{m_\infty} = 4\sqrt{\frac{D t}{\pi h^2}} n p u \frac{m_t}{m_\infty} \leq 0.6, \quad (8)
\]
where \( m_t \) is the mass of OZ desorbed at time \( t \), \( m_\infty \) is the
mass of OZ desorbed at an infinite time (\( t \rightarrow \infty \)), and \( h \)
is matrix thickness.
Since the MP has a spherical shape, the matrix thickness
(\( h \)) is replaced by the particle diameter (\( d \)). After linearization
of the previous equation,
\[
\frac{m_t}{m_\infty} = 4\sqrt{\frac{D t}{\pi d^2}}. \quad (9)
\]
The graphical solution of Equation (9) (Figure 6) allows
us to determine the diffusion coefficient of OZ at the initial
stage of the kinetic curve \((D_1)\). \(D_1\) is equal to 6.1\(\times\)10\(^{-11}\) cm\(^2\)/s for OZ-MP\(_{EM}\) and 2.2\(\times\)10\(^{-9}\) cm\(^2\)/s for OZ-MP\(_{SD}\).

To determine the diffusion coefficient at the second stage \((D_2)\) \((0.4 \leq m_l/m_{\infty} \leq 1.0)\), the following approximation is used:

\[
\frac{m_l}{m_{\infty}} = 1 - \left(\frac{8}{\pi^2}\right) e^{-D_2 t \pi^2/d^2}.
\]  \((10)\)

The graphical solution of Equation (10) in semilog coordinates \((\ln \left[1 - m_l/m_{\infty}\right] - t)\) (Figure 7) allows us to calculate \(D_2\):

\[
D_2 = \frac{t g \alpha \cdot d^2}{\pi^2}.
\]  \((11)\)

\(D_2\) for OZ-MP\(_{EM}\) is 2.7\(\times\)10\(^{-11}\) cm\(^2\)/s and for OZ-MP\(_{SD}\) is 0.5\(\times\)10\(^{-9}\) cm\(^2\)/s. A significant decrease of \(D_2\) for both types of microparticles indicates that the release mechanism of OZ changes.

Livshits et al. [68] obtained similar diffusion coefficients for microparticles of different sizes. For particles with an average size of 0.4 \(\mu\)m, the diffusion coefficients of dipyridamole into the release medium were 0.1\(\times\)10\(^{-11}\) cm\(^2\)/s \((D_1)\) and 0.08\(\times\)10\(^{-11}\) cm\(^2\)/s \((D_2)\) and for particles with an average size of 1.9 \(\mu\)m were 1.5\(\times\)10\(^{-11}\) cm\(^2\)/s \((D_1)\) and 2.0\(\times\)10\(^{-11}\) cm\(^2\)/s \((D_2)\).

Figure 9: LIVE/DEAD assay for films: (a) PHBV film; (b) OZ-F, 0.008% of OZ; (c) OZ-F, 0.016% of OZ; (d) OZ-F, 0.024% of OZ. Blue = live cells; green = dead cells.

Table 4: The cell viability (microparticles).

| Sample   | OZ concentration in PHA (wt.%)\(^a\) | OZ concentration in solution (mmol l\(^{-1}\)) | Cell viability (%) | The number of cells\(\times\)10\(^3\) (cm\(^2\)) |
|----------|--------------------------------------|-----------------------------------------------|--------------------|-----------------------------------------------|
| MP\(_{EM}\) |                                      |                                               |                    |                                               |
| MP\(_{SD}\) |                                      |                                               |                    |                                               |
| OZ suspension |                                    |                                               |                    |                                               |
| OZ-MP\(_{EM}\) | 9.7\%                               |                                               | 10.6 ± 1.4         | 3.8 ± 0.7                                     |
| OZ-MP\(_{SD}\) | 7.1\%                               |                                               | 11.8 ± 0.8         | 4.3 ± 0.2                                     |

\(^a\)Calculated concentration of OZ in particles (drug loading).
Figure 10: LIVE/DEAD assay: (a) MP$_{EM}$; (b) MP$_{SD}$; (c) OZ suspension; (d) OZ-MP$_{EM}$; (e) OZ-MP$_{SD}$. Blue = live cells; green = dead cells.
3.3. In Vitro Cytotoxicity. During the cultivation of HeLa in the presence of OZ-containing samples, it was noted that all depot systems inhibit cell growth and proliferation. The cytotoxicity directly depends on the concentration of OZ. Free OZ, suspended in water, showed pronounced cytotoxicity against HeLa cells.

The presence of OZ in PHA films significantly reduced cell viability (%). A decrease in the number of viable cells (Table 3) was observed in the case of the film with the highest content of OZ (70.5 μmol g⁻¹), when only 2% of viable cells were noted.

OZ-MPEM and OZ-MP SD are slightly less active against HeLa cells (Table 4) apparently due to the not as close sample-cell contact as in the case of depot films.

The results of the LIVE/DEAD assay for films are presented in Figure 9.

The results of the LIVE/DEAD assay for microparticles and free OZ are presented in Figure 10.

As expected, it was noted that with the increase of OZ concentration in the film obtained, the number of viable cells significantly decreases, while the active concentrations of OZ are in the range of extremely low values from 47 to 70.5 μmol g⁻¹. At a concentration of 23.5 μmol g⁻¹, the HeLa cell viability was approximately 20%, which also confirms the high activity of the studied ozonide. Thus, the characteristics of the obtained OZ depot systems provide a basis for scaling up the study of the cytotoxic effects of OZ when it is encapsulated in the PHA matrix with the further development of anticancer, antiparasitic, antiviral, and other depot forms.

4. Conclusion

For the first time, two types of depot systems containing new organic peroxide—casting films and microparticles—based on microbial PHAs were synthesized. The addition of 1,2,4-trioxolane insignificantly changes the main physical characteristics of the PHA matrix: the decrease of melting point and crystallization temperature was noted. Microparticles loaded with OZ were obtained using two methods of micronization of polymer solutions: emulsification and spray drying, with the inclusion of OZ up to 9.7 mass % and its complete sustained release in vitro during 312 h. Casting films containing 0.008%, 0.016%, and 0.024% of OZ caused a significant decrease in the number of living cells of the model cancer culture, which was the result of the high activity of the deposited compound. However, the limitations of the obtained systems are the difficulty in assessing the process of biodegradation of films and particles at this stage, as well as the relatively high sizes of microparticles. Reducing the particle size and increasing the zeta potential can potentially increase their aggregative stability in the studied media. Thus, depot systems containing 1,2,4-trioxolane based on biodegradable natural polymers have been developed.

**Data Availability**

All data included in this study are available upon request by contacting the corresponding author.

**Conflicts of Interest**

There are no conflicts to declare.

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