

## Composite PHB/Chitosan Microparticles Obtained by Spray Drying: Effect of Chitosan Concentration and Crosslinking Agents on Drug Release

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Micropartículas de poli(3-hidroxibutirato) (PHB) contendo cetoprofeno (KET) como fármaco modelo foram preparadas através da técnica de emulsão-evaporação do solvente O/W. Com o intuito de atribuir uma barreira adicional à liberação do cetoprofeno, micropartículas de KET/PHB foram revestidas por um filme de quitosana através da técnica spray drying. O filme de quitosana foi reticulado com glutaraldeído ou genipin. A eficiência de encapsulação, 60%, foi da mesma ordem de grandeza para todas as formulações de micropartículas estudadas. A influência das concentrações de quitosana e do agente reticulante (glutaraldeído e genipin) na quantidade de cetoprofeno liberado após 1 h, e sobre o prolongamento de liberação em 72 h, foi avaliada através de análises estatísticas, indicando que ambas as variáveis influenciaram as respostas. A liberação do cetoprofeno a partir de micropartículas compostas recobertas com quitosana reticulada foi lenta e sustentável, sendo um transportador polimérico muito promissor para a liberação de fármacos.

The purpose of this study was to prepare composite microparticles of poly(3-hydroxybutyrate) (PHB) containing the drug ketoprofen (KET) coated with a layer of crosslinked chitosan (CHI) for application as a controlled drug-release system. Microparticles of PHB containing KET as a model drug were prepared using the emulsion-solvent evaporation technique, and coated with a film of chitosan by spray drying to obtain the composite microparticles. The surface film was modified using glutaraldehyde or genipin as the crosslinking agent. The KET encapsulation efficiency of the PHB microparticle was 60%, and the same value was obtained after inclusion of the CHI film by the spray drying process. The influence of the concentration of chitosan used to obtain the composite microparticles and the crosslinking agent on the amount of drug released after 1 and 72 h was evaluated by statistical analysis, and both variables were found to affect the responses. The drug release from the composite microparticles coated with crosslinked chitosan was slow and sustainable, indicating that this represents a very promising polymeric carrier for drug delivery.

**Keywords:** controlled drug release, chitosan, poly(3-hydroxybutyrate), genipin, glutaraldehyde

### Introduction

The technology of controlled drug release involves multidisciplinary aspects and can greatly contribute to advances in the area of human health. New strategies have been investigated for the preparation of controlled-release drug delivery systems based on composite microparticles,

because of their local and long-term healing ability.<sup>1</sup> The use of polymer microparticles generally leads to a high initial drug release and due to this 'burst effect' the release cannot be sustained for long periods. This burst release may be prevented by developing more complex drug-loaded delivery systems, such as composite microparticles containing multiple cores of one polymer dispersed in a second continuous polymeric matrix (reservoir-type) or core-shell microparticles consisting of a single core

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surrounded by a polymeric layer. These systems present additional barriers to drug diffusion, reducing the burst effect and prolonging the drug release.<sup>2</sup>

Natural polymers, such as poly(3-hydroxybutyrate) (PHB) and  $\beta(1\rightarrow4)$ -2-amino-2-dioxo-*D*-(glucopyranose) (chitosan), have gained increasing importance in the development of controlled-release drug delivery systems and implantable biomaterials. PHB is a biodegradable and biocompatible polyester synthesized by numerous bacteria as an intracellular carbon and energy storage compound, offering a high potential for application in drug-delivery systems such as microparticles. However, its high degree of crystallinity leads to the formation of porous microspheres, which influences the drug release.<sup>3,4</sup>

Chitosan (CHI) is a polysaccharide obtained from chitin, which occurs principally in the exoskeletons of insects and shells of crustaceans. Various drug delivery systems have been developed using chitosan, due to its excellent biocompatibility, biodegradability, bioactivity, mucoadhesive property and non-toxicity.<sup>5,6</sup> However, as it is soluble at pH values below 6, it requires crosslinking in order to modify certain properties of the biopolymer, such as chemical and thermal stability, structural strength, permeability and the ability to modulate the release of active agents.<sup>7,8</sup>

Composite systems are often employed in the design of drug delivery platforms to inhibit the so-called burst effect, in which there is a great amount of drug released in the early phase of the process. In this regard, the combination of the biopolymer PHB with chitosan has been reported as an efficient system for prolonged release.<sup>4</sup>

Several techniques can be used to prepare polymeric microparticles. The choice of the technique depends on the characteristics of the polymer, the drug and the intended use. Emulsion-solvent evaporation is the method most frequently used to prepare microspheres. In this process, the drug and the polymer are dissolved in an organic phase, which is emulsified in an aqueous phase containing a stabilizing agent, under stirring.<sup>3</sup> Spray drying is often used as an encapsulation technique. The principle of spray drying by nebulization is based on the atomization of a solution, containing drugs and carrier molecules, by pumping compressed air through a desiccating chamber, and using a current of warm air for the drying process. Spray drying usually leads to a broad (Gaussian) particle size distribution. The flow rate, nozzle geometry and solution viscosity are the parameters with the greatest influence. In contrast to coacervation, emulsification and freeze-drying methods, the spray drying method is a fast one-step process, and is continuous, easy to scale-up, and inexpensive.<sup>9</sup>

A variety of reagents have been used to crosslink chitosan, including glutaraldehyde, tripolyphosphate,

ethylene glycol, diglycidyl ether and diisocyanate.<sup>10,11</sup> However, studies have shown that the synthetic crosslinking reagents are all cytotoxic (to greater or lesser degrees) and may thus impair the biocompatibility of a chitosan delivery system.<sup>12</sup> Also, some researchers have evaluated the crosslinking of chitosan microparticles with glutaraldehyde as well as glyoxal for the controlled delivery of centchroman, a non-steroidal contraceptive.<sup>8</sup> This study demonstrated that the drug release rates may be affected not only by the degree of crosslinking of the microspheres but also by the type of crosslinker used. While these studies indicate promising results for controlled drug release from chitosan microspheres with the use of crosslinking, concerns remain over the toxicity of the crosslinking reagents used, especially glutaraldehyde, with regard to the biocompatibility of the chitosan delivery system.<sup>13</sup> Hence, it is desirable to provide a crosslinking reagent for use in biomedical applications that has low cytotoxicity and that forms stable and biocompatible crosslinked products.<sup>14,15</sup>

Genipin is a natural water-soluble crosslinker obtained from geniposide, a traditional component in Chinese medicine, and it is isolated from the fruits of the plant *Gardenia jasminoides Ellis*.<sup>16</sup> Sung *et al.* tested genipin as a reagent for the crosslinking of collagen. They found that genipin was 10,000 times less cytotoxic than glutaraldehyde. It has been used as a crosslinking reagent for the fixation of biological tissues in bioprostheses.<sup>17</sup> The biocompatibility of genipin-fixed tissues has been evaluated in several animal studies.<sup>15</sup> It was consistently noted that the inflammatory reaction of the genipin-fixed tissues was significantly less than that of their glutaraldehyde-fixed counterparts, which indicates that it is appropriate for use in biomaterials.

The aim of this study was to obtain PHB/ketoprofen (KET) microparticles coated with a crosslinked chitosan film and compare the drug release of these composite microparticles with samples without the chitosan film coating. The effect of the crosslinking agent (glutaraldehyde or genipin) and concentration of aqueous chitosan solution used in the spray drying on the drug release was also evaluated.

## Experimental

### Materials

Poly(3-hydroxybutyrate) ( $M_n$  of 312,800 g mol<sup>-1</sup> and polydispersity degree of 1.23, determined by gel permeation chromatography) was kindly supplied by PHB Industrial S. A. (Serrana, São Paulo, Brazil). Ketoprofen

(KET) was purchased from All Chemistry (São Paulo, Brazil), chitosan (CHI) (medium molecular weight and deacetylation degree of 75%) from Sigma-Aldrich (USA) and poly(vinyl alcohol) (PVAL) ( $M_n$  of 92,000 g mol<sup>-1</sup> according to the manufacturer) from Vetec (Rio de Janeiro, Brazil). Glutaraldehyde was acquired from Nuclear (São Paulo, Brazil) and genipin (purity > 98%) from Challenge Bioproducts (Taiwan). All chemicals were used without further purification.

#### Preparation of drug-loaded PHB microparticles

PHB (500 mg) and KET (200 mg) were dissolved in dichloromethane (oil phase or internal phase) and then emulsified in 200 mL of an aqueous solution containing 0.1% (m/v) PVAL as a stabilizer and 6% (v/v) isopropanol (aqueous or external phase), selected based on previous studies by our research group.<sup>3</sup> The emulsion was kept under stirring at 600 rpm, at ambient temperature, until the complete evaporation of the organic solvent. The microparticles were washed with distilled water, removed from the water by decantation and dried at room temperature.

#### Preparation of PHB/KET-CHI composite microparticles by the spray drying technique

In order to obtain composite microparticles of PHB/KET-CHI, 1 g of PHB/KET microparticles was dispersed in an aqueous solution of chitosan (1% v/v acetic acid), at 1.0, 1.5 or 2.0% (m/v). The dispersion was then pulverized and dried in a spray dryer (Büchi Mini Spray Dryer B-290, Büchi Inc.) applying the following conditions: inlet temperature of 180 °C, outlet temperature of 50 °C, feed flow of 6 mL min<sup>-1</sup>, drying air flow rate of 35 m<sup>3</sup> h<sup>-1</sup>, and air compressor pressure of 0.7 MPa.<sup>18</sup> Under these conditions, the solvent was removed and the dried powder samples were collected from the base of the cyclone.

#### Crosslinking of the chitosan in the composite microparticles

Composite microparticles of PHB/KET-CHI were immersed in an aqueous solution of glutaraldehyde or genipin, to obtain a final composition ratio of 1:10 (1 mol of crosslinking agent to 10 monomeric units of chitosan), at room temperature.<sup>11</sup> In the glutaraldehyde aqueous solution, the PHB/KET-CHI microparticles were maintained in phosphate buffer pH 7.4, under stirring for 1 h.<sup>4</sup> The genipin aqueous solution was prepared in 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), at pH 10.0.<sup>15</sup> The microparticles were then washed three times with distilled

water to remove the free crosslinking agent and dried at room temperature.

#### Scanning electron microscopy (SEM)

The morphology of the microparticles before and after drug release was analyzed by scanning electron microscopy (SEM), with a Philips XL30 microscope. The samples were coated with gold in a Bal-Tec Sputter Coater SCD005.

#### Particle size determination using a Mastersizer analyzer

The granulometric distribution of the microparticles before and after coating with chitosan was determined by laser diffraction using a Mastersizer 2000 particle analyzer (Malvern Instruments, UK). For the analysis, the sample was pre-dispersed in water and added to the dispersing environment in the Hydro 2000SM apparatus until a laser obscuration index of 10 to 11% was reached. The microparticles were analyzed in triplicate and their size distribution was determined based on the Fraunhofer diffraction theory. This parameter is expressed as equivalent volume diameters at 10% ( $d_{10\%}$ ), 50% ( $d_{50\%}$ ) and 90% ( $d_{90\%}$ ) of the cumulative volume, as the average of the diameter values  $D_{4,3}$  and span. The span value indicates the particle polydispersity and it was calculated according to equation 1:<sup>19</sup>

$$Span = \frac{d_{90\%} - d_{10\%}}{d_{50\%}} \quad (1)$$

#### Determination of encapsulation efficiency (EE%)

The encapsulation efficiency is defined as the percentage difference between the initial active agent concentration of the formulation and the drug concentration retained within the particles.

To determine the amount of encapsulated KET, 10 mg of the microparticles were accurately weighed and maintained in 10 mL of chloroform for 72 h, under stirring.<sup>3</sup> The solution was diluted to obtain a drug concentration equivalent to 10 mg L<sup>-1</sup>, and the absorption band was determined by UV-Vis spectrophotometry (Shimadzu 1601 PC) at 254 nm. The encapsulation efficiency was obtained using equation 2, denoted by EE%. The microsphere drug content was then estimated and expressed as mg %.

$$EE\% = \frac{\text{drug found in microparticle (mg)}}{\text{drug initially added to the formulation (mg)}} \times 100 \quad (2)$$

## Determination of amine groups present in the chitosan macromolecule

Initially, to obtain the ninhydrin solution two different solutions were prepared: (i) 1 mL of concentrated acetic acid, 10 mL of NaOH (1.0 mol L<sup>-1</sup>) and 0.04 g of SnCl<sub>2</sub> were mixed and the volume of 25 mL completed with distilled water; and (ii) 1 g of ninhydrin was added to 25 mL of ethylene glycol and kept under stirring until complete solubilization. The two solutions were then mixed and stored in an amber vial.<sup>13</sup>

For the assay, 10 mg of PHB/KET-CHI microparticles were added to 4 mL of ninhydrin solution (pH 3.5), and maintained for 20 min at 100 °C, after which the microparticles were separated by centrifugation. The absorbance was then measured at room temperature by UV-Vis spectrophotometry at 570 nm. The percentage of free amine groups present in the microparticles after the crosslinking process was calculated based on the ratio between the absorbance values for the free amino groups in the crosslinked microparticle ( $Abs_{cross}$ ) and in the non-crosslinking microparticles ( $Abs_{uncross}$ ), as shown in equation 3:<sup>13</sup>

$$\% \text{ of free amine groups} = \frac{Abs_{cross}}{Abs_{uncross}} \times 100 \quad (3)$$

## *In vitro* drug release

*In vitro* drug release studies were carried out in phosphate buffer solution pH 7.4 at 37 °C, under constant stirring. The *in vitro* release of KET was carried out in order to simulate the intestinal transit.<sup>20</sup> An amount of microspheres containing 10 mg of the drug was placed into 45 mL of phosphate buffer solution and maintained in a thermostated bath for one week. After pre-determined time intervals samples were withdrawn and immediately returned to the dissolution vessels after analysis. The KET concentration was measured by UV-Vis spectrophotometry, at 260 nm. At the end of the assay, the microparticles were washed with distilled water, centrifuged, dried under vacuum and the morphology evaluated as previously described.

## Statistical analysis

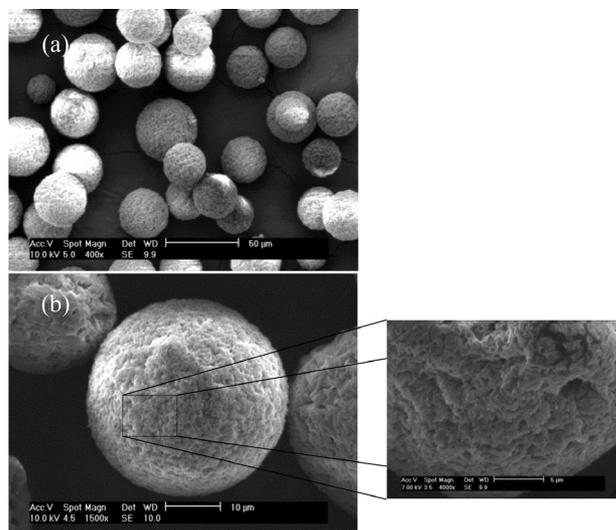
In order to evaluate the influence of the concentration of chitosan used in the preparation of the composite microparticles and of chitosan crosslinking on the drug release process, analysis of variance (ANOVA), followed by application of the Tukey test when significant differences were indicated ( $p < 0.05$ ), was carried out using the software

Graph Pad Prism®. The percentage of drug release in 1 h and the area under the curve (AUC) at 72 h were used to compare the drug release profiles.

## Results and Discussion

### PHB/KET-CHI composite microspheres

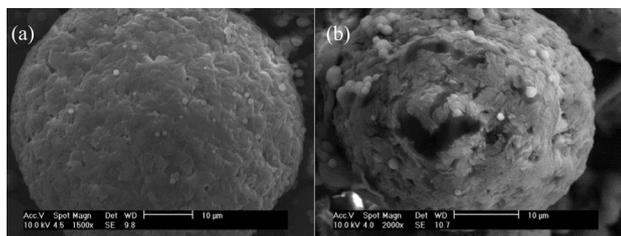
Figure 1 shows the SEM micrographs of the PHB/KET microparticles, which had a spherical shape and rough surface morphology as can be seen in Figure 1b (higher magnification). The roughness of the polymeric matrix is related to the high degree of PHB crystallinity and is normally observed in PHB microparticles prepared through the technique of emulsion-solvent evaporation.<sup>21</sup>



**Figure 1.** SEM micrographs of the PHB/KET microparticles.

The micrographs of composite microparticles obtained after the spray drying process, using chitosan solution at a concentration of 1.5%, show a surface without roughness with small adhered particles of chitosan, as shown in Figure 2a. The surface of the composite microparticles was smoother than that of the PHB/KET microspheres, which is a characteristic of chitosan microspheres,<sup>22,23</sup> indicating that the spray drying process was suitable for coating the PHB/KET microparticles with a chitosan film. This characteristic surface morphology changed after the drug release process, due to the degradation of the chitosan film formed on the PHB/KET microparticle (Figure 2b).

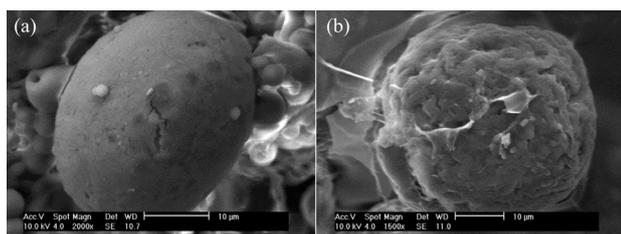
Figure 3 shows the SEM micrographs of the composite microparticles after the crosslinking of the chitosan film formed on the PHB/KET microparticles. As expected, the morphologies of the surfaces of composite microparticles after crosslinking showed roughness. However, the



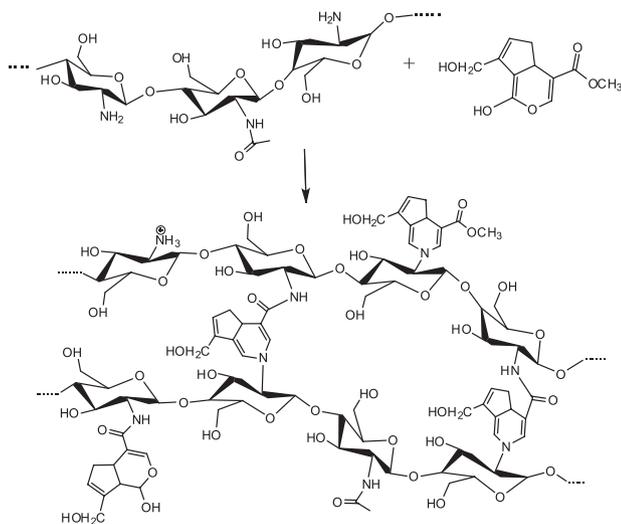
**Figure 2.** SEM micrographs of the PHB/KET-CHI obtained by spray drying using chitosan solution of 1.5 %; (a) before and (b) after drug release.

morphology of composite microparticles after crosslinking shows a reduction in the surface roughness (Figure 3b), due to a strong network formed through the chemical reaction of amine groups of the chitosan unit with genipin, as shown in Figure 4. On the other hand, the crosslinking process promoted the coalescence of the small microparticles of chitosan present in the medium. Analogous behavior was observed for composite microparticles crosslinked with glutaraldehyde.

The adhesion of the PHB to the chitosan macromolecule was due to the formation of hydrogen bonds between the carbonyl groups of the polyester and the hydroxyl and amino groups of the chitosan.<sup>24,25</sup>



**Figure 3.** SEM micrographs of composite microspheres of PHB/KET-CHI, showing the chitosan film (a) before and (b) after crosslinking process with genipin.



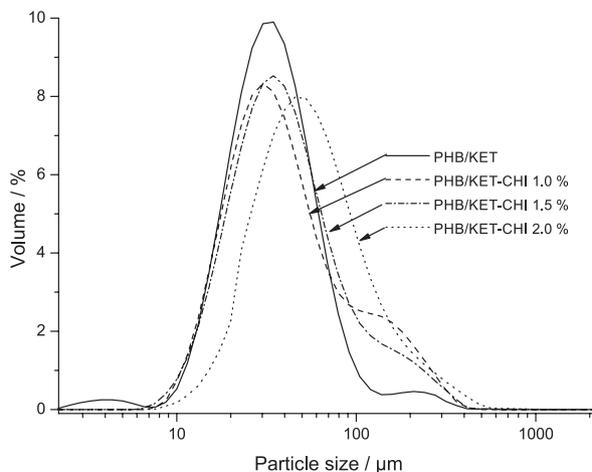
**Figure 4.** Schematic representation of crosslinking reaction between genipin and amine groups of chitosan macromolecule.

The mechanisms of chitosan crosslinking with genipin have been studied by many researchers. Yuan *et al.* proposed that under basic conditions the ring-opening reaction of genipin occurs via a nucleophilic attack by OH<sup>-</sup> in aqueous solution to form intermediate aldehyde groups and subsequent ring-opening polymerization via aldol condensation.<sup>13</sup> Butler *et al.*<sup>25</sup> studied the mechanism of the crosslinking between genipin and chitosan and two mechanisms have been proposed to explain this reaction. One is a slower reaction, which involves a nucleophilic carbon of the genipin ester group being replaced by a secondary amide with the release of methanol. The other mechanism is a reaction that occurs through a nucleophilic attack of the primary amine at the genipin C3 carbon, forming an intermediate aldehyde.<sup>25</sup>

The complete reaction (Figure 4) involves the amine groups of chitosan, where the genipin structure is interconnected with the highly stable intermolecular cross-covalent bonds linking the chitosan chains.

#### Particle size analysis

Figure 5 shows the curves for the size distribution of the PHB/KET microparticles as a function of volume percentage before and after the coating with chitosan at a specific concentration by the spray drying technique. The dimensions and granulometric distribution are important parameters in the characterization of the microparticles since they are directly correlated with the release rate and the conditions of the administration procedure.<sup>26</sup>



**Figure 5.** Microparticle size distributions for the PHB/KET, PHB/KET-CHI 1.0%, PHB/KET-CHI 1.5% and PHB/KET-CHI 2.0% samples, where CHI 1.0, 1.5 and 2.0 % denote the chitosan solution concentration used during the preparation of coated microparticles.

The average size of the microparticles increased with the addition of chitosan, with an average size of 31.33 µm

for particles of PHB/KET and 31.97, 36.02 and 40.34  $\mu\text{m}$  for particles of PHB/KET obtained with aqueous chitosan solutions of 1.0, 1.5 and 2.0%, respectively. These results are consistent with those obtained in the study by He *et al.* involving the preparation of chitosan microparticles by spray drying, in which larger particles were obtained when the concentration of the aqueous chitosan solution was increased from 0.1 to 0.2%.<sup>18</sup>

Table 1 shows the geometric diameters ( $\mu\text{m}$ ), average  $d_{10\%}$ ,  $d_{50\%}$  and  $d_{90\%}$ ,  $D_{4.3}$  and span values, for the microparticles.

The microparticles coated with the 2.0% aqueous chitosan solution show a larger volume than the microparticles coated with the 1.5 and 1.0% chitosan solutions, since under the experimental spray drying conditions the architecture of the drop is fixed and reflects in the structure of the dry particle formed.

The polydispersity was more pronounced in the PHB/KET microparticles coated with 1.0% of chitosan, indicating that there was increased formation of smaller microparticles than those of PHB/KET, which may be attributed to the formation of particles of pure chitosan associated with a higher span value, as shown in Table 1.

#### KET encapsulation efficiency of the microparticles

For the PHB/KET microparticles obtained by emulsion an average encapsulation efficiency of  $64.0 \pm 0.2\%$  was obtained, in agreement with values reported by Bazzo *et al.*,<sup>4</sup> who prepared these microparticles using factorial planning designs to obtain higher levels of the encapsulated drug. The encapsulation efficiency of the drug was less than 100%, indicating that some of the drug was dissolved in the aqueous phase during the preparation of the PHB/KET microparticles.

**Table 1.** Geometric diameters of the microparticles

Chitosan concentration / %	$d_{10\%} / \mu\text{m}$	$d_{50\%} / \mu\text{m}$	$d_{90\%} / \mu\text{m}$	$D_{4.3} / \mu\text{m}$	Span / $\mu\text{m}$
–	14.9	20.5	47.4	31.3	1.58
1.0	10.1	14.6	65.1	31.9	3.75
1.5	10.7	21.2	70.4	36.0	2.76
2.0	11.8	26.4	75.8	40.3	2.42

**Table 2.** Drug encapsulation efficiency for composite microparticles

Chitosan solution / %, m/v	Composite microparticles of PHB/KET-CHI		
	Uncrosslinked / %	Crosslinked with glutaraldehyde / %	Crosslinked with genipin / %
1.0	$52.5 \pm 0.4$	$52.2 \pm 0.3$	$54.5 \pm 0.5$
1.5	$53.2 \pm 0.3$	$53.6 \pm 0.4$	$56.0 \pm 0.3$
2.0	$62.0 \pm 0.1$	$58.4 \pm 0.1$	$61.3 \pm 0.2$

The PHB/KET-CHI composite microparticles obtained by spray drying presented EE% values 2 to 8% lower than those for the microparticles without chitosan film. The solubilization of drugs in acetic acid solution can lead to subsequent loss of the active principle into the external phase during the preparation process. However, encapsulation by spray drying is a rapid process in which KET/PHB microparticles are in contact with the acidic environment for only a short time. Because of this fast process, the spray drying technique has advantages over other techniques in the encapsulation efficiency. Bazzo *et al.* prepared PHB/CHI/KET composite microparticles by the solid-in-water-in-oil emulsion-solvent evaporation technique and obtained low EE% values (34.6 to 23.3%).<sup>4</sup> In this study, the EE% values for the composite microparticles were practically the same as those for the PHB/KET microparticles, indicating that the spray drying procedure was effective in maintaining the drug content.

On comparing the drug encapsulation efficiencies (EE%) before and after the chitosan crosslinking process, no relevant difference was observed in the EE% values. Table 2 shows the drug encapsulation efficiencies for the composite microparticles (uncrosslinked and crosslinked with glutaraldehyde or genipin).

#### Determination of amine group content and crosslinking degree

The product from the reaction of the primary amine groups of CHI and ninhydrin is purple. This chromophore product is not observed when secondary or tertiary amines are formed during the crosslinking reaction. In fact, the difference in the color intensities is related to the amount of free amines in the medium.<sup>13</sup> The positive

reaction of ninhydrin with the PHB/KET-CHI composite microparticles after the reaction with the crosslinking agent permitted the percentage of free amine groups in the microparticles to be calculated, as shown in Table 3. As expected, the amount of amine groups increased when a higher concentration of chitosan in solution was used to obtain the composite microparticles, due to an increase in the chitosan film thickness. On the other hand, the crosslinking reaction occurs from the outside to the inside of the particle and the reaction time remained constant regardless of the chitosan concentration. Thus, it is likely that a gradient in the crosslinking degree occurs from the surface to the interior of the particle.

**Table 3.** Percentage of free amino groups in chitosan backbone of microparticles

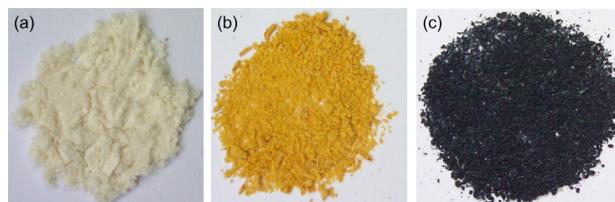
Microparticles	Crosslinking agent	
	Glutaraldehyde	Genipin
Chitosan solution / %, m/v	Free amine group / %	
1.0	43.8 ± 0.9	43.0 ± 2.1
1.5	55.0 ± 1.0	51.0 ± 1.0
2.0	60.0 ± 2.3	56.9 ± 2.5

Based on the ratio between the free amino groups before and after the chitosan crosslinking process it is possible to estimate the crosslinking degree. As expected, for the same concentration of crosslinking agent, the percentage of amino groups increases with the concentration of chitosan solution used in the crosslinking process, and the two crosslinking agents presented the same order of efficiency, where 1 mol of crosslinking agent reacts with 2 mol of the chitosan repeating unit.<sup>25,27</sup>

Studies have shown a variation in the crosslinked chitosan efficiency at different pH values, and with glutaraldehyde crosslinking is carried out at slightly acidic or neutral pH to avoid the complete protonation of the amino groups.<sup>8</sup> Mi *et al.* investigated the crosslinking of chitosan films with genipin under different pH conditions.<sup>15</sup> They observed a crosslinking degree of 45.4% for films with 1% (m/v) of crosslinked chitosan at pH 9.0. Yuan *et al.* reported that the maximum crosslinking degree for crosslinked chitosan microparticles with 0.5 mg of genipin was 32%.<sup>13</sup> The low degree of microparticle crosslinking is attributed by the authors to the fact that the crosslinking reaction mainly occurs in the outer layers, since the microparticles were crosslinked under optimum pH and time conditions,<sup>4,15</sup> as described in the methods section. Thus, we can conclude that no crosslinking occurred in the inner layers.

The color of the composite microparticles changed from orange to tan or blue after treatment with glutaraldehyde

or genipin, respectively, in the chitosan crosslinking process, as shown in Figure 6. This is in agreement with the observations of Yuan *et al.*, where the crosslinking of chitosan increased the unsaturated bonds and changed the final color of the composite microparticle.<sup>13</sup>



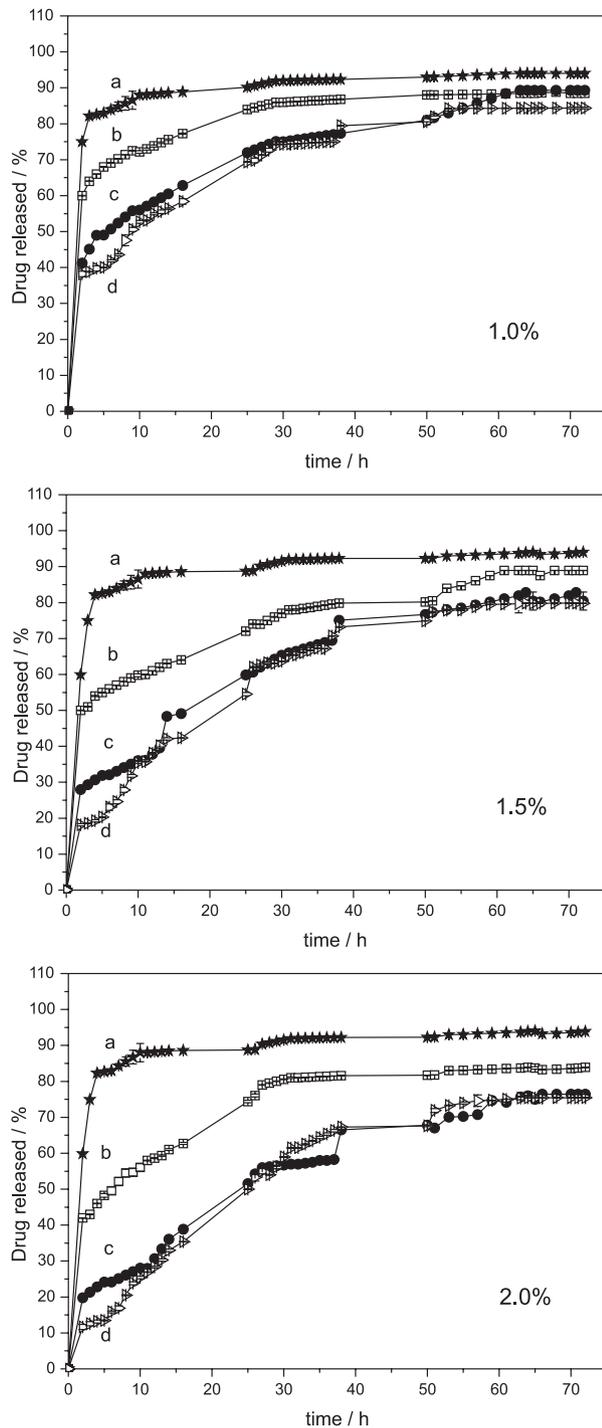
**Figure 6.** Composite microparticles of PHB/KET-CHI before and after treatment with the crosslinking agents. (a) Uncrosslinked, (b) crosslinked with glutaraldehyde, and (c) crosslinked with genipin.

#### *In vitro* drug release

The PHB/KET microparticles showed an initial drug (KET) release in the first hour of 75%, increasing to 85% after 5 h, as shown in Figure 7. The release of a drug from polymeric microparticles occurs through desorption of the drug from the surface of the particles, diffusion of the drug through the pores of the polymeric matrix, erosion of the polymeric matrix, polymer degradation or a combination of different processes.<sup>28</sup> As PHB degrades very slowly, the release profile of a drug from a PHB matrix is generally dependent on drug diffusion, rather than on polymer degradation, which occurs after 20 days.<sup>29</sup> A substantial reduction in the initial burst was observed for the composite microparticles with chitosan film, maybe due to the creation of an additional barrier to drug diffusion. This reduction was even greater after the chitosan crosslinking, as shown in Figure 7. This effect becomes more pronounced with an increase in the chitosan concentration used in the spray drying process. It has been reported that a higher viscosity of the chitosan solution leads to a reduction in the burst effect.<sup>30</sup>

In this study, we used glutaraldehyde in order to evaluate the influence of chitosan crosslinking on the KET release profile. However, due to the toxicity of glutaraldehyde, the effect of using genipin as the crosslinking agent was also compared. The two crosslinking agents showed the same drug-release profile and thus genipin should be applied since it is not damaging to health.

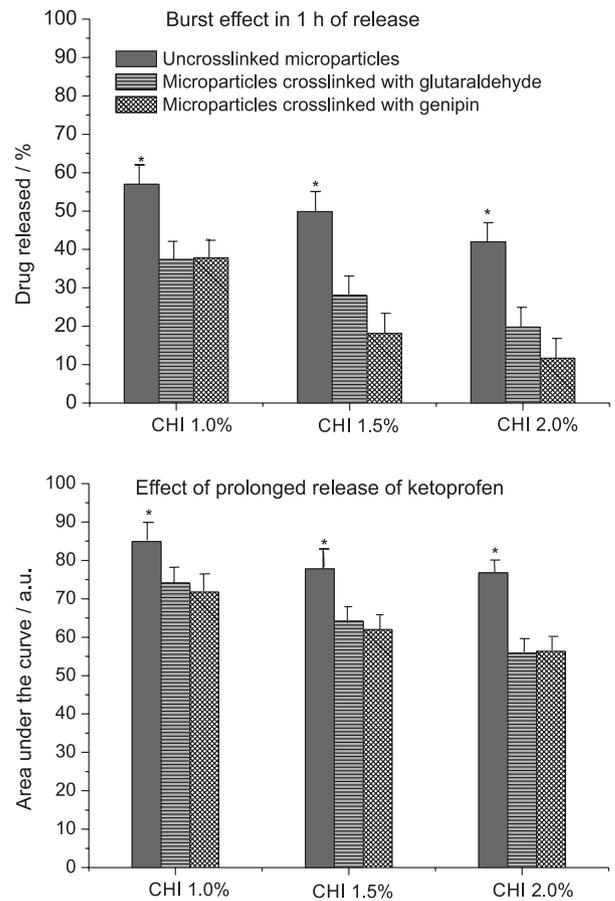
In order to evaluate the effect of the concentration of the chitosan solution and the degree of crosslinking with glutaraldehyde on the capacity of the microparticles to prolong the drug release and decrease the burst effect, the release values in the first hour and the area under the curve (AUC) of the release profiles (Figure 8) were analyzed by ANOVA, indicating that both variables influenced the KET release ( $F_{\text{calculated}} > F_{\text{tabulated}}$ ,  $\alpha = 0.05$ ). The ANOVA results



**Figure 7.** Drug-release profiles for PHB/KET microparticles (a) and PHB/KET-CHI composite microparticles: uncrosslinked (b), crosslinking with glutaraldehyde (c) and crosslinked with genipin (d), at chitosan solution concentrations of 1.0, 1.5 and 2.0% as indicated inside each graph.

verified that there were significant differences between the AUC values for the uncrosslinked composite microparticles and those crosslinked with glutaraldehyde, regardless of the chitosan concentration used.

Through the application of the Tukey test it was shown that the increase in the chitosan concentration from 1.0 to



**Figure 8.** Burst effect and prolongation of the release of ketoprofen from PHB/KET microparticles coated with 1.0, 1.5 and 2.0% chitosan (uncrosslinked and crosslinked with glutaraldehyde or genipin). Data are expressed as means  $\pm$  standard deviation. \* $p < 0.05$ , significantly different from control group.

1.5 or 2.0% significantly decreases the AUC obtained at 72 h. However, the prolonging effect was more significant when the chitosan concentration was increased from 1.0 to 1.5% ( $p < 0.05$ ). As discussed above, the chitosan layer over the PHB microspheres forms an additional barrier to the drug diffusion, with a reduction in the drug delivery rate over time. However, although it is also significant, the prolongation effect (amount of AUC at 72 h) was less prominent when the chitosan concentration was increased from 1.5 to 2.0% ( $p > 0.05$ ).

The Tukey test was applied to evaluate the influence of the chitosan and crosslinker concentrations on the burst effect and the results showed that there was a significant reduction within 1 h of release when the concentration of chitosan was increased from 1.0 to 1.5% and from 1.5 to 2.0% ( $p < 0.05$ ). This indicates that there was a reduction in the burst effect when using a higher chitosan concentration either before or after the crosslinking (Figure 8).

The drug release rates can be controlled by varying the concentration of chitosan film or crosslinking reagent. In

order to study the drug release from the PHB/KET-CHI microparticles using a natural crosslinking agent, genipin was used as an alternative chitosan crosslinking agent. The prolonging effect on the drug release was significant at all concentrations of chitosan solutions and was found to be effective when compared to uncrosslinked microparticles with the same concentrations of chitosan. Figure 8 shows the burst effect and prolongation of the release of ketoprofen from PHB/KET microparticles coated with uncrosslinked chitosan film and crosslinking with glutaraldehyde or genipin. The prolongation release values for the composite microparticles with chitosan film obtained with 1.0, 1.5 and 2.0% of chitosan solution were 13.0, 15.0 and 20.0%, respectively. Tukey's test showed comparable results for the microparticles crosslinked with glutaraldehyde, a more significant drug release prolongation being observed when the chitosan concentration was increased from 1.0 to 1.5% ( $p < 0.05$ ) compared with from 1.5 to 2.0% ( $p > 0.05$ ).

The ANOVA results showed that the chitosan concentration after crosslinking with genipin influenced the amount of drug released in the first 1 h of assay (burst release) ( $F_{\text{calculated}} > F_{\text{tabulated}}, \alpha = 0.05$ ). The application of the Tukey test showed that there was a significant reduction in the drug release in the first hour for the microparticles crosslinked with genipin when the concentration of chitosan increased from 1.0 to 1.5%, indicating an effective reduction in the burst effect ( $p < 0.05$ ). However, when the chitosan concentration was increased from 1.5 to 2.0%, the difference between the values for one hour of release was not statistically significant.

Thus, we can conclude that the release test results indicated that the addition of chitosan film crosslinked with glutaraldehyde or genipin led to a prolongation of the drug release and minimized the burst effect. The use of higher concentrations of chitosan was an important factor in reducing the burst effect and also in prolonging the drug release from the microparticles crosslinked with genipin (Figure 8).

A comparison of the burst effect associated with the two crosslinking reagents showed no significant difference when the concentration of chitosan in the composite microparticles was 1.0%, but when the chitosan concentration was increased, genipin was more effective as a crosslinking reagent. However, in terms of the effect of prolonging release, there was no significant difference between the crosslinking agents.

In view of the chemical reactions associated with the crosslinking of a layer of chitosan on the composite microparticles described above, it is evident that, as a crosslinking agent, genipin is as appropriate as glutaraldehyde, being a natural substitute and much less

toxic than glutaraldehyde. Genipin also offers the advantage of not compromising biodegradability.<sup>15</sup>

## Conclusions

The results obtained in this study suggest that by producing composite microparticles using a second biocompatible and biodegradable polymer, such as a crosslinked chitosan film, it is possible to prolong the release of KET from PHB microparticles. Employing the spray drying technique it was possible to obtain PHB/KET-CHI microparticles and this can be considered a promising method for preparing composite microparticles.

The use of higher concentrations of chitosan and crosslinking resulted in a diminished burst effect and also a prolonged release of the KET. Genipin was as efficient as glutaraldehyde as a crosslinking agent and thus it can be used as a natural and much less toxic substitute for chitosan crosslinking for pharmaceutical and medicinal uses. Furthermore, it is also possible to modulate the release of the active agent from these systems by obtaining different degrees of chitosan crosslinking.

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