Medroxyprogesterone-encapsulated Poly(3-hydroxybutirate-co-3-hydroxyvalerate) Nanoparticles using Supercritical Fluid Extraction of Emulsions

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Abstract

In this work, was investigate the effect of the molecular weight of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) on the preparation of medroxyprogesterone acetate (MPA) -encapsulated polymeric nanoparticles (PNPs) using the supercritical fluid extraction of emulsions (SFEE) technique. Particles with diameters between 850 nm – 183 nm were obtained using PHBV of molar mass reduced and variable between 210 KDa to 14 KDa, thus establishing a direct relation between the size of the PNPs prepared with SFEE and the molar mass of the polymer. These results were contrasted with those obtained with the conventional emulsion solvent evaporation (ESE) technique. PNPs prepared by SFEE were observed to be smaller than those produced by ESE, for all polymer molecular weights (MW) studied. The encapsulation efficiency (EE%) of MPA in the PNPs prepared with the SFEE technique, and with the lowest MW PHBV was determined to be 70%. The *in vitro* release kinetics for this system indicated that the mean time for 35% release of MPA was 18h. Both a first and a second-order kinetics models provide a good fit to the release profile of MPA from the PHBV PNPs. Cellular viability results indicate low toxicity profiles of the PHBV PNPs prepared with the SFEE technique, even at high PNP concentrations.

Keywords: PHBV; nanoparticles; medroxyprogesterone; supercritical fluid extraction of emulsions; drug release; *in vitro* toxicity of nanoparticles.

1. Introduction

Estrus synchronization in cattle by steroid hormones is of great relevance in the production of suckled beef cows as it provides for an opportunity for the herd to return to cyclicity and to begin a new gestation in a reduced period of time[1]. The Artificial Insemination (AI) is one of the most widespread biotechnology used with instrument effective and economic in the reproduction of bovine[2]. There are several protocols in the cattle industry that use progestogens for estrous synchronization prior to AI of cows at a fixed time[3]. Widespread acceptance of AI in beef cattle partly depends on the success of programs that facilitate insemination at a predetermined time[3]. The success of a fixed-time artificial insemination (FTAI) program is dependent on a high ovulation rate during a short interval[4]. Synchronization of ovulation and timed artificial insemination are able to precisely control the time of ovulation and thus avoid the need for estrus detection[5]. Medroxyprogesterone acetate (MPA, **Figure 1a**) is a hormone of particular importance in estrus induction protocols post partum in the context of FTAI[6]. However, MPA is poorly soluble in aqueous solution and has low bioavailability[7].

<< Insert Figure 1 here >>

Polymeric nanoparticles (PNPs) can be used to efficiently encapsulate and to control the rate of dissolution of hydrophobic drugs in aqueous media, to improve their pharmacokinetic profile and biodistribution, and thus to enhance therapeutic efficacy[8]. The encapsulation of MPA within PNPs has the potential, therefore, to overcome the challenges the industry faces today when utilizing MPA in estrus synchronization. There are several considerations, however, with regards to the

preparation of MPA-encapsulated PNPs. The chemistry of the polymeric material is an important consideration as it dictates parameters such as the rate of release[9] and biocompatibility[10] of the formulation. The selected particle formation strategy is also of great relevance as it may impact the size of the PNPs[11], and in turn their biodistribution[12], cellular internalization[13] and also rate of drug release[14]. In the preparation of PNPs using the traditional emulsion solvent evaporation (ESE) technique, the molecular weight (MW) of the polymer has been shown to have a direct influence on the properties of the final particles, including particle size[15], and it is thus another important variable to be considered. As is well known, the molecular weight, viscosity and the size of polymer chain have great influence on the dispersion and aggregation behaviors of nanoparticles in polymer matrix[16].

Based on the challenges and opportunities described above, the goal of this work was to investigate the effect of the MW of a natural (microbial) polyester, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, **Figure 1b**), on the preparation of MPA-encapsulated PNPs, as well as the characteristics of the particles when prepared using supercritical fluid extraction of emulsions (SFEE) technique. The use of supercritical fluids in combination with nano-emulsions, which presents advantages over these two separated technologies is a new promising technology to produce nanometer particles of bioactive compounds in aqueous media [17]. PHBV of varying MW was prepared by acid hydrolysis and characterized by intrinsic viscosity and gel permeation chromatography (GPC). The effect of the MW of PHBV on the size of the PNPs prepared by SFEE was determined by scanning electron microscopy (SEM) and light scattering (LS), and the results contrasted to those obtained with the traditional ESE technique. Was also sought to determine the encapsulation efficiency (EE%), to

characterize and to model the release profile of MPA, and to evaluate the cytotoxicity of the smallest PNPs prepared by SFEE.

2. Materials and Methods

2.1. Materials

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), was kindly provided by PHB Industrial S/A - Brazil. Poly(vinyl alcohol) (PVA, 87-90% hydrolyzed, Mw: 30,000Da to 70,000Da) was purchased from Sigma-Aldrich® - USA. Dichloromethane (DCM, 99.5%) and Ethanol (EtOH, 99.5%) were purchased from EMD Chemicals – USA. Chloroform (CHCl₃, 99.8%) and hydrochloric Acid (HCl, 36.5 – 38.0 %) were purchased from Mallinckrodt Chemicals – USA. Hexane (99.8%) was purchased from Fisher - USA. Carbon Dioxide (CO₂, 99.9%) was from Praxair, Inc. Danbury - USA. Medroxyprogesterone (MPA, Mw: 386.5Da) was kindly provided by DEG – Brazil. Deuterated Chloroform (CDCl₃, 99.9%) was purchased from Cambridge Isotope Lab – USA. Deionized water (NANOpure: Barnstead), with a resistivity of 18.2 MΩ.cm⁻¹, was used in all experiments.

2.2. PHBV Purification

In order to remove impurities present in the polymer, PHBV was solubilized in CHCl₃ (30 mg.mL⁻¹) and the organic solution filtered through cellulose filter paper (150 mm). The polymer was then precipitated in hexane. PHBV was then dried at 40°C to remove the organic solvent. The procedure was repeated three times.

2.3. PHBV Depolymerization

Depolymerization of PHBV was achieved via acid hydrolysis using a 6N HCl acid solution. Approximately 400 mg of PHBV was added to 50 mL 6N HCl acid solution at 105 °C, and depolymerization was carried out for different lengths of time to achieve

different molar masses. After depolymerization, PHBV was washed three times with deionized water and once with EtOH.

2.4.PHBV Molecular Weight Determination

The molecular weight of PHBV was determined by intrinsic viscosity (n), and GPC. The viscosimetric molecular mass (M_v) of the polymer was determined using an Ubbelohde type viscometer and the Mark-Houwink equation[18]. The experiments were conducted in CHCl_3 at 25°C . The viscosity of a polymeric solution is greater than pure solvent. To determine the viscosity of polymer solution the following viscosities are considered: (i) relative viscosity (n_{rel}) - Eq. (1); (ii) specific viscosity (n_{sp}) - Eq. (2); (iii) reduced specific viscosity ($n_{\text{sp.red}}$) Eq. (3); (iv) inherent viscosity (n_{inh}) - Eq. (4); and (v) intrinsic viscosity (n) Eq. (5)[19].

$$n_{\text{rel}} = \frac{t}{t_0} \text{ (dimensionless)} \quad (1)$$

$$n_{\text{sp}} = n_{\text{rel}} - 1 = \frac{(t-t_0)}{t_0} \quad (2)$$

$$n_{\text{sp.red}} = \frac{n_{\text{sp}}}{c} \quad (3)$$

$$n_{\text{inhe}} = \frac{\ln(n_{\text{rel}})}{c} \quad (4)$$

$$n = \lim_{c \rightarrow 0} (n_{\text{inhe}}) = \lim_{c \rightarrow 0} (n_{\text{spe.red}}) \quad (5)$$

Where c is the polymer concentration, t is the flow time of the solution and t_0 the flow time of the pure solvent. Measurements of the flow time of pure CHCl_3 and of the solutions were conducted using a chronometer to calculate the n_{sp} and n_{rel} . The $n_{\text{sp.red}}$ and

n_{inhe} were estimated with values of n_{rel} and solution concentrations. The extrapolation of the $n_{spe,red}$ vs. c and n_{inhe} vs. c curves to zero concentration converge the same intrinsic viscosity, but from different directions — one has a positive slope and the other a negative one. The M_v is determined with Mark-Houwink equation, using the following parameters: $\alpha = 0.82$ and $K = 6.0 \times 10^{-3} \text{ dL/g}$ where $[\eta] = K.M_v^\alpha$ [18].

The molecular weight of PHBV was also determined by GPC, with a Viscotek GPCmax VE2001 system equipped with a triple detector (Refraction index, RI), viscometer and Light Scattering module (270 Dual Detector Viscotek). The samples were injected (200 μL) to a separation column (Waters Styragel HR5E) at 30 °C. HPLC grade CHCl_3 was used as the elution solvent at a flow rate of 1.0 $\text{mL} \cdot \text{min}^{-1}$ [20]. The concentration of the samples was set to 5 $\text{mg} \cdot \text{mL}^{-1}$. The calibration curve was generated with polystyrene (PS) standards. PS with low polydispersity (PolyAnalytik) – (Mw: 93,355 - PDI 1.06) and polydisperse (Mw: 147,700 - PDI 1,687) were used. The integration of three detectors allows the determination of the absolute molecular weight of the polymer [21].

2.5. Integrity of the Molecular Structure

Quantitative one-dimensional ^1H NMR spectra of the PHBV structure after depolymerization were acquired in a solution containing 10 mg polymer / 0.8 mL CDCl_3 , using a Varian Mercury-400 spectrometer. The relative peak intensities of the ^1H spectra were determined using the MestReC Software, and the chemical shifts were referenced to the residual proton peak of CDCl_3 at 7.262 ppm [22]. The mol% of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) in the depolymerization products were determined using the following equations (6) and (7):

$$\% HB = \frac{a(HB)}{a(HB) + a(HV)} \quad (6)$$

and

$$\% HV = \frac{a(HV)}{a(HB) + a(HV)} \quad (7)$$

where $a(HB)$ is obtained from the integral area of the HB peak and $a(HV)$ from the integral area of the HV peak. The peaks are those identified in the 1H spectra, and correspond to the methyl protons signals of two overlapped peaks, a broader peak at higher field, and a narrower one at lower field (Figure 2).

<< *Insert Figure 2 here* >>

These two peaks correspond to methyl groups in the noncrystalline and crystalline region, respectively where HB and HV units are: $\delta(ppm)$: 0.88 and 1.26 (CH_3), respectively[23]. The relative intensities of these two peaks can be obtained by carrying out the deconvolution of the signal as discussed in the literature [24].

2.6. Preparation of MPA/PHBV Reverse Emulsions

A solution of PHBV (16 mg) and MPA (2%W/W) was prepared in DCM (2mL). The organic solution was then added to an aqueous solution of PVA (5 mL at 5% PVA, W/W) dropwise using a syringe and under stirring to form a pre-emulsion. The organic solution was then emulsified under ultrasonication for 2 min (50 W - 10 pulse) in an ice bath, forming an oil-in-water (O/W) emulsion, where MPA and PHBV are incorporated into the dispersed organic phase, with the emulsion stabilized by PVA.

2.7.Preparation of MPA-encapsulated PHBV Nanoparticles using Supercritical Fluid

Extraction of Emulsions (SFEE)

The SFEE process was conducted in an experimental apparatus as shown in [Figure 3](#). The main components of the set up are: (i) a syringe pump (ISCO 260D, Lincoln, USA) that was employed to load the CO₂ into the pressure vessels, and also to maintain the flow during the extraction of the organic solvent from the reverse emulsion; (ii) an HPLC pump (Waters 501) used to feed the reverse emulsion into the high-pressure extraction chamber; (iii) a 40 mL cylindrical pressure vessel (extraction chamber) and backpressure valve (Swargelok-BPR). The CO₂ was first pre-heated in a water bath at 40° C and introduced at controlled mass flow rate of 6.3 g.min⁻¹ (or 10 mL.min⁻¹) to load the main extraction chamber with the help of the ISCO pump until the pressure and temperature equilibrated at 10.0 MPa and 40 °C, these conditions were based in previously works of our group ([6], [25]) and in works available in the literature ([26],[27]). These works use supercritical technology to obtain micro particles of similar polymers. In this way, minor modifications in our procedures were done in function of intrinsic polymer characteristics (molecular mass, solubility, density, etc ...). The O/W emulsion prepared as described above was then introduced into the cylindrical pressure vessel at a constant flow rate of 0.5 mL.min⁻¹ in a co-current mode (flowing parallel to the CO₂ flow) via a 150 µm ID capillary. The system pressure was regulated by a BPR valve. As the emulsion is sprayed coaxially with CO₂, the organic solvent is removed (partitions into the CO₂ phase), giving rise to the PNPs encapsulated with MPA. The latex is collected in a glass test tube at the bottom of the extraction vessel. The PNPs were washed to remove excess PVA. They were then dried and lyophilized for further characterization.

<< *Insert Figure 3 here* >>

2.8. Preparation of MPA-encapsulated PHBV Nanoparticles using Conventional Emulsion Solvent Evaporation (ESE)

The O/W reverse emulsion prepared as discussed earlier was maintained under constant stirring at room temperature overnight for the removal of the organic solvent by evaporation, resulting in the formation of PNPs.

2.9. Morphology and Geometric Size of the Nanoparticles

The morphology and geometric size of the PHBV PNPs were determined by scanning electron microscopy (SEM, Jeol - JSM-6510LV-LGS). Several drops of an aqueous dispersion of the PNPs were transferred onto a cover glass slide (18 mm², Corning Inc.) and allowed to dry. The substrates were then sputter-coated with gold (Ernest Fullam) for 35s, and the SEM micrographs were obtained at 25 kV. Geometric sizes were determined by ImageJ (1.44p software, Wayne Rasband National Institutes of Health, USA) and a Gaussian fit to the size distribution was used to obtain the mean and deviation of sizes. For some samples, particle size distributions (PSD) were measured by dynamic light scattering using a Malver Zeta Sizer instrument (mod. Zetasizer Nano S, Worcestershire, UK). 1 mL of solution containing NPs dispersed in deionized water was used for each test.

2.10. Cytotoxicity of the PHBV PNPs

2.10.1. Cell culture

Caco-3 human intestinal epithelial cells were obtained from American Type Culture Collection (ATCC#: HTB-55, Manassas, VA). Cells were maintained in a controlled atmosphere at 37 °C with 95% of relative humidity and 5% CO₂. Cells were grown in

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta biologicals, Lawrenceville, GA) and 2% penicillin G/streptomycin solution (Gibco). Culture media was changed every 3–5 days. Cells were passaged at 70-90% confluency using 0.25% trypsin/ethyl-enediaminetetraacetic acid (EDTA) solution. For the experiments, cells with passage numbers between 20 and 30 were used.

2.10.2. In vitro cytotoxicity

The toxicity of PHBVPNPs was assessed by determining cell viability of Calu-3 cell cultures as a function of the concentration of the PNPs using a colorimetric method (MTS, Promega), which determines the intracellular dehydrogenase activity. Cells were seeded on 96 well plates with a cell density of 1.65×10^4 cells.cm⁻² and maintained in a controlled atmosphere at 37 °C with 95% of relative humidity and 5% CO₂ until a confluent cell monolayer was obtained. The medium was replaced by a suspension of PNPs at concentrations ranging between 0.075 and 0.6 mg.mL⁻¹. PHBVPNPs and controls were contacted with the monolayers for 24 hours. The medium was then removed and cells were washed with PBS two times. To each well, 100 µL of DMEM was added. 2.0 mL of MTS were mixed with 100 µL of PMS, and 20 µL of the mixed solution was transferred to each well. Cells were incubated in mixture for 4 h at 37°C and 5% CO₂. MTS is bio-reduced into formazan (which is soluble in the culture medium) by dehydrogenase enzymes found in metabolically active cells. Absorbance of formazan at 490 nm was measured directly from the 96-well plate, and it is proportional to the number of living cells. Cell viability was calculated as the ratio between the absorbance of treated (incubated with PNPs) and untreated (free of PNPs) cells.

2.11. Encapsulation Efficiency

The encapsulation efficiency of MPA within PHBV PNPs prepared by the conventional ESE and SFEE methods was determined spectrophotometrically. First, a calibration curve for MPA was constructed in ethanol using a UV-visible spectrophotometer (Shimadzu, model UV-2601) at 240 nm. To measure encapsulation efficiency (EE%), known amounts of PHBV PNPs (3.5 mg) were dispersed in 15 mL of ethanol. The solution was maintained under stirring for 24 hours at room temperature, and then centrifuged at 10,000 rpm for 30 min. The concentration of MPA in the supernatant was determined by UV spectroscopy. While MPA is soluble in ethanol, PHBV is not, and the drug is thus extracted into ethanol. The procedure was repeated three times. EE% was determined using the following equation:

$$EE\% = \frac{C_{rec}}{C_{the}} \times 100\% \quad (8)$$

where C_{rec} is the experimental drug concentration recovered after encapsulation and C_{the} is the theoretical drug concentration in the formulation [28].

2.12. *In vitro* drug release

The *in vitro* drug release experiment was carried out using a solution containing ethanol-water (30:70). A known amount of PHBV PNPs containing MPA (10 mg) was added to 50 mL of ethanol-water solution. The system was maintained at 37 °C. The PNP dispersion was centrifuged at 10,000 rpm at different pre-determined times, and 2.5 mL of supernatant was collected for the UV analysis at 240 nm. The UV spectrum of the solution was collected, and the solution was returned to maintain constant total volume. The amount of drug released was quantified with the MPA calibration curve.

3. Results and Discussion

3.1. PHBV Depolymerization and Characterization

The size of the PNPs is a key parameter in terms of their application as drug carriers, as it influences how the particles interact with the physiological environment [29]. One important characteristic governing the size of PNPs prepared by emulsification is the molecular weight of the polymer [30, 31]. In this work, we investigated the effect of the molecular weight of PHBV on the size of PNPs prepared by both the conventional ESE technique and by SFEE. GPC and intrinsic viscosity were employed to determine the molecular weight of the polymer as received and after acid hydrolysis/depolymerization. The molecular weight for PHBV was determined for depolymerized products obtained at 2h, 3h, 4h and 8h. The number average (M_n), viscosity average (M_v) and weight average (M_w) molecular weights in Daltons (Da) are shown in **Figure 4** as a function of the depolymerization time.

<< *Insert Figure 4 here* >>

The results (summarized in Table 1) show that the molecular weight of PHBV can be reduced from 210 kDa down to 14 kDa with acid hydrolysis. The reduction in molecular weight is seen to happen most precipitously up until 4h of acid hydrolysis, and little further reduction in molecular weight is seen at longer times. The polydispersity as measured by M_w/M_n is seen to increase with time, and continues to increase even after 4h of depolymerization, as indicated in Table 1. The molecular weight values reported here are observed to be in agreement with literature results [18], where depolymerization of

PHBV was achieved with a sodium borohydride treatment. In that work, a 4h incubation/depolymerization resulted in a 14-fold decrease in molecular weight (from 297,192 Da to 20,753 Da).

¹H NMR spectroscopy was employed to evaluate the molecular structure of PHBV after depolymerization by acid hydrolysis. The % of HB and HV of the different PHBV samples obtained by acid hydrolysis are summarized in Table 1. The results indicate that the structure of resulting depolymerized chains is similar to PHBV as received. The %HV of PHBV as received was determined to be 5.4%, while the %HV after depolymerization was found to be between 5.2% and 6.0%.

3.2. Preparation of PHBV Nanoparticles

In this study, PHBV PNPs were prepared by SFEE, and the results compared and contrasted with those from the ESE technique. The PNPs were prepared using the same emulsion batch, so as to evaluate the effect of the different drying strategies. Was also investigated the effect of the molecular weight of PHBV obtained by acid hydrolysis on the characteristics of the PNPs produced under both strategies.

In the ESE, the organic solvent is removed by simple evaporation, while the emulsion droplets, stabilized with PVA in an aqueous solution, are stirred over-night at ambient conditions [32]. One of the potential disadvantages of the conventional ESE process is that it takes long periods of time for the solvent to evaporate from the nanoparticle suspension, a process that can induce degradation of thermolabile compounds or aggregation of the particles [33].

In the SFEE technique, instead of the non-forced organic solvent removal, the organic phase is removed upon contact with CO₂. The organic phase is highly soluble in CO₂, and fresh CO₂ is flown co-currently with the emulsion, thus quickly carrying away the organic solvent. The O/W emulsion containing both the PHBV polymer and the drug

(MPA) solubilized in the organic phase is pumped inside the pressure vessel co-currently with CO₂, which was held at 313 K and 10.0MPa(Figure 3).Based on the high-pressure vapor-liquid equilibrium diagram (VLE) of the dichloromethane + CO₂ system[34], the operating point is maintained above the mixture critical point[35].

The single emulsion was introduced in the system using an HPLC pump and high pressure CO₂ was introduced using a syringe pump, both with a pre-determinate flow rates. Considering that DCM (the dispersed phase of the emulsion) is soluble in scCO₂ under these conditions, the organic solvent is expected to be easily extracted, thus leading to the precipitation of both the PHBV and nucleation of MPA, thus resulting in the formation of the PNPs of PHBV with encapsulated MPA (Figure 5). The solid PNPs formed were collected as latex in water, inside the extraction vessel, as shown in Figure 3, and subsequently washed with deionized water and lyophilized – same strategy used for the processing of the PNP prepared using the conventional ESE technique.

<< *Insert Figure 5 here* >>

3.3.Characterization of the PHBV PNPs

The PHBV PNPs formed by the conventional ESE and SFEE were characterized with regards to their shape and geometric size by SEM. The average diameter and deviation were determined from a Gaussian fit to the particle distributions, which were obtained with ImageJ. The SEM images and the Gaussian fits are shown in Figure 6 and 7 for particles obtained with ESE and SFEE, respectively. The results are shown as a function of molecular weight of the polymer.

<< *Insert Figure 6 and 7 here* >>

LS was conducted for some of the samples to corroborate the size determined by SEM. A summary of the geometric diameters obtained by SEM, and the hydrodynamic diameters obtained by LS, is shown in Table 2.

<< *Insert Table 2 here* >>

The results shown in Table 2 indicate a strong correlation between the diameter of the PNPs with the molecular weight of PHBV. The lower the molecular weight of the polymer, the smaller the size of the particles. Similar results are seen for both SFEE and the traditional ESE technique. Particles with diameters as small as 215 ± 52 nm (ESE) and 183 ± 32 nm (SFEE) were obtained for PHBV of molecular weight of 14,080 Da, while for PHBV with 210,379 Da, the diameter of the particles was as large as 932 ± 367 nm (ESE) and 850 ± 266 nm (SFEE). The sizes from LS are seen to corroborate the SEM results.

Besides the smaller sizes of the PNPs obtained with the SFEE process, there may be other advantages in preparing PNPs with the supercritical technology, when compared to the traditional ESE method. The evaporation of the organic solvent from the NP suspensions prepared by conventional ESE is a very long process. It may induce degradation of thermolabile compounds during the slow replacement of the non-solvent [33]. In contrast, the rate of removal of the organic solvent with the SFEE is much higher than for the conventional methods [36]. While not the focus of this work, it

is also likely that a fast precipitation of drug and polymer can lead to a better dispersibility of the drug within the PNP, and thus the ability to slow the recrystallization of the therapeutic, which is expected to lead to faster dissolution rates of such non-water soluble drugs.

3.4. Encapsulation Efficiency and in vitro Release Profile

The encapsulation of MPA in PHBV PNPs was tested for PNP prepared by SFEE and ESE, and with the polymer with the lowest molecular weight (14,080 Da). This was the combination that resulted in the smallest particle diameter and lowest size polydispersity. The encapsulation efficiency of MPA in the PHBV PNPs under those conditions was 70.3%. The performance of both techniques (ESE and SFEE) was very similar. This high loading capacity can be rationalized based on the fact that little migration of the drug is expected from the primary emulsion to the external aqueous phase during the emulsification and organic solvent extraction.

The release kinetics of MPA from the PHBV PNPs was determined *in vitro*. Considering the encapsulation efficiency discussed above, the mean time for 35% release of MPA was 18 h, and 70% in 144 h. This profile suggests that the release could be mediated by two different processes: (i) the slow polymer matrix degradation promotes a significant retardation of the release of MPA; (ii) this is a diffusion-controlled process, where the PHBV particles do not significantly degrade during the time that drug is released [37]. The slow degradation rate of PHBV can be attributed to its high crystallinity, which results from its repeat units having a short methyl side group—**Figure 1b** [38]. In contrast, MPA loaded in crosslinked polyvinyl-pyrrolidone (PVP) particles show different release kinetics which is mainly governed by dissolution [8].

To better understand the release kinetics of the MPA, the release profile and fit using first and second order kinetic models are shown in Figure 8, the release profile was fit to mathematical models[39].

<< *Insert Figure 8 here* >>

A first order kinetic model is shown in Eq. (8):

$$F_R = F_{max}(1 - e^{-(k_R/F_{max})t}) \quad (8)$$

Where k_R is the release constant F_R is the drug released fraction (y axis of Figure 8) and F_{max} is the maximum fraction released. A second order model was also applied. In this case, the “partition parameter” (α) and a release parameter (k_L) were fit to the experimental data using Eq. (9):

$$F_R = \frac{F_{max}(e^{2(k_L/\alpha)t}-1)}{1-2F_{max}+e^{2(k_L/\alpha)t}} \quad (9)$$

Both models were capable of fitting the experimental very well, with R^2 values of 0.975 for the first order model, and 0.957 for the second order model.

3.5.Toxicity of the PHBV PNPs

The cytotoxicity of the PHBV NPs prepared using the SFEE technique and the depolymerized PHBV at 14,080Da molecular weight, that resulted in particles with the smallest hydrodynamic diameter (183nm) were tested against Calu-3 cells. Cellular

viability was determined at 24h post-exposure, using the MTS assay. The results as a function of concentration of NPs, and their comparison with the control where no NPs were used are shown in Figure 9.

<< Insert Figure 9 here >>

It can be observed that the NPs have a very low toxicity profile, with cellular viability being smaller than control only at very high concentrations (0.6 mg.mL^{-1}). This suggests that PHBV NPs have a toxicity profile even lower than PLGA NPs, as PLGA NPs of similar size and also prepared with PVA, show an impact in cellular viability at 24h at concentrations as low as 1.0 mg.mL^{-1} [40].

4. Conclusions

In this work, was studied the effect of the molecular weight of PHBV on the properties of PNPs prepared with the SFEE technique. Were also investigated the ability to encapsulate MPA within such PNPs, and their release rate, as well as the toxicity of the PHBV PNPs. The results showed a strong function of the molecular weight of PHBV on the geometric diameter of the PNPs, with the lowest molecular weight studied (14,080Da) resulting in the smallest diameter (183nm), while micron-sized particles were obtained at the highest molecular weight (210,379Da). The diameter of PNP's were lightly smaller when prepared using traditional ESE. However, the time of solvent elimination using traditional ESE is huge on compare with the SFEE technique. Was also observe very high encapsulation efficiency of the hydrophobic therapeutic MPA (70%), a steroid hormone of great relevance in estrus synchronization, with a first order release kinetics fitting the drug release profile. The PHBV PNPs have

a very low toxicity profile, even lower than those of PLGA produced using similar conditions and excipients, when tested against a model human airway epithelial cell line (Calu-3).

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Tables

Table 1: PHBV molecular weight from intrinsic viscosity (η) and gel permeation chromatography (GPC), and the % HB/HV in PHBV from $^1\text{H-NRM}$.

<i>Depolymerization time (h)</i>	<i>M_v Viscosity (Da)</i>	<i>M_n GPC (Da)</i>	<i>M_w GPC (Da)</i>	<i>M_w/M_n</i>	<i>% HB</i>	<i>% HV</i>
0	210,379	199,626	228,515	1.145	94.6	5.4
2	69,024	63,045	92,466	1.467	94.7	5.3
3	32,031	22,453	61,068	2.720	94.6	5.4
4	17,452	15,134	40,665	2.687	94.8	5.2
8	14,080	10,143	44,890	4.426	94.0	6.0

M_v: viscosity average; *M_n*: number average; *M_w*: weight average.

Table 2: Effect of the PHBV molecular weight on the size of the PHBV nanoparticles prepared by the conventional emulsion solvent evaporation (ESE) and the supercritical fluid extraction of emulsion (SCFEE) techniques. Geometric diameter from SEM and hydrodynamic diameter from LS.

<i>Diameter of the PNPs (nm)</i>				
PHBV Mw (Da)	<i>ESE</i>		<i>SFEE</i>	
	<i>SEM</i>	<i>LS</i>	<i>SEM</i>	<i>LS</i>
210,379	932 ± 367	916	850 ± 266	N/A
69,024	635 ± 124	511	421 ± 62	N/A
32,031	470 ± 82	488	377 ± 61	N/A
17,452	426 ± 99	N/A	348 ± 72	327
14,080	215 ± 52	N/A	183 ± 32	263

PNPs: Polymeric nanoparticles; ESE: Emulsion Solvent Evaporation; SFEE: Supercritical Fluid Extraction of Emulsions.

Figures

Figure 1.Chemical structure of (a) medroxyprogesteroneacetate(MPA); and (b) poly(3-hydroxybutyrate-*co*-hydroxyvalerate) (PHBV).

Figure 2. ^1H -NMR spectra of the PHBV as received (0h) and those for the samples after depolymerization under acid hydrolysis at 2h, 3h, 4h, and 8h.

Figure 3. Schematic diagram of the supercritical fluid extraction of emulsion (SFEE) set up.

Figure 4. Molecular weight of PHBV as received and after depolymerization, as determined by GPC and intrinsic viscosity (n).

Figure 5. Schematic diagram of the particle formation in the supercritical fluid extraction of emulsion (SFEE) technique.

Figure 6. Morphology and average geometric size (SEM) of the PHBV PNPs prepared using the conventional emulsification-solvent evaporation (ESE) technique, as a function of the molecular weight of PHBV. Insets: *lower* = Gaussian fits to the size distribution obtained with ImageJ; *upper*: higher resolution SEM of the same.

Figure 7. Morphology and average geometric size (SEM) of the PHBVPNPs prepared using the supercritical fluid extraction of emulsions (SFEE) technique, as a function of the molecular weight of PHBV. Insets: *lower* = Gaussian fits to the size distribution obtained with ImageJ; *upper*: higher resolution SEM of the same.

Figure 8. Release profile of MPA loaded in PHBV PNPs prepared using the supercritical fluid extraction of emulsions (SFEE) technique, and PHBV with molecular weight of 14,080Da, and the first and second order kinetic model fits.

Figure 9. Calu-3 cellular viability (MTS) profile upon 24h exposure to PHBV nanoparticles prepared using the supercritical fluid extraction of emulsions (SFEE) technique. Nanoparticles prepared with 14,080Da molecular weight PHBV. The bars represent $\pm$ SD (n=5). * = $p < 0.05$, one-way ANOVA, with Dunnett's post hoc test, the only condition where cellular viability was statistically lower than the control.