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# PHBHV/PCL microspheres as biodegradable drug delivery systems (DDS) for photodynamic therapy (PDT)

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**Abstract** Unloaded microspheres were prepared from polyhydroxybutyrate-co-valerate (PHBHV) and poly(ε-caprolactone) (PCL) polymers using the emulsification-solvent evaporation method (EE). The study was conducted to determine the ideal polymeric composition and ideal molecular weight for the microspheres preparation to be used as a Drug Delivery System (DDS) for cancer therapy. In this work, NzPC, a new photosensitizer, has been investigated when incorporated into microspheres of PHBHV/PCL evaluating its application for Photodynamic Therapy (PDT) of neoplastic tissue. The biodegradation studies were conducted to analyze the effects of the incorporation of the NzPC and also to determine the release profiles *in vitro* condition. We also evaluated the dark toxicity and the photobiological effect of the PHBHV-PCL microspheres in cutaneous melanoma cell line (B-16-A1) used as a biological neoplastic medium.

## Introduction

Photodynamic therapy (PDT) is a viable treatment modality for a variety of tumors, as well as for selected non-oncologycal diseases [1]. The therapy starts with the administration of the drug (known as a photosensitizer), which then accumulates selectively in the tumor tissue. Posterior illumination with visible light conducts the photosensitizer to its first excited singlet state and then by intersystem crossing, reaches the triplet excitable state, by the classic photochemistry pathway described in the Jablonski Diagram. From this excitable state the molecules could react to two different types of mechanism; a Type I (electron transfer process with biological subtracts to form radicals or radicals ions) or by a Type II (energy transfer process) interacting with the ground triplet state of molecular oxygen, resulting in highly reactive singlet oxygen species which is regarded as the key reactive oxygen species (ROs) that kills tumors [2, 3].

The efficiency of a photosensitizer in sensitizing cells to photoinactivation is dependent upon its ability to be taken up by cells, as well as by its photochemical properties. In order to induce a more specific localization of the photosensitizer in the tumor target tissue, DDS are currently used in PDT, such as micro and nanospheres, which can improve the selectivity of anti-tumor sensitizers [4].

Polymer blends containing biodegradable components or hydrolytically unstable components are of great interest because of the way in which their physical properties and degradation characteristics can be tailored. These materials have attracted quite a lot of attention, especially in relation to their applications in devices and biomedical uses [5].

Aliphatic polyesters such as poly(glicolic acid), D–L and poly(L-lactic acid), poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(ε-caprolactone) are the most important biodegradable polymers with frequent application as biodegradable products in prosthesis and controlled drug release products [6]. Embleton and Tighe [7] demonstrated how some characteristics of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

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microspheres such as yield, shape, and surface morphology were affected by the methodology of preparation and from some variables such as molecular weight and 3-hydroxyvalerate content used in the polymer synthesis, together with the temperature at which the organic solvent evaporation was conducted. Furthermore, Re et al. [8] showed that PHBHV microspheres porosity changed by blending the developed poly(3-hydroxybutyrate-co-3-hydroxyvalerate) polymer with poly(ε-caprolactone) PCL, concluding that PHBHV/PCL microspheres are a good approach to achieve biodegradable microspheres with controlled porosity.

The use of microspheres as DDS *in vivo* studies allows the understanding of the influence of physical–chemical, photobiological and photochemical factors which will control the *uptake* of photosensitizer by tissues, their mechanism of action and the subsequent photoinduced necrosis or apoptosis in neoplastic tissue. In this study, we have investigated the use of biodegradable PHBHV/PCL microspheres to encapsulate a phthalocyanine drug, NzPC, to be applied for PDT. We also evaluate its photobiological activity in cells, B-16 neoplastic cell line was used as biological model.

## Experiment

### Preparation of NzPC-loaded PHBHV/PCL microspheres

The choice of a specific DDS is critical to maximize the photodynamic action and pharmacokinetic properties of many photosensitizer dyes used in academic and clinical trials. PHBHV/PCL microspheres were synthesized for an EE previously described by Maia et al. [9] using different blend compositions: 90%(PHB-9.8%HV):10%PCL 273 kDa (A); 80%(PHB9.8%HV):20%PCL 23 kDa (B) and 90%(PHB-9.8%HV):10%PCL 23 kDa (C). Figure 1 shows the scheme of the preparation used from PHBHV/PCL microspheres.

### Biodegradation studies

The stock solution of NzPC/PHBHV/PCL used was prepared at a final concentration of 1.0 mM in phosphate buffer (PBS), pH 7.4. Fluorescence spectra were recorded with a Fluorolog-3 SPEX Ivon spectrofluorimeter. NzPC was excited with 612 nm and the fluorescence emission was recorded in the 650–800 nm range. Bandwidths were fixed at 5 nm for excitation and emission.

The measurements of kinetics of degradation of the microspheres were made under constant agitation and controlled temperature (37 °C) in human plasma serum obtained from a healthy donor from the Hemocentro of the

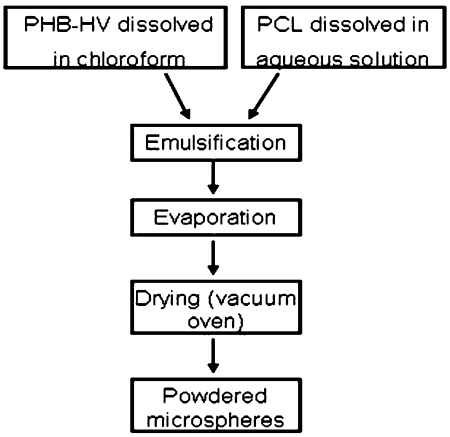


**Fig. 1** Scheme of preparation using the emulsification-solvent evaporation (EE) method from polyhydroxybutyrate-co-valerate/poly(ε-caprolactone) (PHBHV/PCL) microspheres

São Paulo University Hospital. The choice of this environment for studies is that this medium mimics very well some of the biological conditions in vivo. The solutions of NzPC/PHBHV/PCL in human plasma remained 72 h under the conditions described above.

Fluorescence measurements in the maximum wavelength of the compound (located at 682 nm, Fig. 2) were realized at intervals of two in 2 h (first 12 h) and six in 6 h (the last 36 h).

### Growth of B-16 cells

The cell line used in this study was the mouse melanoma B-16 supplied by ATCC. B16 is a melanoma cell line derived from B6 mice. Monolayer cultures of B-16 were grown in Dubelco Eagle's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (Gibco), 1% L-glutamine, and 1% penicillin–streptomycin (Gibco). The cells were used in the logarithmic phase of growth and maintained at 37 °C in humidified 5% $CO_2$ and 95% air.

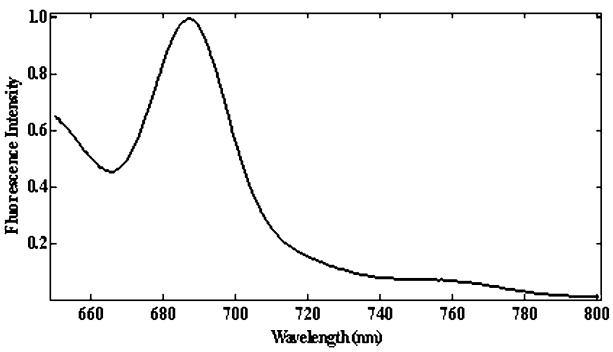


**Fig. 2** Fluorescence emission spectra of NzPC/PHBHV/PCL 3.0 mM in human plasma

### Cytotoxicity and photobiological assays

For the biological test, 96 wells microplates were employed. The methodology used to evaluate the toxicity and phototoxicity was the classic MTT assay. The tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] produced the highly colored formazan dye upon NADH reduction, which reflects a living cellular dehydrogenase [10].

To investigate the toxicity, suspension of $5 \times 10^3$ B-16 cells in 200 μL of medium were inoculated into each well in a 96-well microplate. After culturing in a $CO_2$ incubator for 24 h, monolayer cultures of B-16 were treated with the blends A, B, and C at a concentration of 3.0 μM. After incubation for 3 h, the cells were washed twice and the volume was completed with the addition of 200 μL DMEM in each well for 24 h. The 1.0 mg/mL MTT solution (50 μL per well) was added to the cells in 96-well plates, followed by incubation for 4 h, at 37 °C. Consequently, the crystals formed due to the interaction between the mitochondrial dehydrogenases and the MTT reagent was dissolved with 2-propanol and the samples were shaken until complete dissolution of the formed product. After the color reaction was finished, the absorbance at 560 and 690 nm of each well was measured with Safire II (TECAN). The percentage of cellular viability was calculated with respect to the control cells incubated without photosensitizer.

In the phototoxicity test, three different doses (1.0, 5.0 and 10.0 J/cm$^2$) were used of visible light irradiation at 675 nm using a diode laser *Eagle* (Quantum Tech, Brazil). After incubation for 3 h, the cells were washed twice with PBS and fresh DMEM was added before cell irradiation. Results are given as the percentage of the results obtained from the control cultures exposed to samples (A, B, and C) alone (in absence of irradiation).

All experiments were done in duplicate and the statistical significance ($p$-value) was determined using Student's $t$-test. $p$-Values <0.05 mean the results are significant.

## Results and discussion

### Production of NzPC-loaded PHBHV/PCL microspheres

The microspheres were obtained by the emulsification-solvent evaporation method. The particles obtained starting from the different blends presented medium size of 30 μm and a spherical form as shown in Fig. 3. The drug release inside the cells is dependent on the interaction of these with the DDS [11, 12]. Considering our result with these DDS and comparing with the classic liposomal system, the microspheres produced showed an increase in size, however, this is not a problem considering that

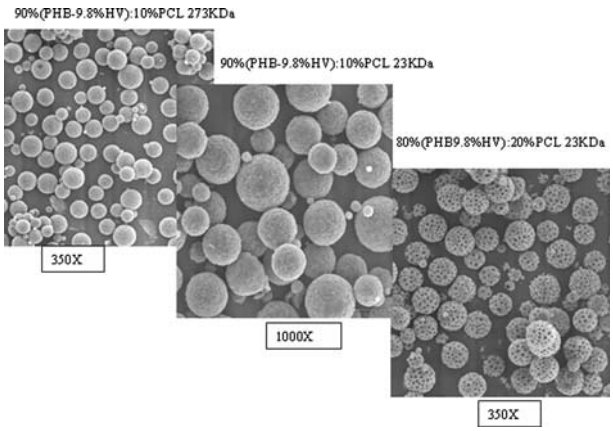


**Fig. 3** PHBHV/PCL microspheres obtained by emulsification-solvent evaporation method using different blend percentages

microparticles such as DDS still present a size in the range of cellular dimension. Therefore, we believe that the DDS proposed in this work will be efficient in *uptake* in all the cellular processes, allowing maximizing of the extension of the photodynamic damage with a sustainable release of the drug that was usually requested for cancer therapy.

Analyzing these data, it can be observed that a larger percentage of PCL induces a larger porosity, an important factor that will influence the release profile presented by these DDS.

### Biodegradation measurements

The stock solutions of NzPC/PHBHV/PCL in human plasma were set into quartz cuvettes under constant agitation and controlled temperature at 37 °C. Fluorescence emission measurements were made at predetermined intervals of 2 h.

From a comparative study between the samples A and B we observed that the sample with smaller molecular weight presented a release profile showing small initial bursts with increased release, reaching the maximum at 24 h (Fig. 4a). In another study between the samples B and C we could observe the best blend composition with a better release profile in the sample B. This sample presented a good initial burst with an increased release reaching the maximum at 24 h (Fig. 4b).

All these studies lead to the ideal composition for microspheres used as DDS in PDT: low molecular weight and high blend percentage. These results confirm that in the biological medium the amount of dye and time releasing was accelerated reaching higher drug level in the target tissue as expected for a drug delivery system proposed for PDT treatment. It also provides basic

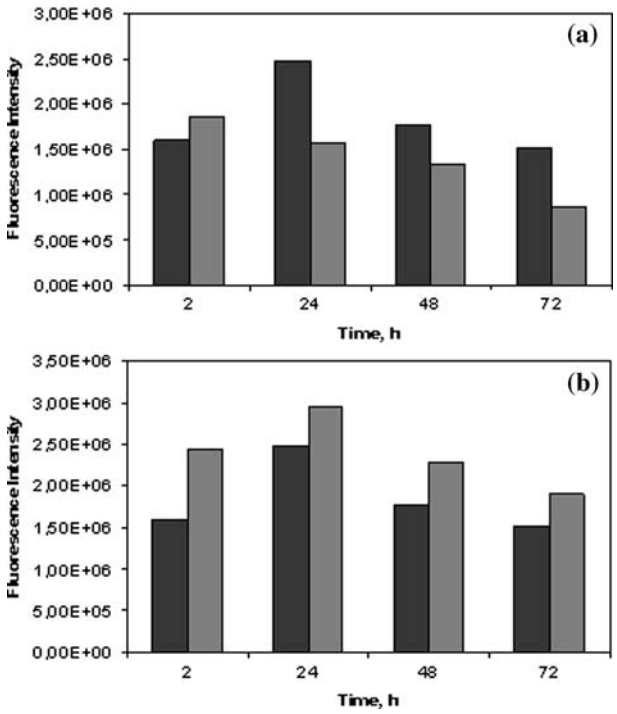


**Fig. 4** Fluorescence measurement of the drug release (at 682 nm) from NzPC/PHBHV/PCL microspheres in human plasma serum

information about the action of NzPC after its incorporation into the PHBHV/PCL microspheres. This is another important characteristic presented by microsphere systems when compared with other DDS, since liposomal systems have been shown to present a very short circulation time due to the fast clearance process from the body considering the endothelial system (RES) phagocytosis activity [13].

Biologicals assays

From the in vitro dark toxicity assay by MTT for the NzPC-loaded PHBHV/PCL microspheres, a dark toxicity around 5–8% was found, values well accepted as a basal level for this kind of cell. This suggested no cytotoxicity of the NzPC-loaded PHBHV/PCL microspheres. The data of dark toxicity is reduced by approximately 10% when compared with other DDS used in PDT [14], a factor that reinforces the use of the system in studies as a promising DDS with good applications in this therapy when combined with light. The phototoxicity assay (after light irradiation at three different doses normally used in clinical trials), showed that the system that presented a better response in the light treatment was the sample B, indicating the ideal composition for microspheres in agreement with the results obtained in the biodegradation studies (Fig. 5).

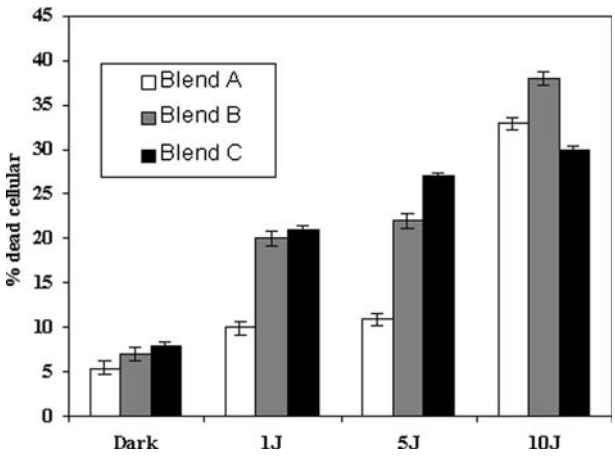


**Fig. 5** Cellular viability from B-16 cells after the treatment with PHBHV/PCL microspheres in the absence and presence of the light

## Conclusions

By the conventional administration routes in vivo it is not possible to use very high doses of drugs due to their normally secondary toxic effects. Therefore, in order to improve the accumulation of drugs in the target tissue (at the tumor level), different DDS, such as microspheres present good results. PHBHV/PCL microspheres suspensions are suitable for entrap drugs using the emulsification-solvent evaporation method and produce a biodegradable DDS and could be used for fast and reliable initial cancer detection (photodiagnostic) and PDT profiles. The in vitro studies indicated the low cytotoxicity of the DDS and good cellular response of this system in the light treatment.

All results confirm that in the biological medium the amount of dye time release was accelerated reaching a higher drug level in the target tissue after 24 h, as expected for a good drug delivery system proposed for PDT treatment, and also providing basic information about the action of NzPC after its incorporation into the PHBHV microspheres.

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