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POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYVALERATE) NANOPARTICLES PREPARED BY A MINIEMULSION/SOLVENT EVAPORATION TECHNIQUE. EFFECT OF PHBV MOLAR MASS AND CONCENTRATION

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Abstract - Miniemulsification and emulsion/solvent evaporation techniques were combined to produce poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) nanoparticles. Those nanoparticles were prepared with different PHBV molar masses and PHBV concentrations to verify their effect on the final particle size. Nanoparticles with an average diameter of 133 nm were obtained when a low molar mass (Mw = 44,350 g/mol) polymer was used. On the other hand, when high molar mass PHBV (Mw = 369,900 g/mol) was used under the same operational conditions, nanoparticles with a 300 nm average diameter and a broader particle size distribution were formed. Results also showed that increasing the PHBV concentration led to an increase of the particle size and, when the polymer/costabilizer (PHBV/hexadecane) weight ratio was close to 1, nanocapsules (hexadecane core surrounded by a PHBV shell) were formed.
Keywords: PHBV; Miniemulsion; Nanoparticles; Molar mass; Morphology.

INTRODUCTION

Polymer nanoparticles are a promising tool for drug delivery since their use can increase the efficiency of drugs, protecting them from degradation or metabolization after administration, and also creates new routes of administration, like for example, oral application or transdermal delivery of drugs [Mailander and Landfester, 2009]. Nanoparticles made from natural polymers are interesting drug delivery systems due to their characteristics of biodegradability and biocompatibility [Pich *et al*., 2006; Reis *et al*., 2006; Poletto *et al*., 2008]. PHBV belongs to the group of polyhydroxyalkanoates (PHAs), polyesters naturally synthesized by microorganisms and deposited intracellularly as insoluble spherical inclusions [Grage *et al*., 2009]. Its characteristics of biocompatibility and biodegradability make PHBV attractive for numerous applications in medicine, including drug delivery systems [Reis *et al*., 2006; Poletto *et al*., 2008; Grage *et al*., 2009; Franceschi *et al*., 2008; Gangrade and Price, 1991; Sendil *et al*., 1999]. PHBV is a more attractive polyester than PHB because it has a lower

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degree of crystallinitty and this property can be changed as a function of the hydroxyvalerate content [Modi *et al*., 2011]. Furthermore, PHBV is produced by microorganisms, another advantage when compared with PLLA and PLGA, which are usually prepared with the use of catalysts under high temperature conditions [Park *et al*., 2012; Thomas and Lutz, 2011].

Different techniques can be employed to obtain polymeric nanoparticles and they can be divided into two main groups depending on the origin of the polymeric material, that can be: a) synthesized *in situ* using miniemulsion, microemulsion or emulsion polymerization techniques, or b) prepared from preformed polymers (natural or synthetic polymers). The last group comprises different techniques such as emulsification/solvent evaporation, emulsification/ solvent diffusion and nanoprecipitation (or solvent displacement method).

In the emulsion/solvent evaporation technique, the organic phase, composed of a solution of the preformed polymer in an organic solvent, is emulsified in water containing a surfactant. The solvent is removed from the dispersion by evaporation, inducing polymer precipitation as submicrometric particles [Reis *et al*., 2006]. According to Maia *et al*. (2004), some processing conditions such as temperature, solvent composition and surfactant concentration in the aqueous phase affect the physical properties of the resultant microspheres produced by this technique.

Miniemulsion polymerization and the miniemulsification technique are based on the pioneering work of Ugelstad *et al*. (1973) demonstrating that submicrometric monomer droplets (miniemulsions, though this term only began to be used a few years later), prepared with combinations of an anionic emulsifier and a fatty alcohol and stable for two weeks, could be efficiently nucleated (droplet nucleation) and thus serve as the main *locus* of polymerization (miniemulsion polymerization). Since this early work, numerous studies involving miniemulsion polymerization were published. Miniemulsions that remain stable for periods ranging from hours to months [Asua, 2002; Antonietti and Landfester, 2002; El-Asser and Sudol, 2004] are prepared using a high efficiency homogenization equipment (e.g., sonicator, rotor-stator systems or high pressure homogenizers), a surfactant to minimize droplet coagulation and a costabilizer to retard droplet diffusional degradation (Ostwald ripening). Usually, this costabilizer is a very hydrophobic molecule such as hexadecane.

It is possible to combine miniemulsification and emulsion/solvent evaporation techniques to produce polymeric nanoparticles using preformed polymers. The organic phase (preformed polymer, organic solvent, costabilizer) is dispersed in water containing a surfactant with the use of a sonicator, forming stable nanometric droplets. The solvent is removed from the miniemulsion by evaporation. The first results on the combination of these techniques were published in the late 70s [El-Asser *et al*., 1977; Vanderhoff *et al*., 1978; Vanderhoff *et al*., 1979], only a few years after the first works involving miniemulsion polymerization, and resulted in one of the earliest commercial products making use of miniemulsification technology, Aquacoat® [El-Asser and Sudol, 2004]. Recently, this combination of techniques was used to prepare poly(L-lactide) (PLLA), poly[(D,L-lactide)-*co*-glycolide] (PLGA), and poly(ε-caprolactone) (PCL) nanoparticles with controlled size and size distribution from solutions of the preformed polymers in chloroform [Musyanovych *et al*., 2008]. Nanoparticles of poly(3-hydrohybutyrate-*co*-3-hydroxyvalerate) (PHBV) were prepared using a combined approach of oil-in-water (O/W) emulsion followed by gel formation in toluene/PHBV droplets and toluene extraction. Due to the thermoreversible gelation of PHBV in toluene, the O/W dispersion was performed by means of ultrasonic agitation with heating (70^{o}C). Gel beads swollen in toluene were formed after cooling the dispersion to room temperature and the solvent was extracted with ethanol [Pich *et al*., 2006]. The authors obtained particles with a relatively broad particle size distribution and observed that the particle shape was strongly affected by the ultrasonic power and the mode of toluene extraction.

The release kinetics of the encapsulated drug is strongly affected by nanoparticle size and size distribution [Poletto *et al*., 2008]. Therefore, the control of these characteristics during nanoparticle production is very important. Parameters such as molar mass and polymer concentration in the formulation can affect size, size distribution and morphology of the nanoparticles. Some of these parameters were already investigated for other polymers. Musyanovych *et al*. (2008) evaluated the effect of the ratio between the organic and aqueous phases (O/W ratio) on the average size of polymer-chloroform miniemulsion droplets and solid polymeric nanoparticles, as well as the effect of molar mass of the polymers (PLLA and PCL). Manea *et al*. (2007) investigated the effect of the viscosity on the droplet size caused by the alkyd resin content in the organic phase of a miniemulsion prepared with a high pressure homogenizer system. They concluded that an increase in the viscosity influenced the droplet break up mechanism, leading

to an increase in the droplet and particle sizes.

The objective of this work was to prepare PHBV nanoparticles by the miniemulsification/solvent evaporation technique using chloroform as solvent, hexadecane as costabilizer and sodium lauryl sulfate as surfactant. The PHBV molar mass and concentration and organic phase concentration were varied to evaluate their effects on the initial miniemulsion droplet and final nanoparticle sizes and polydispersity indexes.

MATERIAL AND METHODS

Material

Poly(3-hydroxybutyrate-*co*-hydroxyvalerate) (PHBV, Weight Average Molar Mass – Mw = 369,900 g/mol containing 8.71% hydroxyvalerate) was kindly supplied by PHB Industrial S.A. (Serrana, Brazil). Chloroform, hexane, methanol and sodium borohydride ($NaBH_4$) (Nuclear, P.A. Diadema, Brazil) were used for PHBV purification and reduction of its molar mass. Sodium lauryl sulfate (SLS) was used as the miniemulsion surfactant (Vetec, ultrapure; Duque de Caxias, Brazil) and hexadecane (HD, Vetec, P.A.) as costabilizer. All the reagents were used as received, except PHBV.

Purification of PHBV

A solution of PHBV (5 wt%) in chloroform was prepared by heating the solution for 4 h at 50 °C under magnetic stirring. The solution was kept under stirring for an additional 16 h. In sequence, the solution was filtered under vacuum and precipitated in hexane. Finally, the precipitated PHBV was dried at 60 °C until no mass variation could be detected.

Preparation of Low Molar Mass PHBV

The procedure adopted was described by Baran *et al*. (2002). PHBV (15 g) was dissolved in chloroform (400 mL). $NaBH_4$ (130 mg) was dissolved in methanol (44 mL) and mixed with the PHBV solution. The solution was continuously stirred during 6 h at room temperature. The solution was then precipitated in cold methanol, filtered under vacuum and dried at 60 °C until no mass variation could be detected.

Molar Mass of PHBV

An Ubbelohde type viscosimeter was used to estimate the viscosimetric molar mass (Mv) of PHBV. Five solutions of PHBV in chloroform were prepared with concentrations varying from 0.17 to 0.25 g/dL for purified PHBV and from 0.9 to 2.1 g/dL for low molar mass PHBV. Measurements of the flow time of pure chloroform and of the solutions, by using a chronometer, were employed to estimate relative viscosities. The relative viscosities and solution concentrations were used to calculate inherent, specific and reduced viscosities. The intrinsic viscosity (η_{int}) was obtained by graphical extrapolation to infinite dilution (concentration *versus* reduced viscosity) and applied in the Mark-Houwink Equation (1). The constants for PHBV solutions in chloroform are α=0.82 and K=7.7x10^{-5} dL/g (Baran *et al*., 2002).

$$\bar{M}_V = \left(\frac{\eta_{int}}{k} \right)^{1/a} \quad (1)$$

Gel permeation chromatography (GPC) was used to determine the molar mass distribution and the weight-average molar mass (Mw) and number-average molar mass–(Mn). The measurements were carried out with an apparatus consisting of a Spectra System P2000 pump, an Agilent 1100 autosampler, and a Shodex refractive index RI101 detector. Polymer samples were dissolved in HPLC-grade chloroform at a concentration of 5 mg/ml, filtered through a 0.45 μm Teflon filter and separation was carried out with three columns (0.8 x 30 cm, 10 μm) with different porosities (500 Å, 104 Å, 106 Å) from SDV (PSS, Germany) at room temperature and a flow rate of 1 ml/min. The molar masses were calculated using polystyrene standards.

Nanoparticle Preparation

The formulations of all experiments are presented in Table 1. The organic phase (solution of PHBV in chloroform at 3, 5, 10 e 20 wt%) was prepared by heating the solution to 50 °C for 4 h under magnetic stirring. The solution was kept under stirring for an additional 16 h (T = 25 °C). The chloroform amount was checked after that and completed if evaporation was detected. Hexadecane (amounts varying from 0 to 4 wt% in relation to the organic phase) was added to the organic phase and the stirring was kept during 15 min. The aqueous phase was prepared with water (25 g) and SLS (amounts varying from 1 to 2.5 wt% in relation to the aqueous phase) under magnetic stirring in a 100 mL borosilicate jacketed reactor for 10 min. Then the reactor was connected to a thermostatic water bath to maintain the temperature at 10 °C and the organic phase was added to the reactor under vigorous stirring for 10 min.

Table 1: PHBV nanoparticle formulations as a function of the organic phase (OP) amount and PHBV concentration.

OP (wt%)[a]	PHBV (wt%)[b]	PHBV (g)	$CHCl_3$ (g)	HD (g)	SLS (g)	H_2O (g)
20	LPHBV (0)	0.000	6.250	0.250	0.625	25.000
20	LPHBV (3)	0.188	6.062	0.250	0.625	25.000
20	LPHBV (5)	0.312	5.938	0.250	0.625	25.000
20	LPHBV (10)	0.625	5.625	0.250	0.625	25.000
20	LPHBV (20)	1.250	5.000	0.250	0.625	25.000
30	LPHBV (20)	1.875	7.500	0.250	0.625	25.000
20	HPHBV (3)	0.188	6.062	0.250	0.625	25.000
20	HPHBV (5)	0.312	5.938	0.250	0.625	25.000

[a] wt% relative to the total mass (aqueous and organic phases)
[b] wt% relative to the organic phase mass

Subsequently, the coarse emulsion was subjected to ultrasonication for 10 min at 60 % intensity using a Fisher-Scientific – Ultrasonic Dismembrator 500. The miniemulsion was transferred to an open erlenmeyer flask and maintained at 35 °C for 6 h with gentle agitation to evaporate chloroform and produce PHBV nanoparticles. A schematic representation of the experimental procedure for the preparation PHBV nanoparticles is shown in Figure 1.

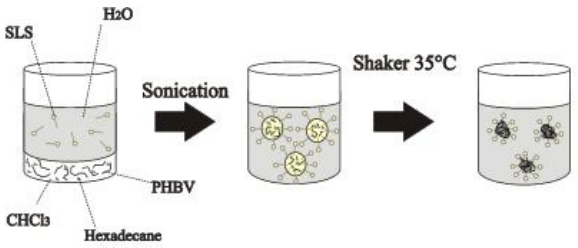


Figure 1: Experimental procedure for PHBV nanoparticles preparation by the miniemulsification/ solvent evaporation technique. (Adapted from Musyanovych *et al*., 2008)

Miniemulsion and Nanoparticles Characterization

The z-average diameter and polydispersity index (PDI) of miniemulsion droplets and of the final polymer nanoparticles were determined by Dynamic Light Scattering (Malvern – Zetasizer - Nano Series) without dilution. These values, measured in duplicates, were calculated from the cumulants analysis and the intensity size distributions by applying a multiexponential fit to the autocorrelation function using a nonnegative least squares (NNLS) algorithm, General Purpose Algorithm. In addition, final polymer particle diameters were also calculated based on the initial average droplet diameters, Equation (2), considering that the particle number remains constant during solvent evaporation and that all solvent is removed:

$$D_P^c = D_D^m \left(\frac{\left(m_{PHBV} / \rho_{PHBV} + m_{HD} / \rho_{HD} \right)}{\left(\begin{matrix} m_{PHBV} / \rho_{PHBV} + m_{HD} / \rho_{HD} \\ + m_{CHCl3} / \rho_{CHCl3} \end{matrix} \right)} \right)^{1/3} \quad (2)$$

where D_P^c and D_D^m are, respectively, the calculated final average particle diameter and the measured initial miniemulsion average droplet diameter and m_i and ρ_i are, respectively, the mass and density of compound i, which is equal to PHBV, HD or chloroform.

Transmission Electron Microscopy (TEM, JEOL JEM-1011) and Field Emission Gun Scanning Electron Microscopy (FEG-SEM, JEOL JSM-6701F) were used to study the morphology of the PHBV nanoparticles. The nanoparticle dispersions were applied to the parlodium-covered TEM grids (300 mesh cooper grids) and on FEG-SEM stubs without dilution. The TEM was operated at a voltage of 80 kV and the FEG-SEM was operated at a voltage of 15 kV.

RESULTS AND DISCUSSION

Effect of PHBV Molar Mass

The average molar masses of PHBV were determined by viscosimetry (Mv) and by GPC (Mn and Mw) prior to and after the molar mass reduction of PHBV using $NaBH_4$. The high and low molar mass PHBV will be referred to in the text, respectively, as HPHBV and LPHBV. As shown in Table 2, the treatment with $NaBH_4$ led to a strong

reduction of the molar mass. This reduction allowed dissolution of a greater amount of PHBV in chloroform, as shown in the next sections.

Table 2: Number-average molar mass (Mn), weight-average molar mass (Mn) and viscosity-average molar mass (Mv) of PHBV.

PHBV	Mn (g/mol)	Mw (g/mol)	Mv (g/mol)
HPHBV	130,330	369,900	214,600
LPHBV	22,600	44,350	22,200

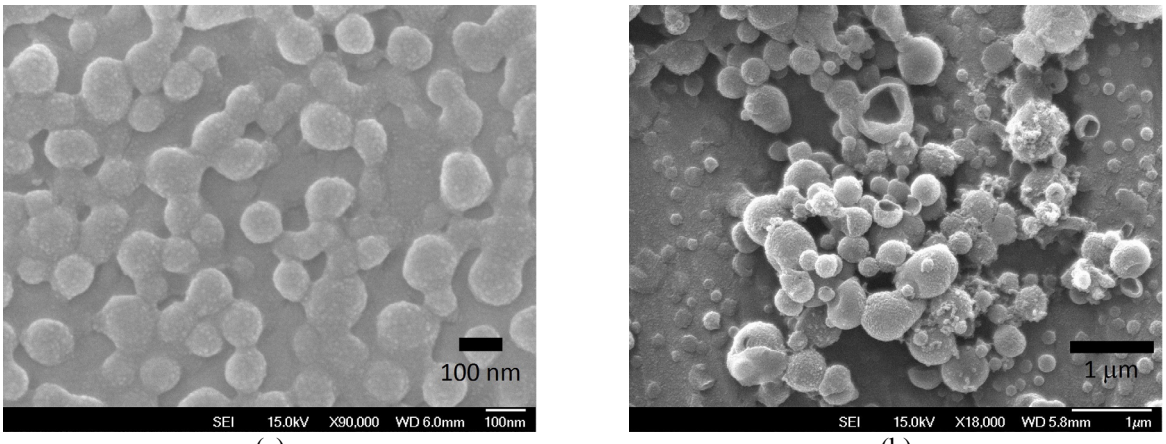
HPHBV and LPHBV were used to prepare nanoparticles, evaluating the effect of the molar mass of the polymer on the nanodroplet dispersion and on the final nanoparticles. The polymer molar mass affects the viscosity of the organic phase, as can be seen from the intrinsic viscosity of dilute chloroform solutions of HPHBV and LPHBV (1.81 and 0.27 dL/g, respectively). When LPHBV was used, the viscosity of the organic phase was lower and considerably smaller particles with a narrower size distributions were obtained for both droplets and nanoparticles, as shown in Table 3.

The high co-stabilizer/polymer (HD/PHBV) ratio, 4/3 and 4/5 (wt/wt) for, respectively, 3 and 5 wt% of PHBV, led to phase separation inside the polymer particles during solvent evaporation, resulting in the formation of nanocapsules (a core-shell morphology with a liquid HD core and a PHBV shell [Romio *et al*., 2009a; Romio *et al*., 2009b]). This can be observed for the larger particles that appear as hollow capsules in the FEG-SEM images of nanoparticles produced with 5 wt% of HPHBV in Figure 2(b). Agreeing with the FEG-SEM images (Figure 2), the particle size distribution obtained by DLS, Figure 3, of HPHBV nanoparticles was composed of two populations, one more pronounced composed of submicrometric particles (around 250 nm) and a second smaller population of considerably larger particles. This result is related to the higher viscosity of the organic phase due to the high molar mass of HPHBV when compared to LPHBV, making the dispersion more difficult [Baran *et al*., 2002]. The particle size distribution of LPHBV was shifted towards smaller sizes (slightly above 100 nm) and does not show a second population of larger particles. This result indicates that, using LPHBV, it is possible to prepare small nanoparticles with a monomodal size distribution.

Table 3: Effect of PHBV molar mass on nanoparticle average diameters and polydispersity (PDI) (20 wt% organic phase, 4 wt% HD, 2.5 wt% SLS).

PHBV (wt%)[a]	PHBV	D_D^m (nm)	PDI_D	D_P^m(nm)	PDI_P
3	LPHBV	246 ± 2	0.20 ± 0.02	116 ± 1	0.13 ± 0.01
	HPHBV	424± 4	0.22± 0.02	222± 2	0.15± 0.01
5	LPHBV	251 ± 2	0.19 ± 0.02	133 ± 1	0.16 ± 0.01
	HPHBV	489± 6	0.32± 0.03	300± 4	0.44± 0.03

[a] Relative to the organic phase; subscript D - Droplets (miniemulsion); subscript P - Final nanoparticles.



(a) (b)

Figure 2: FEG-SEM images of PHBV nanoparticles (5 wt% PHBV, 20 wt% organic phase, 2.5 wt% SLS and 4 wt% HD). (a) LPHBV and (b) HPHBV.

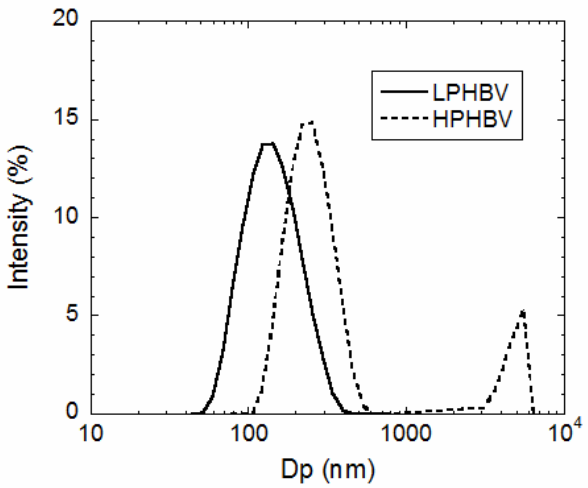


Figure 3: Particle size distribution of LPHBV and HPHBV nanoparticles measured by DLS (5 wt% PHBV, 20 wt% organic phase, 2.5 wt% SLS and 4 wt% HD).

Alternatively, if high molar mass PHBV nanoparticles are desired, the population of larger particles can be reduced considerably with HPHBV if a lower polymer concentration is used, as shown by the particle size distribution results in Figure 4. With the smaller amount of HPHBV (3 wt%), the organic phase viscosity is reduced, demanding less energy to break up the droplets. In addition, the lower viscosity of the dispersed phase with 3 wt% of HPHBV also makes the droplets less tacky, thus reducing the coalescence efficiency.

TEM images of the nanoparticles prepared with 3 and 5 wt% of HPHBV and 4 wt% of HD in Figure 5 show that the particles formed are not completely spherical, confirming the morphology observed in the FEG-SEM analyses in Figure 2(b). The low glass transition temperature of PHBV (Tg = 1 °C [Nanda *et al*., 2011]) could lead to a deformation of the nanoparticles during solvent evaporation or during TEM analysis.

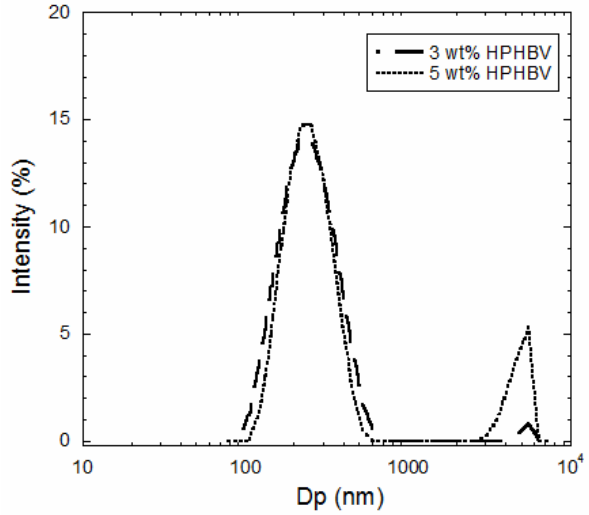
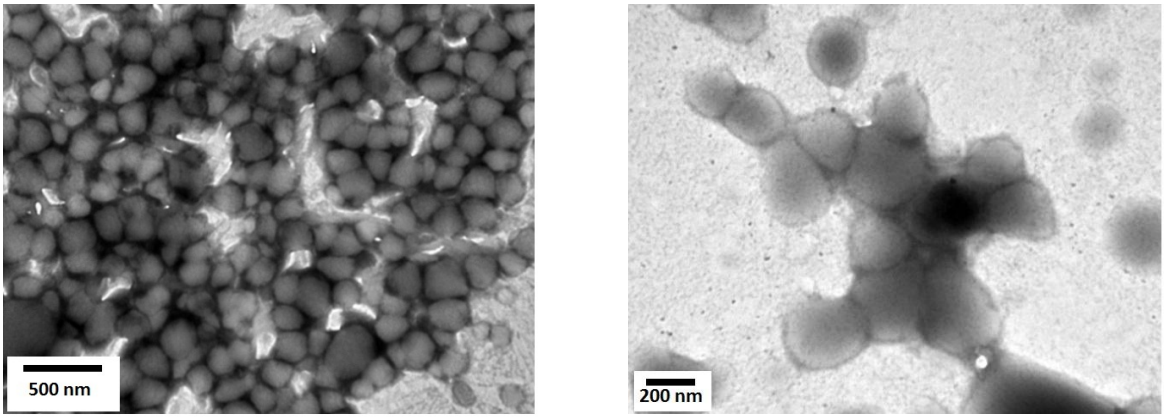


Figure 4: Particle size distribution of 3 and 5 wt % HPHBV nanoparticles measured by DLS (20 wt% organic phase, 2.5 wt% SLS and 4 wt% HD).



(a) (b)

Figure 5: TEM images of HPHBV nanoparticles (20 wt% organic phase, 2.5 wt% SLS and 4 wt% HD) at different magnifications (a) 3 wt% HPHBV; (b) 5 wt% HPHBV.

Effect of LPHBV Concentration and Organic Phase Fraction

The reduction of the molar mass of PHBV allowed the incorporation of larger amounts of polymer into the organic phase (10 and 20 wt%) due to the lower viscosity of the dispersed phase. The results of different LPHBV concentrations (3, 5, 10 and 20 wt%) and the amount of organic phase (OP) on the average diameters of the miniemulsion droplets and the final nanoparticles are presented in Table 4. A "blank" miniemulsion was also prepared without PHBV, using only chloroform (with HD as co-stabilizer).

A general trend observed in the results shown in Table 4 and in Figure 6 is that the increase of the concentration of LPHBV leads to increases in the average particle diameters (Dp) and polydispersity indexes of both the miniemulsion droplets and polymer particles, being more pronounced in the latter. This effect is due to the increase of the viscosity of the organic phase when higher polymer concentrations are used. Similar results were observed in the preparation of PHBV nanocapsules by a double emulsion-solvent evaporation procedure (w/o/w) [Vanderhoff *et al*., 1978] and in the preparation of PLLA nanoparticles by the miniemulsification/solvent evaporation technique [Musyanovych *et al*., 2008]. Compared to the droplets, nanoparticles presented smaller diameters due to chloroform evaporation and the volume reduction of the droplets. In addition, nanoparticles prepared with lower LPHBV concentrations resulted in narrower particle size distributions (as indicated by the polydispersity indexes) than the original miniemulsions. Nanoparticles prepared with higher LPHBV concentrations (20 wt%), on the other hand, had broader particle size distributions than the original miniemulsions. This result possibly indicates that these nanoparticles prepared with higher LPHBV concentrations (20 wt%) coalesced during solvent evaporation due to the higher viscosity of the organic phase that turned the droplets/particles more tacky increasing the coalescence efficiency. In fact, for 20 wt % of LPHBV, the final measured average particle diameter (D_p^m) was 10% higher than the final calculated average particle diameter (D_p^c) shown in Table 4, confirming the coalescence of droplets/particles during solvent evaporation. On the other hand, for up to 10 wt % of LPHBV, the calculated (D_p^c) and measured (D_p^m) final polymer average particle diameters agree quite well, confirming that, for these concentrations, particle number remained constant during solvent evaporation and, thus, coalescence was negligible.

When the organic phase amount was increased from 20 to 30 wt%, there was an increase in PHBV total amount in the formulation, generating a higher concentration of droplets favoring coalescence and generating larger and more polydisperse particles.

Table 4: Effect of the organic phase (OP) amount and LPHBV concentration on nanoparticle average diameters and polydispersity (4 wt% HD, 2.5 wt% SLS).

OP (wt%)	LPHBV [a] (wt%)	D_D^m (nm)	PDI_D	D_p^m (nm)	PDI_P	Calculated D_p^c (nm)	Relative error (%)
20	0	210 ± 1	0.20 ± 0.02				
	3	246 ± 2	0.20 ± 0.02	116 ± 1	0.13 ± 0.01	119	-2.75
	5	251 ± 2	0.19 ± 0.02	133 ± 1	0.16 ± 0.01	131	1.91
	10	232 ± 1	0.22 ± 0.03	137 ± 1	0.23 ± 0.01	137	0.00
	20	267 ± 1	0.24 ± 0.02	203 ± 3	0.42 ± 0.03	184	10.5
30	20	341 ± 3	0.24 ± 0.03	240 ± 2	0.54 ± 0.02	235	2.25

[a] Relative to the organic phase; subscript D - Droplets (miniemulsion); subscript P - Final nanoparticles; superscript m – Measured; superscript c - Calculated final nanoparticle diameters based on the initial average droplet diameters considering that the particle number remains constant during solvent evaporation and that all solvent is removed.

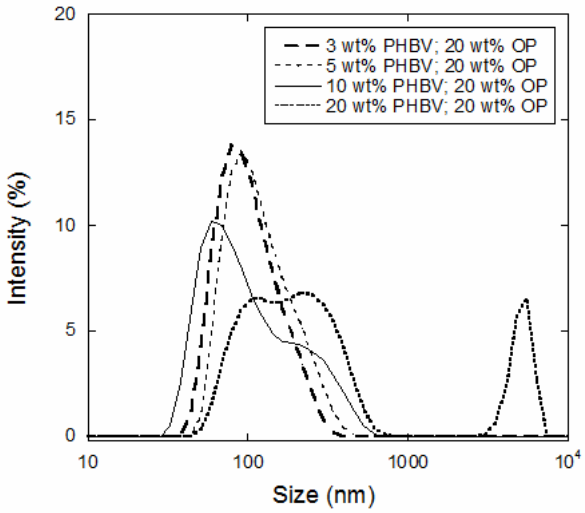


Figure 6: Effect of LPHBV concentration on the particle size distribution of nanoparticles measured by DLS (20 wt% organic phase, 2.5 wt% SLS and 4 wt% HD).

CONCLUSIONS

Submicrometric PHBV particles were prepared with success by the combination of mini-emulsification and solvent evaporation techniques. The effects of PHBV molar mass and concentration, costabilizer concentration and organic phase amount on the formation of PHBV nanoparticles were evaluated. Low molar mass PHBV (Mw = 44,350 g/mol) was obtained by treating high molar mass PHBV (Mw = 369,900 g/mol) with sodium borohydride. It was possible to obtain nanoparticles with narrow size distribution and increase the amount of organic phase in the experiments when PHBV of low molar mass was used, allowing the incorporation of a larger amount of polymer in the miniemulsions. Particle sizes increased with increasing PHBV concentration due to viscosity effects. Furthermore, nanocapsules were formed when 4 wt% of costabilizer and 5 wt% PHBV were used, resulting in a different type of morphology.

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