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Ceftiofur-loaded PHBV microparticles: A potential formulation for a long-acting antibiotic to treat animal infections

Cristian Vilos[1,2,3] · Luis Constandil[1,2,3] · Natalia Herrera[1] · Paula Solar[1] · Jorge Escobar-Fica[4] · Luis Alberto Velásquez[1,3] ✉

1 Universidad Andrés Bello, Facultad de Medicina, Center for Integrative Medicine and Innovative Science, Santiago, Chile

2 Universidad de Santiago de Chile, Facultad de Química y Biología, Santiago, Chile

3 Centro para el Desarrollo de la Nanociencia y Nanotecnologia, Santiago, Chile

4 Pontificia Universidad Católica de Valparaíso, Facultad de Ciencias, Instituto de Química, Laboratorio de Química Biológica, Curauma, Valparaíso

✉ Corresponding author: luis.velasquez@unab.cl
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Abstract

Background: The infectious diseases in the livestock breeding industry represent a significant drawback that generates substantial economic loss and have led to the indiscriminate use of antibiotics. The formulation of polymeric microparticles loaded with antibiotics for veterinary use can: reduce the number of required doses; protect the drug from inactivation; and maintain a sustained-release of the antibiotic drug at effective levels. Accomplishing all of these goals would have a significant economic and animal health impact on the livestock breeding industry. **Results:** In this work, we formulated ceftiofur-loaded PHBV microparticles (PHBV-CEF) with a spherical shape, a smooth surface and diameter sizes between 1.65 and 2.37 µm. The encapsulation efficiency was 39.5 ± 1.1% w/w, and we obtained a sustained release of ceftiofur in PBS-buffer (pH 7.4) over 7 days. The antibacterial activity of ceftiofur was preserved after the encapsulation procedure, and toxicity of PHBV-CEF microparticles evaluated by MTS was represented by an IC50 > 10 mg/mL. **Conclusions:** Our results suggest that PHBV-CEF particles have a potential application for improving the treatment of infectious diseases in the livestock breeding industry.

Keywords: ceftiofur, drug delivery, livestock breeding industry, PHBV, polymeric microparticles

INTRODUCTION

Antibiotics are therapeutic agents produced by bacteria or fungi that destroy or inhibit the growth of susceptible organisms. Antibiotics have been used to treat a variety of infectious diseases, both in humans and animals (Barton, 2000; Demain and Sanchez, 2009). In animals, the indiscriminate use of antibiotics in the livestock breeding industry is a global problem due to their impact on the environment, human health and the generation of antibiotic resistance (Wallmann et al. 2003; Knezevic and Petrovic, 2008). Moreover, issues associated with the current antibiotic administration paradigm include the necessity of a large staff specialized in evaluating and dosing many animals; the requirement of repeated drug administration to maintain plasma concentrations at an effective level; and in some cases, the use of high doses to reach therapeutic concentrations at the site of action. The latter two requirements can lead to toxic drug levels in the plasma as well as to adverse reactions (Ahmed and Kasraian, 2002; Sun et al. 2004). In order to promote the productive development of the livestock

breeding industry, and to reduce the negative impact of the over-use of antibiotics it is necessary to develop novel drug delivery systems (DDS). The main role of those DDS is provide support to reduce the number of doses, improve the effectiveness of treatments, and maintain the plasma concentrations of antibiotics within a safe therapeutic-window for extended-time periods.

Biodegradable and biocompatible polymers such as poly (lactic-co-glycolic acid) (PLGA), poly (ε-caprolactone), and poly (sebacic acid), provide a multifunctional platform for the sustained release of drugs based on microencapsulation technology (Edlund and Albertsson, 2002; Vilos and Velasquez, 2012). Although a large number of human therapeutic agents have been encapsulated into polymeric microparticles (p-MPs), the development of microparticles for veterinary applications has been reported only in a limited number of studies (Atkins et al. 1998; Blanco-Príeto et al. 2002).

Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a natural biodegradable and biocompatible polymer obtained from large-scale bacterial systems (Mitomo et al. 1999; Park et al. 2001; Loo and Sudesh, 2007), and has showed promising biomedical applications in tissue engineering and drug delivery (Li and Chang, 2005; Huang et al. 2010). Ceftiofur is a broad-spectrum cephalosporin (third-generation) used to treat a variety of infections in animals such as bovine and porcine respiratory diseases associated with *Pasteurella haemolytica, Pasteurella multocida, Haemophilus somnus, Actinobacillus pleuroneumonia, Salmonella choleraesuis* and *Streptococcus suis* (Brown et al. 1999; Hibbard et al. 2002; Tang et al. 2010). The mechanism of action of ceftiofur is through inhibiting the synthesis of the bacterial cell wall, and its site of action is the penicillin-binding protein (PBP), which is involved in cell wall synthesis, and is located on the inside of the bacterial cell membrane. In actively growing bacteria, the ceftiofur binds to the PBP in the cell wall and leads to the interference of the production of the cell wall peptidoglycans. This binding leads to the subsequent bacterial cell lysis in an isotonic environment due to the inadequately constructed bacterial cell wall (Hornish and Kotarski, 2002).

The low commercial cost of PHBV, and the need for novel drug delivery systems for livestock breeding industry exhibits a strong potential to generate a long-acting veterinary antibiotic formulation based on pMPs. In this work, we synthesized and characterized ceftiofur-loaded PHBV microparticles, evaluated their antimicrobial activity and cytotoxicity in cells cultures as a potential system for sustained release of antibiotic to treat animal infections.

MATERIALS AND METHODS

Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), with 12% PHV by weight and polyvinyl alcohol (PVA) with an average mol. wt. of 30.000-70.000 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (DCM), chloroform and methanol were purchased from Merck (Germany). Ceftiofur hydrochloride was kindly provided by Centrovet (Santiago, Chile).

Formulation of ceftiofur-loaded PHBV microparticles

Ceftiofur-loaded PHBV microparticles (PHBV-CEF) were formulated by a double emulsion ($w_1/o_1/w_2$) solvent evaporation method. Briefly, 15 mg of ceftiofur was dissolved in 450 µL of liquid containing a 2:1 mixture of water: methanol. This solution was added to one mL of PHBV (25 mg/mL) dissolved in dichloromethane. The first emulsion (w_1/o_1) was prepared by homogenization using a tissue-tearor at 35.000 rpm for 40 sec, Biospec Products (Bartlesville, OK, USA). After this first homogenization, the water-in-oil emulsion was further emulsified under the same conditions in 4 mL of an aqueous solution of polyvinyl alcohol (PVA) (0.5% w/v) (w_2). This $w_1/o_1/w_2$ emulsion was immediately poured into a beaker containing 25 ml of PVA (0.1% w/v) solution and was stirred in an orbital shaker at 120 rpm for 12 hrs in a hood to evaporate the solvent. The solidified ceftiofur-loaded PHBV microparticles were washed three times with distilled water and harvest by centrifugation at 5,000 rpm for 10 min. The microparticles were either stored at 4ºC for immediate use, or lyophilized for storage at -80ºC for later use.

Particle size and zeta potential

Dynamic light scattering (DLS) was used to characterize particle size (diameter, µm), particle size distribution and zeta potential. Each preparation was suspended in 1 ml of distilled water or phosphate buffered saline (PBS), pH 7.4. The particle size was obtained from three independent measurements on a Zetasizer Nano S90 (Malvern Instruments, UK), and the zeta potential (mV) was determined from three repeated experiments using a Zeta Pals instrument (Brookhaven, USA) at 25ºC.

Scanning electron microscopy (SEM)

The morphology of the PHBV-CEF microparticles was analyzed by scanning electron microscopy (SEM) DSM 960 (Carl Zeiss, Germany). The samples were lyophilized and deposited onto a 300-mesh, carbon-coated copper grid that had been previously hydrophilized under UV light (Electron Microscopy Sciences, Hatfield, PA) and coated with gold prior to sample examination.

The drug loading and encapsulation efficiency

The experimental loading and efficiency of ceftiofur encapsulation was analyzed by an extraction method described by Coimbra et al. (2008). Experimentally, 10 mg of MPs-CEF was dissolved in 1 mL of chloroform, followed by the addition of 9 mL of methanol to precipitate the polymer. The resulting suspension was centrifuged, and the supernatant was filtered and analyzed by ultra-performance liquid chromatography (UPLC) (Coimbra et al. 2008). Triplicate measures were performed for each batch. The experimental drug loading was calculated using the formulas as follow:

Experimental drug loading = (weight of detected drug) / (weight of drug loaded microparticles).

Theoretical drug loading = (weight of added drug) / (weight of added drug + weight of added polymer).

The encapsulation efficiency (EE%) was calculated as the ratio between the experimental drug loading and the theoretical drug loading.

Ultra-performance liquid chromatography (UPLC)

UPLC was performed using an Acquity system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an auto sampler and a tunable UV detector. The chromatographic separation was performed using a Waters Acquity BEH 50 x 2.1 mm, 1.7 µm C18 column. The mobile phase was a 78:22 (v/v) mixture of 20 mM disodium hydrogen phosphate dihydrate buffer (pH 6.0, pH adjusted by the addition of 85% ortho-phosphoric acid) and acetonitrile loaded at a flow rate of 0.6 mL/min. Peak detection was carried out at a wavelength of 292 nm. The injection volume was 0.5 µL, and the mobile phase was used as a diluent, while the column was maintained at 27ºC. An amount of ceftiofur reference standard equivalent to 25 mg of ceftiofur was accurately weighed and transferred to a 25-mL volumetric flask. The mobile phase was added to give a final concentration of 1000 µg/mL. Chromatographic standard solutions (0.25, 50, 100 and 150 µg/mL) were prepared fresh every day in a volumetric flask, along with the mobile phase.

***In vitro* release assay**

The *in vitro* release of ceftiofur was performed by a dialysis system. Experimentally, 20 mg of PHBV-CEF and ceftiofur non-encapsulated were incorporated into dialysis membrane bags with a molecular weight cut off 14.000 g/mol. The bags were tied and dropped into 50 ml of PBS-buffer, pH 7.4. After, the preparations were placed in an orbital shaker (120 rpm) and maintained at 25ºC. At predetermined time intervals, 2 mL of medium was taken for analysis and fresh medium of an equal volume was replaced. The *in vitro* release of ceftiofur was evaluated in triplicate, and the concentration of ceftiofur in the medium was determined by UPLC.

Antibacterial activity

Antimicrobial activity was measured by bacterial growth of *Escherichia coli* (ATCC 25922) on a microplate during a 24 hrs time interval. All tests were conducted in triplicate and according to the National Committee for Clinical Laboratory Standards (NCCLS). The bacterial inoculum was prepared from a Mueller-Hinton plate that had been streaked with a single colony from an initial subculture plate, and incubated for 18 to 24 hrs. The culture growth was carried out in 96 well ELISA plates after the inoculum was adjusted to 10^5 UFC/mL in Mueller-Hinton liquid medium in a total volume of 300 µL per well. The cultures were treated with 1000, 100, 10, 1, 0.1, and 0.01 µg/mL of PHBV-CEF in a total volume of 100 µL of Mueller-Hinton liquid medium. In addition, as control we used PHBV-empty microparticles and ceftiofur non-encapsulated at 1000, 100, 10, 1, 0.1 and 0.01 µg/mL in Mueller-Hinton liquid medium. The plates were incubated at 37ºC, and during 24 hrs at 1 hr intervals, the optical density was determined at 400 nm using a Labsystem Multiskan MS Type 352 (Helsinki, Finland). These experiments were performed in triplicate from three independent formulations. The optical density results were expressed as bacterial growth curves (Log CFU/mL versus time).

Hep-G2 cell cultures

Hep-G2 cells (ATCC No. HB-8065) were obtained from the American Type Culture Collection (ATCC). The cells cultures were maintained in DMEM supplemented with 10% FBS (v/v), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37ºC under a humidified atmosphere of 5% CO_2. The cells were passaged once every 2-3 days.

Cytotoxicity assays for microparticles

Cell viability was examined by the MTS CellTiter 96 AQ Non-Radioactive Cell Proliferation assay (Promega, Madison, USA), following the manufacturer's protocol. Experimentally, Hep-G2 cells were seeded into 96-well plates (75,000 cells per well), grown to 80% confluence, and treated with different concentrations (1, 10, 100, 1000, or 10000 µg/mL) of PHBV-empty microparticles, PHBV-CEF, or with vehicle (PBS-buffer, pH 7.4) alone as a negative control for 24 hrs at 37ºC under a humidified atmosphere of 5% CO_2. After this treatment, the cells were carefully washed with Hank's Medium and treated according to the MTS manufacturer's protocol. The light absorbance of the solution in the wells was determined at 450 nm using a 96-well plate reader (ThermoScientific, ON, Canada). The cell viability (%) relative to the control wells containing cell culture medium without microparticles or PBS as a vehicle was calculated by (OD-test/OD-control x 100), where OD-test is the light absorbance (450 nm) of the test sample and OD-control is the light absorbance (450 nm) of the control sample. Each sample was measured in triplicate from three independent experiments.

Statistical analysis

The results are presented as means ± SE. Overall statistical analysis was performed by the Kruskal-Wallis test, followed by a Mann-Whitney test for pair-wise comparisons when overall significance ($p < 0.05$) was detected. The Two-way ANOVA was used to analyze the data from the groups in the cell viability assay.

RESULTS AND DISCUSSION

The double emulsion solvent evaporation method described by Ogawa et al. (1988), is a highly reproducible method that provide support to create particles with a uniform size and inner core-shell structure (Ogawa et al. 1988). Figure 1 shows a scheme of synthesis of PHBV microparticles loaded with ceftiofur (PHBV-CEF). The size (diameter, µm) and particle size distribution measure by DLS was 1.89 ± 0.24 µm to PHBV-empty and 2.2 ± 0.17 µm to PHBV-CEF. The size of particles of PHBV-empty and PHBV-CEF was not statistically significant ($p > 0.05$) and revealed that the incorporation of the drug did not affect the size, as is shown in Figure 2. The particle size obtained from our formulations was smaller and with less dispersion of size than previous reports of PHBV microparticles loaded with others antibiotics such as rifampicin, gentamicin and tetracycline (Sendil et al. 1999; Li and Chang, 2005; Duran et al. 2008).

Studies about the inner distribution of hydrophilic and hydrophobic dyes-loaded polymeric microparticles synthesized by double emulsion method, and characterized by confocal microscopy have showed a localization of hydrophilic dye in the core of particles and the hydrophobic dyes in the shell structure of microparticles (Lamprecht et al. 2000; Mao et al. 2007). These antecedents together with the physicochemical structure of ceftiofur suggest that distribution of ceftiofur inside of PHBV microparticles is uniform in both, core and shell structure.

The zeta potential of microparticles was calculated for preparations in distilled water and phosphate buffered saline (PBS, pH 7.4). The results obtained showed that PHBV-empty microparticles and PHBV-CEF microparticles exhibited a negative zeta potential in water and PBS, as illustrated in Figure 3. The microparticles suspended in PBS (pH 7.4) showed a less negative zeta potential than in water, which could be explained by the adsorption of ions to the surface of the microparticles when they were suspended in PBS due to carboxyl groups of PVA and PHBV presents in their structure. It has been described that micro and nanoparticles with negative zeta potential are more advantageous as drug delivery systems because prolong the circulation of the particulate into the bloodstream and decrease their less RES uptake by the reticuloendothelial system (RES) (Sustronck et al. 1995).

The SEM micrographs of microparticles showed spherical shaped particles with a smooth surface (Figure 4a). The morphology of the particles suspended in phosphate buffered saline (PBS, pH 7.4) was analyzed after 7 days by SEM and did not show apparent differences compared to the fresh microparticles. After 30 days, the particles were examined again and structures like flowers with highly rough surfaces and small particles with a broad size distribution were found agglomerated, as illustrated in Figure 4b.

The polymer-drug interactions are highly specific generating a diverse morphology of microparticles. In previous reports, flurbiprofen, metformin and diazepam loaded PHBV microparticles exhibited a surface thoroughly rough and with large porous (Chen and Davis, 2002; Coimbra et al. 2008; Farago et al. 2008). In contrast, Wang et al. (2007) showed a formulation of PHBV microparticles loaded with ibuprofen that presented a smooth surface without pores.

The experimental ceftiofur loading of PHBV-CEF microparticles was achieved as triplicate measurements from three different batches were analyzed and parameters were calculated as described in the Materials and Methods section. The experimental drug loading of PHBV-CEF microparticles was 6.6 ± 0.2%, w/w. The theoretical drug loading was kept constant (37.5%), and the encapsulation efficiency (EE%) was 39.5 ± 1.1%, w/w.

The *in vitro* release of ceftiofur from PHBV-CEF microparticles and ceftiofur non-encapsulated was measured during 7 days in phosphate buffered saline (PBS) at pH 7.4 and 25ºC. The samples were incorporated into dialysis bags and maintained in an orbital shaker during the 7 days. At predetermined time intervals, aliquots were taken for analysis and medium that was removed was replaced with fresh medium equal to the volume taken. The PHBV-CEF released was compared with the measurement of ceftiofur non-encapsulated directly added to the dialysis bags. The cumulative release of ceftiofur is illustrated in Figure 5. The results showed that 100% of ceftiofur non-encapsulated was delivered at 12 hrs, while almost 60% of PHBV-CEF was delivered during the first 2 days.

The degradation mechanism of microparticles synthesized from biodegradable poly (alpha-hydroxy esters) has been well established, and the bulk-degrading is the most common mechanism that occurs in PLGA, PLC and other polyesters (Göpferich, 1996; von Burkersroda et al. 2002). Bulk-degrading is a homogeneous process that occurs throughout the polymer matrix and include four stages: (i) the polymer absorbs water and undergoing soft swelling, which is followed by the penetration of water in the amorphous region stabilized by van der Waal's forces and hydrogen bonds; (ii) begin the cleavage of the covalent/ester bonds in the polymer backbone (by hydrolysis), and are generated carboxylic end groups which may auto-catalyze the hydrolysis; (iii) in this stage, exist a massive cleavage of the backbone covalent bonds, which generates a decreased of molecular weight with a significant mass loss, and loss of physical and mechanical integrity; (iv) finally, the polymer loses substantial mass due to solubilisation of their oligomers into the surrounding medium and breaks down to many small fragments, which will be further hydrolyzed into free acids (Lao et al. 2011).

The PHBV is a copolymer formed by ester bounds, and the profile release of ceftiofur from PHBV-CEF microparticles fits with the mathematical models of release described by Batycky et al. (1997) and

Siepmann et al. (2002) from PLGA microparticles. The chemical nature of PHBV and the profile release of ceftiofur from PHBV-CEF suggest that as well PLGA, the mechanism of degradation of PHBV microparticles correspond to bulk-degrading (Lao et al. 2011).

The antimicrobial activity of microparticles was analyzed by the bacterial growth in microplate cultures of *Escherichia coli* (ATCC 25922). Our results are shown in Figure 6 and illustrate that the minimum inhibitory concentration (MIC) of ceftiofur free was 1 µg/mL. The cultures treated with PHBV-CEF at the same concentration (1 µg/mL) showed a slight bacterial inhibition during the first 5 hrs and a delay in the onset of the bacterial exponential growth curve. The MIC of PHBV-CEF was determined to be at 10 µg/mL. This result is well correlated with the experimental loading obtained (~7%), and the *in vitro* release assay. The antimicrobial activity of PHBV-CEF confirms that ceftiofur maintains antibacterial activity after the microencapsulation procedure.

In the preclinical development of a new pharmaceutical formulation, the toxicological characterization is a critical point. We examined the viability of Hep-G2 cultures treated with different amount of ceftiofur non-encapsulated, PHBV-empty and PHBV-CEF microparticles using the MTS Cell Proliferation assay, as illustrated in Figure 7. The results were analyzed by a two-way ANOVA test, showed that PHBV-empty, PHB-CEF microparticles and ceftiofur non-encapsulated did not affect the viability of these cells at the doses used. In addition, the cells were challenged up to 10 mg/mL of PHBV-empty and PHBV-CEF microparticles, and do not reduced the viability of cells (data not show), thus confirmed that the toxicity microparticles is very low and the IC50 > 10 mg/mL.

CONCLUDING REMARKS

The aim of this work was the development of a drug delivery system to treat infectious diseases observed in the livestock breeding industry based on the antibiotic ceftiofur, and the biodegradable and biocompatible poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). This polymer has a low commercial price that makes it a good candidate for large-scale applications. In addition, PHBV has shown suitable properties for the production of micro and nanoparticles loaded with drugs. Our PHBV-CEF microparticles had a diameter of 2.2 ± 0.17 µm. The SEM studies showed freshly made particles with a spherical shape and smooth surface. The experimental drug loading obtained was 6.6 ± 0.2% w/w, and the encapsulation efficiency reached 39.5 ± 1.1% w/w. The *in vitro* release experiment showed a sustained release profile of ceftiofur during 7 days, and the antimicrobial test demonstrated that ceftiofur kept its antibiotic activity after the encapsulation process. Finally, the cell proliferation assay showed a very low cytotoxicity of PHBV-CEF on Hep-G2 cells with an IC_{50} > 10 mg/mL. The results obtained support PHBV-CEF particles as a suitable sustained release system of ceftiofur, and provide evidence to continue the research of its pharmacokinetic and toxicological assessment in animal models. The PHBV-CEF particles have a potential application for improving the treatment of infectious diseases in livestock compared to current practices in the breeding industry.

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FIGURES

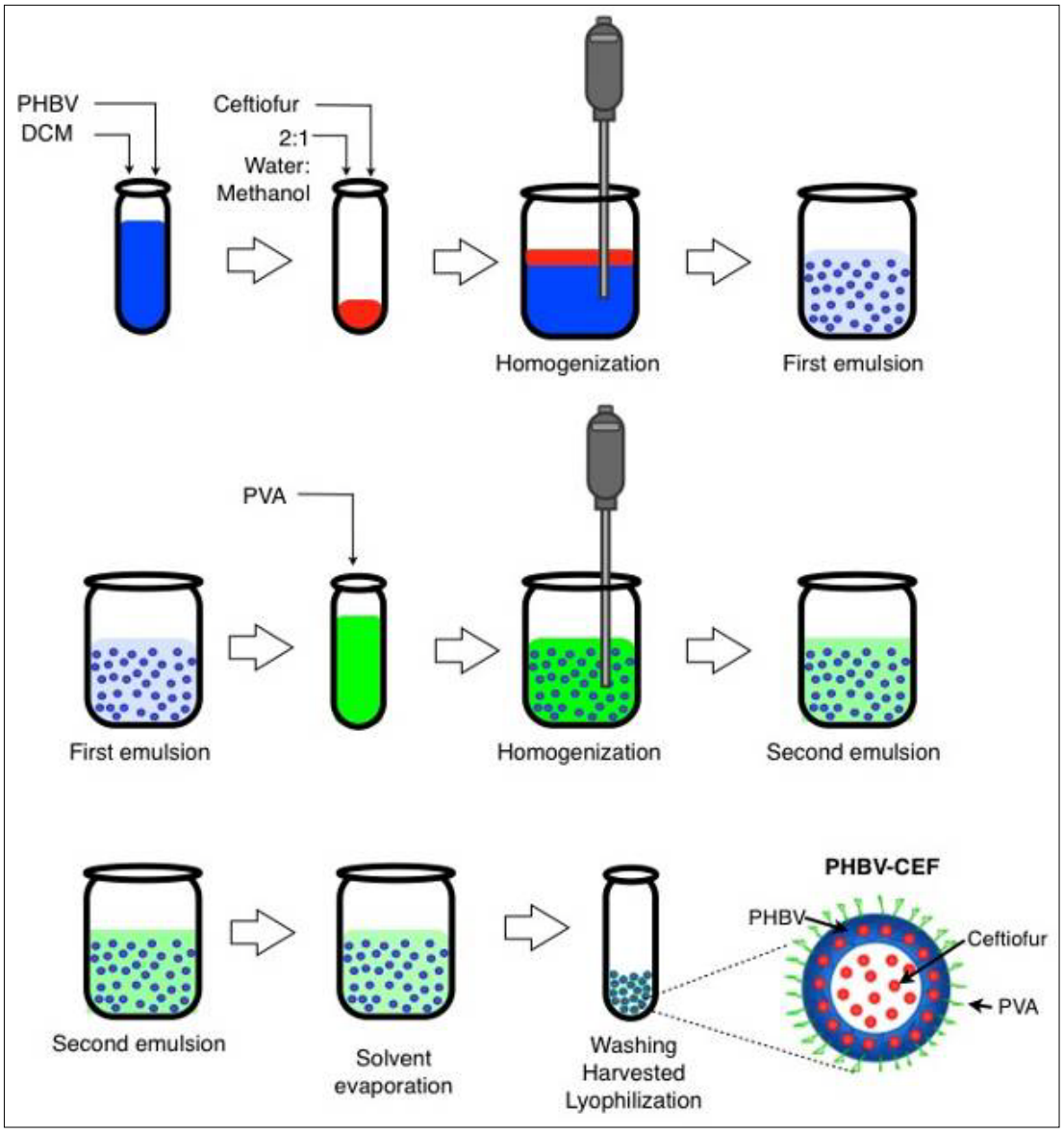

Fig. 1 Schematic representation of the double emulsion evaporation method ($w_1/o_1/w_2$) used to synthesize microparticles of PHBV loaded with ceftiofur (PHBV-CEF). A first water-in-oil emulsion (w_1/o_1) was prepared by the addition of ceftiofur (dissolved in water:methanol 2:1) in a larger volume of PHBV polymer (dissolved in dichloromethane). Both phases were homogenized to obtain the first emulsion. This emulsion was added to a larger volume of an aqueous solution containing the stabilizer agent (PVA) to form the second emulsion ($w_1/o_1/w_2$) by homogenization. The particles formed were arranged in a shaker system to evaporate the solvent, then washed, harvested by centrifugation and lyophilized.

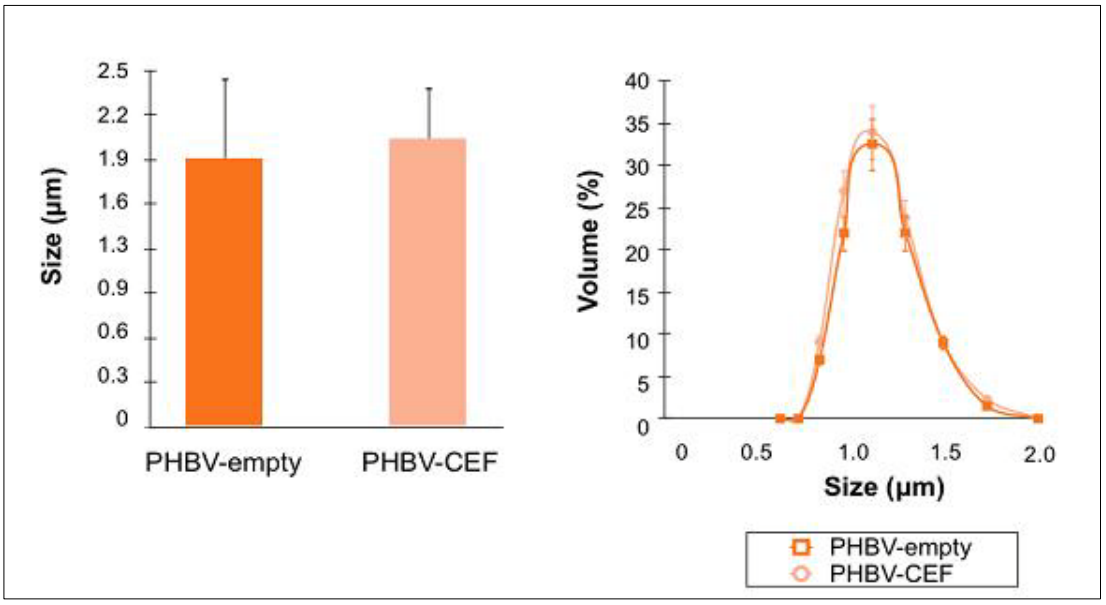

Fig. 2 Size (diameter, µm) and particle size distribution of PHBV-empty and PHBV-CEF microparticles by dynamic light scattering (DLS). The average diameter of PHBV-empty (1.89 ± 0.24 µm), and PHBV-CEF (2.2 ± 0.17 µm) not exhibited significant difference (n = 7).

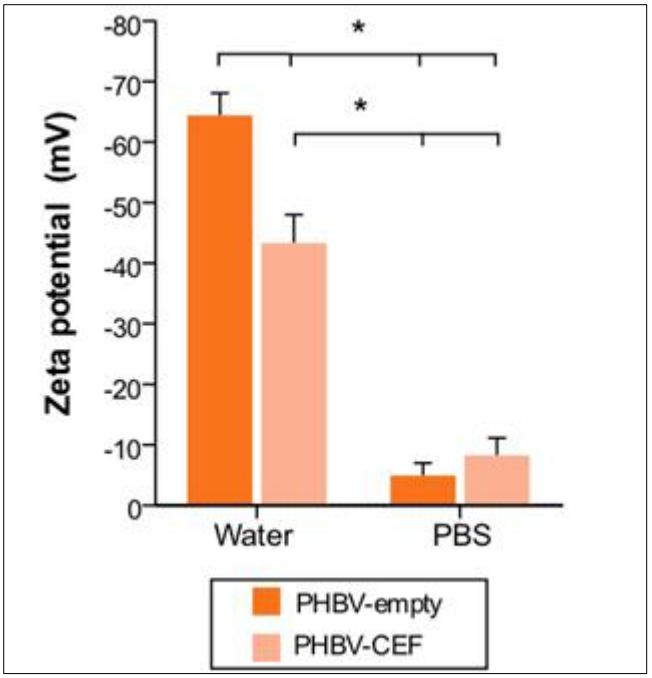

Fig. 3 Zeta potential (mV) of PHBV-empty and PHBV-CEF microparticles determined from three repeated experiments in distilled water and phosphate buffered saline (PBS) pH 7.4 at 25ºC (n = 3). Kruskal-Wallis $p < 0.05$, and (*) = $p < 0.05$ Mann Whitney post test.

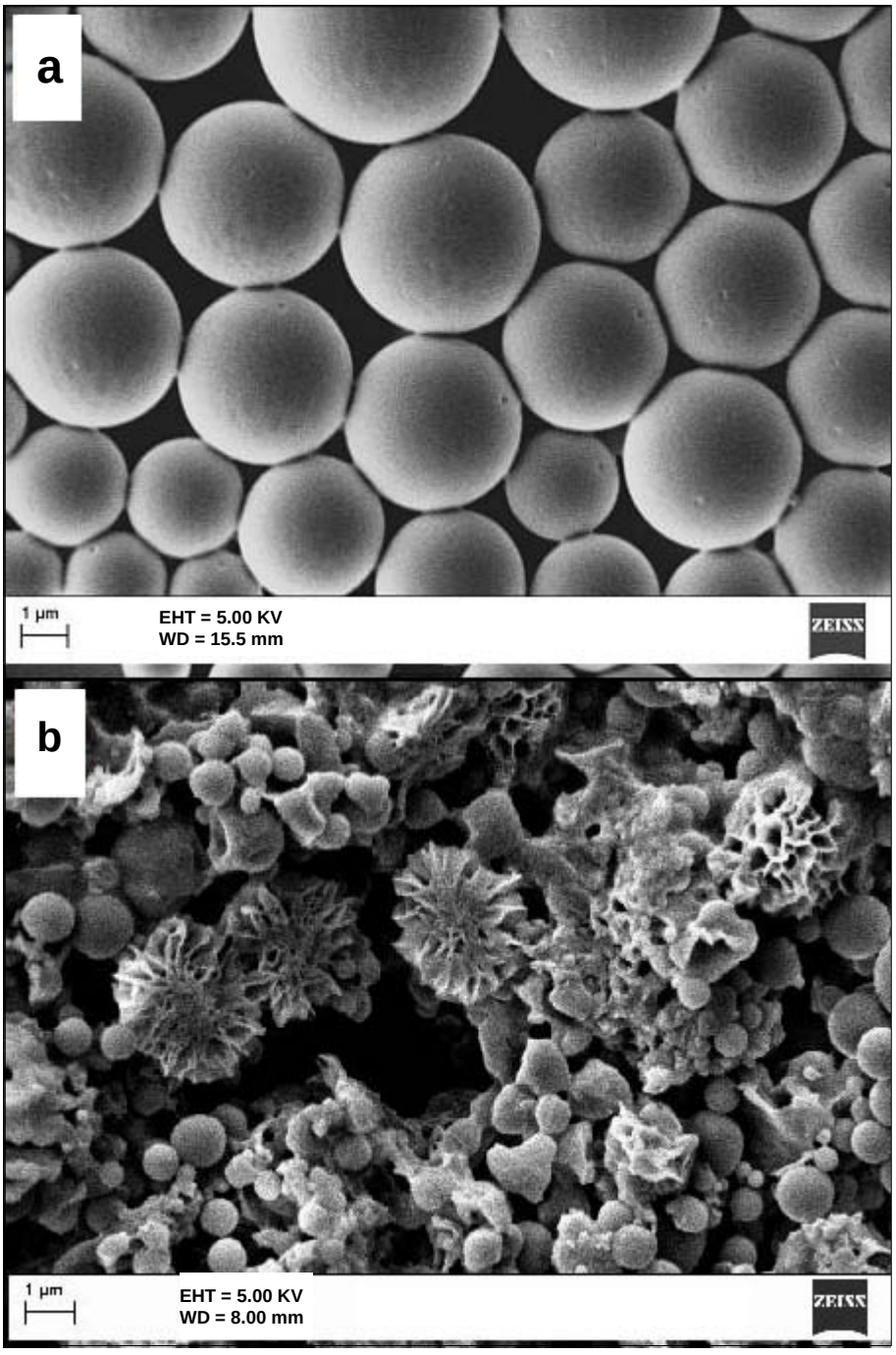

Fig. 4 Representative micrographs of fresh PHBV-CEF microparticles by SEM (a), and after 30 days suspended in phosphate buffered saline (PBS) pH 7.4 at 25ºC (b).

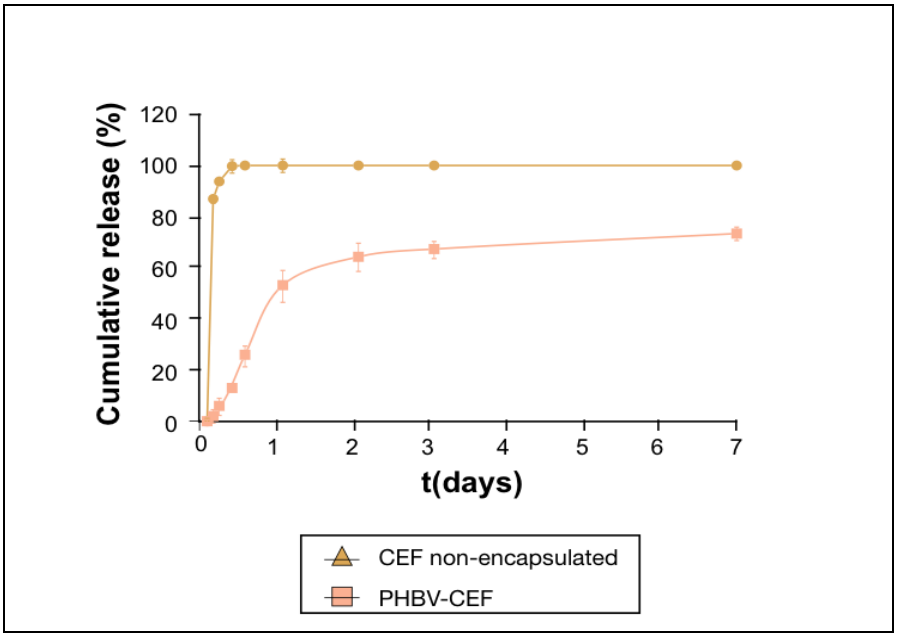

Fig. 5 Cumulative release of ceftiofur from PHBV-CEF microparticles, and CEF non-encapsulated in a dialysis system suspended in PBS pH 7.4 at 25ºC for 7 days.

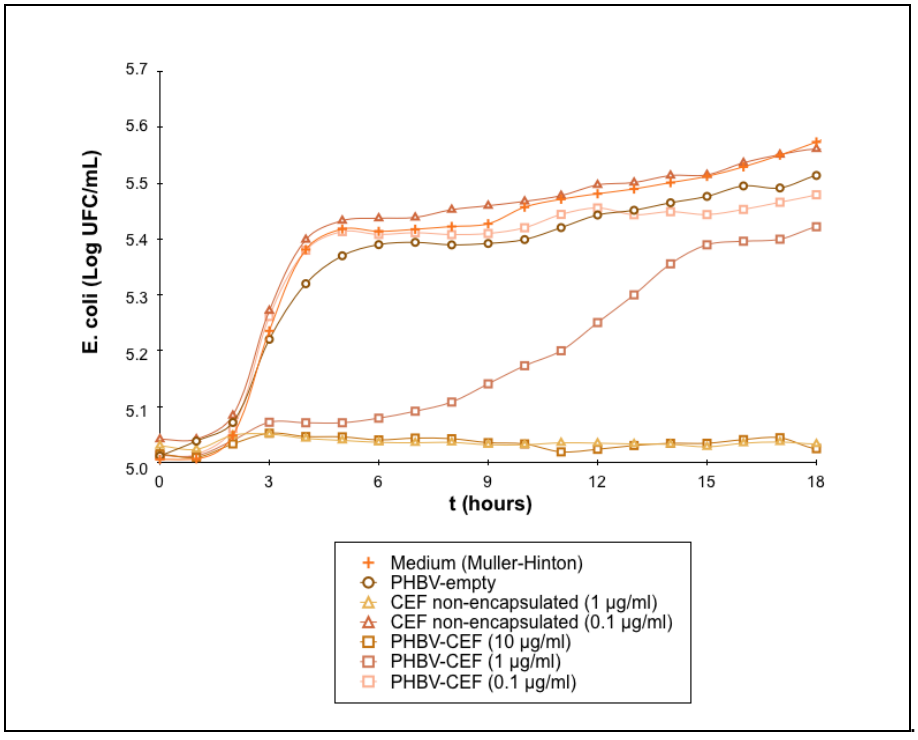

Fig. 6 Antimicrobial susceptibility measured by the kinetic of growth of *Escherichia coli* (ATCC 25922) in microplate during 24 hrs at 25ºC.

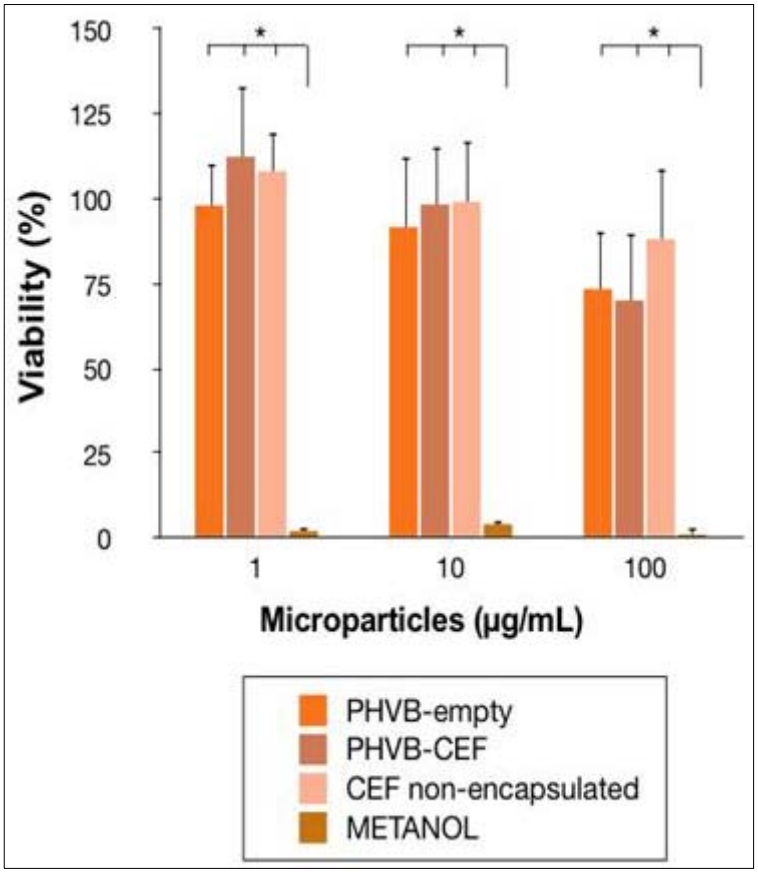

Fig. 7 Cell viability of Hep-G2 determined using MTS Cell Proliferation assay challenged for 24 hrs with 1, 10 and 100 µg/mL of PHBV-empty and PHBV-CEF loaded microparticles, ceftiofur non-encapsulated and methanol. Note that only the cells treated with methanol showed statistic difference ($p < 0.05$) with the other groups.