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Preparation and Characterization of Rifampicin-Loaded poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Microparticles

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Abstract. In this work, polyhydroxyalkanoates-based microparticles containing antibacterial drug rifampicin were obtained. The main characteristics of the microparticles were investigated: the particles yield, encapsulation efficiency and drug loading, average radius, surface morphology, polydispersity index, zeta potential and cytotoxicity towards HeLa cells. Loading of RIF into microparticles leads to the substantial decrease of its cytotoxicity.

Keywords: polyhydroxyalkanoates, microparticles, rifampicin.

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Получение и свойства микрочастиц поли(3-гидроксибутирата-со-3-гидроксивалерата), нагруженных рифампицином

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Аннотация. В настоящей работе описан процесс получения микрочастиц поли(3-гидроксибутирата-со-3-гидроксивалерата), содержащих антибактериальный препарат рифампицин. Определены основные параметры процесса получения и свойства микрочастиц: выход, эффективность инкапсулирования и включение препарата, средние размеры, морфология поверхности, индексы полидисперсности, дзета-потенциал, цитотоксичность в модельной культуре клеток HeLa. Установлено, что цитотоксичность полученной системы существенно ниже таковой для чистого РИФ.

Ключевые слова: полигидроксиалканоаты, микрочастицы, рифампицин.

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Introduction

Rifampicin (RIF) is a semi-synthetic antibiotic used as a first-line anti-tuberculosis drug. However, due to low cell permeability, degradation of the drug before reaching target cells and the emergence and rise of the resistance to RIF in many *M. tuberculosis* strains the efficacy of rifampicin treatment may significantly decrease [1]. In order to increase the efficiency of RIF therapy nano- and microparticulate drug delivery systems can be used owning to their ability to effectively combat bacterial pathogens [2, 3].

The use of nanoencapsulated RIF is a promising approach as RIF-loaded polymeric nanoparticles provide sustained drug release [4, 5], decrease its toxicity and dosage frequency [6], and exhibit increased macrophage uptake [7]. Many polymeric materials have been employed for the preparation of RIF-loaded nano- and micro-particles including poly(D, L-lactide-co-glycolide), chitosan, alginates, polycaprolactone [8], poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P3HBV) and other polyhydroxyalkanoates (PHAs) [9]. RIF-loaded P3HBV-based microparticles possess excellent properties including reduced cytotoxicity, sustained drug release and high encapsulation efficiency [10]. However, to the best of our knowledge, there are no comprehensive studies on the effect of various factors, in particular, the 3-hydroxyvalerate (3HV) content in the polymeric matrix on the efficiency of RIF loading and the properties of the resulting particles.

Materials and methods

Materials

Microbial P3HB (2000 kDa), $P3HBV_{15.7}$ (15.7 % of 3HV, 826 kDa) and $P3HBV_{82.0}$ (82.0 % of 3HV, 432 kDa) were produced at the laboratory of Biotechnology of new biomaterials of Siberian Federal University in Krasnoyarsk, RF [11]. Poly(vinyl alcohol) (PVA, 31–50 kDa), RIF and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA).

All chemicals were used as received without any further purification.

Preparation of RIF-loaded P3HB and P3HBV microparticles

RIF-loaded P3HBV microparticles with different 3HV and RIF content ($P3HBV_{15.7}$-RIF_{50}-MPs and $P3HBV_{15.7}$-RIF_{250}-MPs for matrix containing 15.7 % of 3HV and $P3HBV_{82.0}$-RIF_{50}-MPs and $P3HBV_{82.0}$-RIF_{250}-MPs for matrix containing 82.0 % of 3HV) and P3HB microparticles (P3HB-RIF_{50}-MPs and P3HB-RIF_{250}-MPs) were prepared using double-emulsion solvent evaporation technique. In brief, the solution of 0.4 g of P3HBV or P3HB in 40 ml of $CHCl_3$ was mixed with the solutions of different amounts of RIF in deionized water (50 mg of RIF or 250 mg of RIF) and sonicated for 5 min (Misonix Sonicator S 3000, USA). The resulting primary emulsion was added dropwise to the solution of 0.5 g of PVA in 100 ml of deionized H_2O and mechanically stirred at 24000 rpm for 5 min (Heidolph SilentCrusher M, Germany). Then, the resulting secondary emulsion was magnetically stirred at 1000 rpm for 24 h until the complete $CHCl_3$ evaporation. The obtained microparticles were collected by centrifugation at 8000 rpm for 5 min, washed multiple times with deionized water and freeze-dried.

Microparticles without RIF ($P3HBV_{15.7}$-MPs, $P3HBV_{82.0}$-MPs and P3HB-MPs) were prepared using the same technique.

Yield of microparticles

Yield of the obtained microparticles (Y) was determined according to the formula:

$$Y = \frac{M_{MPs}}{M_{polymer}} \cdot 100\%,$$

where and M_{MPs} is a mass of the obtained MPs and $M_{polymer}$ is a mass of polymer used.

Hydrodynamic particle size and zeta potential

Zetasizer Nano ZS (Malvern, UK) was used to determine the average hydrodynamic particle size, polydispersity index (PDI) and zeta potential. 5 mg of each sample was suspended in 2 ml of deionized water and sonicated at 30 W for 1 min before the measurements.

Morphological analysis

In order to assess shape and surface morphology of the obtained microparticles scanning electron microscopy (SEM) was carried out using SU 3500 (Hitachi, Japan). The samples were positioned on a specimen stub and sputter-coated with platinum using Leica EM ACE 200 (Leica Microsystems, Germany) to increase conductivity and promote heat dissipation from polymer matrix.

Determination of RIF encapsulation efficiency and drug loading

The amount of RIF loaded into polymeric matrix was determined spectrophotometrically using Genesys 10S UV–Vis (Thermo Scientific, USA) after dissolution of the obtained P3HBV$_{15.7}$-RIF$_{50}$-MPs, P3HBV$_{15.7}$-RIF$_{250}$-MPs, P3HBV$_{82.0}$-RIF$_{50}$-MPs, P3HBV$_{82.0}$-RIF$_{250}$-MPs, P3HB-RIF$_{50}$-MPs and P3HB-RIF$_{250}$-MPs in $CHCl_3$. Due to the shifts of RIF absorbance maxima in the $CHCl_3$ solutions containing P3HBV or P3HB standard curves for RIF determination were plotted on different wavelengths: 343.6 nm, 350.0 nm, 349.1 nm and 348.8 nm for RIF solutions in pure $CHCl_3$ and $CHCl_3$ containing P3HBV$_{15.7}$, P3HBV$_{82.0}$ and P3HB respectively.

The encapsulation efficiency (EE) was calculated according to the formula:

$$EE = \frac{M_1}{M_2} \cdot 100\%,$$

where M_1 is a mass of RIF in MPs and M_2 is an initial mass of RIF.

Drug loading (DL) was calculated according to the formula:

$$DL = \frac{M_1}{M_{MPs}} \cdot 100\%,$$

where M_1 is a mass of RIF in MPs and M_{MPs} is a mass of the obtained RIF-containing MPs.

In vitro cell viability assay

HeLa cells, as a model adhesive culture for the evaluation of cytotoxicity, were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) with the addition of 10 % fetal bovine serum (HyClone, USA) and standard antibiotic-antimycotic supplement (Sigma-Aldrich, USA) and planted at a density of 2×10^4 cells per 1 cm^2 in 96-well plates. After seeding and 72 h of incubation with RIF, blank P3HB-MPs or P3HB-RIF$_{250}$-MPs the cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorption of the resulting formazan dissolved in DMSO was recorded using iMark Microplate Reader (Bio-Rad Laboratories, USA) after 4 h of incubation with MTT at 37 °C (λ = 550 nm). Cell viability was calculated relative to untreated cells according to the formula:

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

where [A]test is the absorbance of the test sample and [A]control is the absorbance of the control sample.

To visualize cell viability, 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.

Results and discussion

In this work, both blank and rifampicin-loaded microparticles of poly-3-hydroxybutyrate and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 15.7 % and 82.0 % of 3-hydroxyvalerate were obtained.

The obtained microparticles have smooth spherical shape. The average hydrodynamic sizes of blank particles of P3HB-MPs, P3HBV$_{15.7}$-MPs and P3HBV$_{82.0}$-MPs are 384±16 nm, 510±15 nm and 1272±32 nm, respectively. The average hydrodynamic sizes of RIF-loaded particles are 427±23 nm, 389±9 nm, 1022±22 nm, 1008±11 nm and 668±8 nm for P3HB-RIF$_{50}$-MPs, P3HB-RIF$_{250}$-MPs, P3HBV$_{15.7}$-RIF$_{50}$-MPs, P3HBV$_{82.0}$-RIF$_{50}$-MPs and P3HBV$_{82.0}$-RIF$_{250}$-MPs, respectively. Due to the pronounced aggregation process correct determination of the hydrodynamic radius, polydispersity index and value of the zeta potential of P3HBV$_{15.7}$-RIF$_{250}$-MPs could not be carried out.

The loading of RIF into the PHA matrix has an impact on the particle size in different ways which is consistent to the results of the researches of *Durán et. al.* [10] and *Labuschagne et. al.* [12]. The authors of the article [10] noted the increasing of the P3HBV$_{9.8}$-MPs size with increasing the amount of the loaded RIF. The opposite dependence was shown in the article [12]. PLGA microparticles' size decreases with the increase of the amount of RIF. According to the data presented the change of the particles' size depends on the polymer matrix composition.

The obtained microparticles possessing such average sizes are suitable for inhalable administration of RIF for tuberculosis treatment. It's shown that particles within size range from 0,5 to 10 μm possess the most appropriate accumulation in alveoli [13].

It was determined that the value of the zeta potentials of all the obtained microparticles are in the range from –25.0±0.3 mV to –13.4±0.3 mV. It was also noted that the encapsulation of RIF reduces the modulus of the zeta potential that leads to the decrease of the hydrolytic stability of the obtained MPs. A decrease in the zeta potential of drug-loaded microparticles compared to blank microparticles is also noted when other drugs are encapsulated in PHAs matrix. *Vilos et. al.* reported that ζ-potential of P3HBV$_{12}$-MPs decreases from –8,9±2,7 mV to –6,0±1,4 mV after encapsulation of 5 μmol of Paclitaxel [14]. In the articles [15] and [16] the encapsulation of Cl-containing ozonide and ellipticine into the P3HB and P3HBV$_{15}$ matrices, respectively, also decreases the value of the zeta potential.

According to SEM analysis results microparticles from all the obtained samples have spherical shape (Fig. 1), smooth surface and uniform size distribution. Meanwhile, P3HB-RIF$_{250}$-MPs have the lowest average size among all the samples containing RIF.

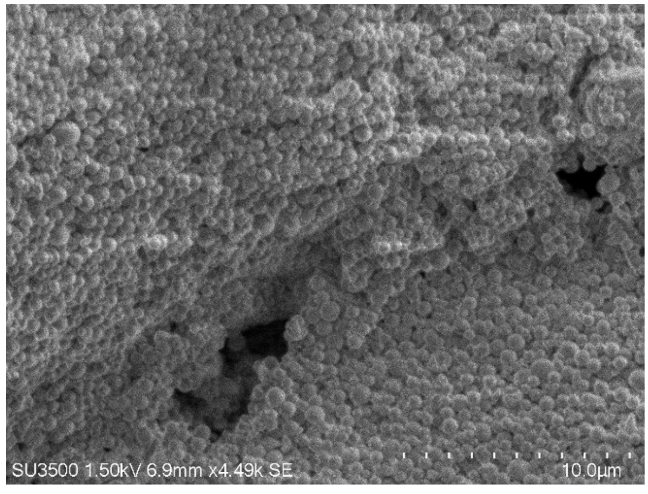

Fig. 1. SEM image of P3HB-RIF$_{250}$-MPs

Encapsulation efficiency of RIF into MPs is in range of 0.8±0.1 % to 16.4±0.3 % and the drug loading value is in range of 0.3±0.1 % to 10.4±0.4 %. With an increase in the RIF content in the primary emulsion, the drug loading increases in all the studied compositions. This is consistent with the results of *Petkar et. al.* [8], who encapsulated RIF into chitosan matrix (DL in range of 1.64±0.16 % to 13.51±0,81 %).

The characteristics of the obtained microparticles are presented in the Table 1.

The viability of HeLa cells (human cervical cancer) decreases in a dose-dependent fashion with the increase of concentration of both pure RIF and RIF-containing P3HB microparticles (Fig. 2, 3). The loading of RIF into microparticles leads to the substantial decrease of cytotoxicity of the obtained formulations towards the model cell culture used, that is possibly due to RIF sustained release from the polymeric matrix. Blank P3HB microparticles don't affect cell viability significantly.

Table 1. Characteristics of the obtained microparticles

Sample	Y, %	Radius, nm	ζ, mV	EE, %	DL, %	PDI
P3HB-MPs	92.9±0.3	384±16	-22.6±0.2	_	_	0.269
P3HB-RIF$_{50}$-MPs	96.0±0.7	427±23	-20.8±0.3	14.8±0.3	2.0±0.2	0.148
P3HB-RIF$_{250}$-MPs	85.1±0.4	389±9	-20.6±0.1	14.2±0.4	10.4±0.7	0.198
P3HBV$_{15.7}$-MPs	91.9±0.1	510±15	-19.7±0.2	_	_	0.153
P3HBV$_{15.7}$-RIF$_{50}$-MPs	92.1±0.3	1022±22	-13.4±0.3	16.4±0.3	2.2±0.3	0.130
P3HBV$_{15.7}$-RIF$_{250}$-MPs	97.5±0.4	_	_	6.0±0.2	4.3±0.4	_
P3HBV$_{82.0}$-MPs	94.7±0.4	1272±32	-25.0±0.3	_	_	0.174
P3HBV$_{82.0}$-RIF$_{50}$-MPs	92.8±0.5	1008±11	-24.8±0.2	1.4±0.2	0.3±0.1	0.082
P3HBV$_{82.0}$-RIF$_{250}$-MPs	91.5±0.3	668±8	-22.2±0.4	0.8±0.1	0.8±0.1	0.282

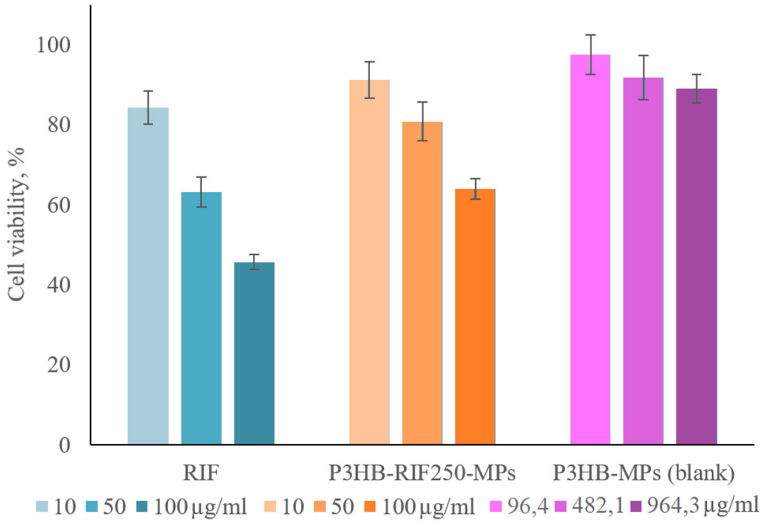

Fig. 2. Cytotoxicity of RIF, P3HB-RIF$_{250}$-MPs and P3HB-MPs towards HeLa cells in different concentrations, 72 hours of incubation, MTT assay

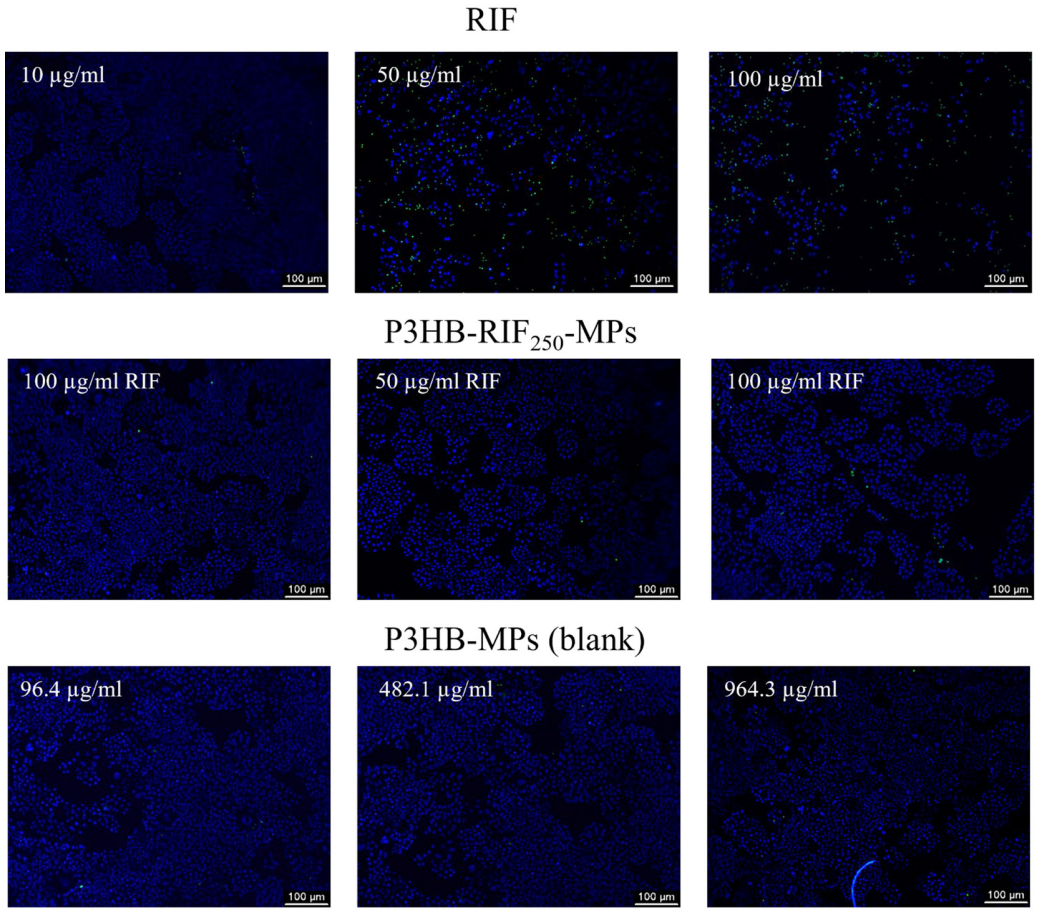

Fig. 3. Fluorescence images of HeLa cells incubated with RIF, P3HB-RIF_{250}-MPs and P3HB-MPs. Live cells are blue, dead cells are green

Conclusion

RIF-loaded PHAs-based microparticles were obtained and analyzed. The effect of 3HV content on RIF encapsulation in PHAs microparticles was evaluated. It's found that the increase in 3HV percentage in the matrix leads to the decrease of RIF loading and encapsulation efficiency. The MPs of $P3HBV_{82.0}$ possess larger average sizes and lower melting temperatures. It was also noted that the addition of RIF into the initial emulsion leads to a decrease in zeta-potentials of the resulting microparticles. The loading of RIF into P3HB microparticles leads to the substantial decrease of the obtained formulations cytotoxicity (compared to pure RIF) towards HeLa cells.

References

[1] Ishikawa A.A., Salazar J.V., Salinas M., Gaitani C.M., Nurkiewicz T., Negrete R.G., Garcia C.D. Self-Assembled Nanospheres for Encapsulation and Aerosolization of Rifampicin. *RSC Advances 2016.* 6(16), 12959–12963. https://doi.org/10.1039/C 5RA25044G

[2] Trousil J. et al. System with embedded drug release and nanoparticle degradation sensor showing efficient rifampicin delivery into macrophages. *Nanomedicine: Nanotechnology, Biology and Medicine 2017.* 13(1), 307–315. https://doi.org/10.1016/j.nano.2016.08.031

[3] Labuschagne P.W., Adami R., Liparoti S., Naidoo S., Swai H., Reverchom E. Preparation of rifampicin/poly(d, l-lactide) nanoparticles for sustained release by supercritical assisted atomization technique. *The journal of Supercritical Fluids 2014.* 95, 106–117. https://doi.org/10.1016/j.supflu.2014.08.004

[4] Kr**ö**ger A.P., Hamelmann N.M., Juan A., Lindhoud S., Paulusse J.M. Biocompatible single-chain polymer nanoparticles for drug delivery – A dual approach. *ACS Applied Material Interfaces 2018.* 10(37), 30946–30951. https://doi.org/10.1021/acsami.8b07450

[5] Thomas D., Latha M.S., Thomas K.K. Synthesis and in vitro evaluation of alginate-cellulose nanocrystal hybrid nanoparticles for the controlled oral delivery of rifampicin. *Journal of Drug Delivery Science and Technology 2018.* 46, 392–399. https://doi.org/10.1016/j.jddst.2018.06.004

[6] Rawal T., Parmar R., Tyagi R.K., Butani S. Rifampicin loaded chitosan nanoparticle dry powder presents an improved therapeutic approach for alveolar tuberculosis. *Colloids and Surfaces. B, Biointerfaces 2017.* 1(154), 321–330. https://doi.org/10.1016/j.colsurfb.2017.03.044

[7] Basha R.Y., Kumar S., Doble M. Dual delivery of tuberculosis drugs via cyclodextrin conjugated curdlan nanoparticles to infected macrophages. *Carbohydrate Polymers 2019.* 15(218), 53–62. https://doi.org/10.1016/j.carbpol.2019.04.056

[8] Petkar K.C. et al. Development of Novel Octanoyl Chitosan Nanoparticles for Improved Rifampicin Pulmonary Delivery: Optimization by Factorial Design. *AAPS PharmSciTech 2018.* 19(4), 1758–1772. https://doi.org/10.1208/s12249–018–0972–9

[9] Farrag Y., Montero B., Rico M., Barral L., Bouza R. Preparation and characterization of nano and micro particles of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) via emulsification/solvent evaporation and nanoprecipitation techniques. *Journal of Nanoparticle Research 2018.* 20. https://doi.org/10.1007/s11051–018–4177–7

[10] Duran N., Alvarenga M.A., Da Silva E.C., Melo P.S., Marcato P.D. Microencapsulation of antibiotic rifampicin in poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Archives of pharmacal research 2008.* 31(11), 1509–1516. https://doi.org/10.1007/s12272–001–2137–7

[11] Патент № 2439143 Российская Федерация, МПК C 12N 1/20, C 12P7/62. Штамм бактерий Cupriavidus eutrophus ВКПМ B-10646 – продуцент полигидроксиалканоатов и способ их получения: № 2439143: заявл. 15.11.2010: опубл. 10.01.2012 / Волова Т.Г., Шишацкая Е.И. 13. [RU Patent 2439143 Bacterial strain Cupriavidus eutrophus B-10646 – producer of polyhydroxyalkanoates and method for their production. Published 10.01.2012. Volova T.G., Shishatskaya E.I. 13. (In Russ.)]

[12] Pandey R., Sharma A., Zahoor A., Sharma S., Khuller G.K., Prasad B. Poly(DL-lactide-co-glycolide) nanoparticles-based inhalable sustained drug delivery system for experimental tuberculosis. *The Journal of antimicrobial chemotherapy 2003.* 52(6), 981–986. https://doi.org/10.1093/jac/dkg477

[13] Qiao H. et al. The transport and deposition of nanoparticles in respiratory system by inhalation. *Journal of Nanomaterials 2015.* 2015. https://doi.org/10.1155/2015/394507

[14] Vilos C. et al. Paclitaxel-PHBV nanoparticles and their toxicity to endometrial and primary ovarian cancer cells. *Biomaterials 2013.* 34(16), 4098–4108. https://doi.org/10.1016/j.biomaterials.2013.02.034

[15] Lipaikin S.Y., Yaremenko I.A., Terent'ev A.O., Volova T.G., Shishatskaya E.I. Development of Biodegradable Delivery Systems Containing Novel 1,2,4-Trioxolane Based on Bacterial

Polyhydroxyalkanoates. *Advances in Polymer Technology 2022.* 2022. https://doi.org/10.1155/2022/6353909

[16]Masood F., Chen P., Yasin T., Fatima N., Hasan F., Hameed A. Encapsulation of Ellipticine in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) based nanoparticles and its in vitro application. *Materials science and engineering. C, Materials for biological applications 2012.* 33(3), 1054–1060. https://doi.org/10.1016/j.msec.2012.11.025.