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Enzyme-loaded PIBCA nanoparticles (SOD and L-ASNase): optimization and characterization

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Abstract

Some parameters of the emulsion polymerization process were studied in order to optimize the incorporation of the enzymes L-asparaginase (L-ASNase) and superoxide dismutase (SOD) into polyisobutylcyanoacrylate (PIBCA) nanoparticles and to make a contribution to the characterization of enzyme-loaded nanoparticles. Optimization criteria were the maximization of enzyme load and the minimization of the lost of catalytic activity. The effect of enzyme molecular weight, enzyme concentration and enzyme substrate on the loading performances were studied and discussed considering the mechanism of emulsion polymerization. The effect of the polymerization process on the characteristics of immobilized L-ASNase and SOD, macromolecules with very different three-dimensional structures, were studied. The incorporation of enzymes in PIBCA nanoparticles must be strongly dependent on the enzyme structure. The performances of the incorporation of SOD in PIBCA nanoparticles can be compared with results regarding other colloidal carriers.

Keywords: Enzyme-loaded nanoparticles; L-Asparaginase; Superoxide dismutase; Polyalkylcyanoacrylate nanoparticles; Nanoparticles characterization

1. Introduction

The therapeutic use of enzymes is considered to be a very promising field due to the large number of enzymes with potential for medical therapy: antitumoral, thrombolitic, hydrolytic or anti-inflammatory enzymes, and enzymes useful for replacement therapy and treatment of inherited storage diseases or active in some bacterial and viral infections (Torchilin, 1991). Meanwhile, their effectiveness is limited by the low stability of the majority of enzymes and the different unwanted reactions that occur after in vivo administration.

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In order to overcome these problems, greater attention is being given to the development of immobilization methods in order to create systems with improved properties (Torchilin, 1991; O'Mullane et al., 1987; Kossovsky et al., 1993; Kreuter, 1994). Some of the modifications in enzyme properties that can be achieved for medical therapy via immobilization are: increased stability and prolonged activity in the body; decreased antigenicity; decreased affinity to natural inhibitors; natural targeting of the enzyme to different body compartments; or reduction of the therapeutic dose.

An extensive discussion of the state of immobilized enzymes used in medicine was previously published (Torchilin, 1991). Several studies were devoted to enzyme immobilization in liposomes and semipermeable polymeric microparticles, mainly microcapsules (Klei et al., 1985; Kennedy and Cabral, 1987). A detailed review on synthetic carriers intended for medical enzyme immobilization, evidencing the polymer effect on the proprieties of the system and the effect of various polymers on the half-life of circulating enzymes was previously published (Abuchowski and Davis, 1981). Many polymers are being used in a number of approaches for the synthesis of polymers capable of biodegradability under physiological conditions (Linhardt, 1989; Passl, 1989; Puisieux and Roblot-Treupel, 1989). The polymer particles are obtained by different processes based on polymerization reactions or on the use of preformed polymers. A review of the technologies used for obtaining nanoparticles and nanocapsules to be used as drug carriers was previously reported (Vauthier-Holtzcherer et al., 1991).

Polyalkylcyanoacrylates (PACA) are synthetic, biologically degradable polymers (Grangier et al., 1991; Scherer et al., 1994) and easily polymerize to give nanoparticulate structures by an emulsion polymerization process developed by Couvreur (1988). However, no published work on the incorporation of enzymes in PACA nanoparticles was observed (Kreuter, 1994), in spite of the efficient incorporation of a large number of drugs (mainly anticancer agents and antibiotics) and of some peptides or antibodies to this type of nanoparticle (Couvreur et al., 1980; Damgé et al., 1988; Kubiak et al., 1988; Grangier et al., 1991; Gautier et al., 1992; Kreuter et al., 1994).

The structural characteristics of enzymes make their immobilization difficult. Conformational changes of the enzyme structure with the consequent partial inactivation or strong modification of the kinetics and other enzyme properties are the main limitations of the immobilization technology (Kennedy and Cabral, 1987). Mild conditions are required and the parameters of each process must be optimized for each enzyme. Comparing the process of emulsion polymerization developed by Couvreur (1988) with other processes used for the preparation of colloidal carriers, the absence of organic solvents is the more obvious advantage, although other limitative parameters can be the low pH, the high reactivity of the monomer and the presence of stabilizers. Studies identifying some process parameters for the incorporation of peptides in PACA nanoparticles are reported in the literature (Grangier et al., 1991).

In the present work, the study of technological parameters influencing the process of emulsion polymerization of isobutylcyanoacrylate (IBCA) was performed in order to optimize the incorporation of the enzymes L-asparaginase (L-ASNase) and superoxide dismutase (SOD) into polyisobutylcyanoacrylate (PIBCA) nanoparticles. Optimization criteria were the maximization of enzyme load and the minimization of the loss of catalytic activity. The catalytic activity was quantified in intact nanoparticles suspended in the initial polymerization medium as well as in disrupted nanoparticles previously separated. The effect of increasing the pH after initialization of polymerization on the characteristics of PIBCA nanoparticles and on the reduction of activity in immobilized enzyme was reported in previous work (Martins et al., 1996a). Taking this approach, we furnished a contribution to the characterization of enzyme-loaded nanoparticles and studied the effect of initial enzyme concentration on the characteristics of enzyme-loaded nanoparticles.

2. Materials and methods

2.1. Materials

Isobutylcyanoacrylate (IBCA) was a gift from Loctite (Dublin, Ireland). Superoxide dismutase (SOD) (EC 1.15.1.1.) and esterase (EC.3.5.1.1.) were from Sigma (St. Louis, MO, USA). L-Asparaginase (L-ASNase) (EC. 3.5.1.1.) was from Merck Sharp and Dohme (Darmstadt, Germany). Other reagents were analytical grade.

2.2. Methods

2.2.1. Preparation of nanoparticles

Nanoparticles were prepared by emulsion polymerization of IBCA, according to the method described by Couvreur et al. (1982), with a slight modification concerning the change in pH (Martins et al., 1996a). In brief, the monomer (25 μl) was added under stirring to 2475 μl of the polymerization medium (aqueous solution containing: citric acid, 1 mM; glucose, 5%; dextran 40, 0.5%; Synperonic F68, 1%; and adjusted to pH 3 with hydrochloric acid), in which an amount of enzyme was added to obtain concentrations from 100 to 1200 μg/ml. For the incorporation assays of L-ASNase, 3.4 mg of L-asparagine was also added to the polymerization medium. Sodium hydroxide (0.2 N) was added to the polymerization medium 1 h after beginning the polymerization, to increase the pH from 3 to 5. The preparation was maintained under magnetic stirring until final polymerization and the polymerization was considered complete 4 h after initiating the dispersion of the monomer.

2.2.2. Particle size analysis

The size distribution of nanoparticles was measured by photon correlation spectroscopy (PCS) using laser light scattering equipment (ZetaSizer 3, Malvern Instruments, UK). Samples were diluted with polymerization medium previously filtered through membranes with a pore size of 0.02 μm.

2.2.3. Separation of nanoparticles from polymerization medium

Nanoparticles (600 μl of nanoparticles suspension added to 2900 μl of water) were separated from polymerization medium by ultracentrifugation at $100\,000 \times g$ for 2 h at 5°C, using an ultracentrifuge Beckman XL90, equipped with a swinging bucket rotor SW 55 Ti.

2.2.4. Quantification of nanoparticles enzyme loading

The enzyme content in nanoparticles was calculated from the difference between the enzyme concentration in the polymerization medium before polymerization and the enzyme concentration that remains in the polymerization medium after separation from nanoparticles (Martins et al., 1996b). In brief, both quantifications were performed using an SE-HPLC method with a spectrophotometric determination. The column, Licrospher 300 Diol, 10 μm (250×4 mm) from Merck, was protected by a pre-column Licrospher 100 Diol, 5 μm (4×4 mm), also from Merck. An HPLC equipment from Beckman, System Gold, equipped with a spectrophotometric detector was used. The mobile phase was phosphate buffer 0.1 M, NaCl 0.1 M, pH 7, flow rate 0.3 ml/min, with detection at 220 nm.

The efficacy of incorporation was obtained by the ratio: $(E_f/E_i) \times 100$, E_f being the enzyme content in nanoparticles and E_i the enzyme quantified in the polymerization medium before addition of the monomer.

2.2.5. Separation of nanoparticles by sucrose gradient

Nanoparticles previously separated from the polymerization medium and resuspended in fresh polymerization medium were placed on the top of the sucrose gradient with the following composition: 5, 10, 20 and 30% sucrose in water. The gradient had volumes of 2.4 ml at each concentration, and samples were in volumes of 1 ml.

The separation was performed using a ultracentrifuge Beckman XL90, equipped with a swinging bucket rotor.

2.2.6. Turbidity measurements

Turbidity measurements were accomplished by light transmittance of nanoparticles suspensions at the wavelength 450 nm, after appropriate dilution.

2.2.7. Disruption of nanoparticles

Enzymatic hydrolysis of nanoparticles was performed by the incubation of esterase with nanoparticles (30 IU of esterase per mg of polymer, according to Pinto-Alphandary et al. (1994)), in phosphate buffered saline (PBS) (pH 7.4) at 37°C for 12 h. Several types of sample preparation were used: a suspension of nanoparticles in the polymerization medium (as they are obtained after polymerization); nanoparticles separated from the polymerization medium by ultracentrifugation and resuspended in PBS; nanoparticles separated from the polymerization medium, frozen at −70°C, defrosted to room temperature and resuspended in PBS; a suspension of nanoparticles dialyzed against PBS for 48 h (with substitution of buffer after 6 and 24 h from the beginning of dialysis).

2.2.8. Retention of catalytic activity on intact nanoparticles

The catalytic activity on intact nanoparticles was evaluated on nanoparticles suspensions without previous separation of the non-incorporated enzyme eventually presented in the polymerization medium.

The assay of activity for SOD is based on measuring the ability of this enzyme to decrease the rate of autoxidation of epinephrine to adrenochrome. The extent of inhibition is taken as a measure of the catalytic activity of the enzyme (Misra and Fridovich, 1972; Sun and Zigman, 1978).

L-ASNase activity is measured according to the assay of Abuchowski et al. (1979), which is a modification of the coupled enzyme system of Jayaram et al. (1974).

The retention of catalytic activity is represented by the ratio: $(A_i/A_t) \times 100$, A_i being the catalytic activity on intact nanoparticles and A_t the catalytic activity of an enzyme solution (in the polymerization medium containing all the components except the monomer) at a concentration equal to the initial enzyme concentration present in the polymerization medium before the addition of the monomer.

In the case of L-ASNase, loaded nanoparticles, were used with substrate included in the polymerization medium, the reference also including substrate.

2.2.9. Retention of catalytic activity on disrupted nanoparticles

The catalytic activity of the incorporated enzyme was quantified after disruption of nanoparticles with esterase as described in Section 2.2.7.

The retention of catalytic activity is represented by the ratio: $(A_d/A_t) \times 100$, A_d being the catalytic activity of disrupted enzyme-loaded nanoparticles and A_t the catalytic activity of an enzyme solution (in the polymerization medium) with the same concentration as if all the loaded enzyme were in solution.

3. Results

3.1. Disruption of enzyme-loaded nanoparticles by esterase

The efficiency of the disruption process as a function of the type of sample was studied.

Table 1 shows the turbidity of different samples of nanoparticles in the presence of esterase, just before the incubation at 37°C and after 12 or 24 h of incubation. The samples used were: nanoparticles in the polymerization medium; nanoparticles separated from the polymerization medium by ultracentrifugation or nanoparticles separated from the polymerization medium and treated by a frozen/defrozen process, resuspended in PBS in both cases; and nanoparticles in PBS (polymerization medium replaced by PBS through a dialysis procedure).

Table 1
Evolution of turbidity as function of process parameters

Sample	Turbidity (absorbance: 450 nm)		
	Initial time	After 12 h	After 24 h
Nanoparticles in polymerization medium			
Enzyme loaded	0.320	0.303	0.315
Unloaded	0.338	0.304	0.321
Nanoparticles resuspended in PBS			
Enzyme loaded	0.305	0.181	0.202
Unloaded	0.305	0.180	0.202
Nanoparticles frozen/defrozen			
Enzyme loaded	0.096	0.079	0.092
Unloaded	0.125	0.158	n.d.
Nanoparticles in PBS			
Enzyme loaded	0.78	0.24	n.d.
Unloaded	—	—	—

Loaded nanoparticles prepared with an initial SOD concentration of 200 μg/ml. Incubation at 37°C.

No significant reduction of turbidity after 12 h of incubation was observed either using nanoparticles in the polymerization medium or nanoparticles previously frozen/defrozen. A significant reduction of turbidity after 12 h was observed using nanoparticles previously separated from the polymerization medium or nanoparticles in PBS (polymerization medium replaced by PBS by dialysis).

3.2. Incorporation of L-ASNase and SOD in nanoparticles

The results of the incorporation of L-ASNase and SOD in nanoparticles performed using the procedure described in Section 2.2 are presented in Table 2. In the case of L-ASNase, the substrate, L-asparagine, was included in the polymerization medium.

In order to elucidate the discrepancy between the results of the retention of activity on intact and disrupted L-ASNase-loaded nanoparticles, the retention of activity on intact nanoparticles was analyzed after separation from the polymerization medium (Table 3). The effect of the disruption process on the activity of L-ASNase and SOD was also studied (Table 4).

As shown, the separation and disruption processes have a great influence on the final results (in terms of retention of activity).

Table 2
Characteristics of L-ASNase- and SOD-loaded nanoparticles

Characteristics	Nanoparticles	
	L-ASNase loaded	SOD loaded
Size [nm (% of population)]	250 (90); 325 (10)	55 (30); 170 (30); 430 (40)
Efficiency of incorporation (%)	20	87
Retention of activity (%)[a]		
Intact nanoparticles[b]	90	15
Disrupted nanoparticles[c]	0.1	32

Nanoparticles prepared with an initial enzyme concentration of 200 μg/ml.
[a]100% activity corresponds to enzyme in polymerization medium (200 μg/ml).
[b]Suspension of nanoparticles in the polymerization medium.
[c]Nanoparticles separated from polymerization medium, resuspended in buffer and disrupted by incubation with esterase.

Table 3
Comparison between the retention of activity on intact L-ASNase-loaded nanoparticles with and without separation from polymerization medium

L-ASNase-loaded nanoparticles	Retention of activity (%)[a]
In polymerization medium	90
Resuspended in fresh polymerization medium	2.5

Nanoparticles prepared with an initial enzyme concentration of 200 μg/ml.
[a]100% activity corresponds to enzyme in polymerization medium (200 μg/ml).

3.3. Effect of initial enzyme concentration on the incorporation of SOD in nanoparticles

3.3.1. Effect of initial SOD concentration on the efficiency of incorporation

The efficiency of SOD incorporation as a function of the initial enzyme concentration was studied (Fig. 1).

SOD incorporation increased as function of initial SOD concentration (Fig. 1a); however, a decrease in the efficiency of incorporation was observed with the increase of SOD added before polymerization (Fig. 1b).

3.3.2. Effect of initial SOD concentration on the size distribution of loaded nanoparticles

The influence of initial enzyme concentration in the size of SOD-loaded nanoparticles as a function of the initial enzyme concentration was evaluated (Fig. 2).

With the increase of initial enzyme concentration, a decrease in the percentage of smaller-size nanoparticles and an increase in the size of larger particles of each population, was observed.

Table 4
Effect of the disruption process on the retention of activity of the enzymes

Enzyme	Retention of activity (%)
L-ASNase	50[a]
SOD	100[b]

[a]Retention of activity after 12 h of incubation in the disruption medium.
[b]Retention of activity after 12 h of incubation in the disruption medium, either with or without unloaded nanoparticles.

3.3.3. Effect of initial SOD concentration on the retention of activity of incorporated enzyme

The retention of activity of the incorporated enzyme was studied on either intact or disrupted nanoparticles with different enzyme load. Fig. 3 represents the retention of activity on intact and disrupted nanoparticles as a function of the SOD load. For each preparation, a solution of enzyme in the polymerization medium with a concentration equal to the initial enzyme concentration was used as a reference.

The evolution in the retention of activity after disruption, as a function of the increase in the enzyme load, points to a decrease in the efficiency of the disruption process when the enzyme load is increased.

3.4. Effect of the enzyme load on the aggregation of resuspended nanoparticles

Ultracentrifugation is an efficient separation process, as evidenced by the control of nanoparticles in the supernatant (results not shown), although after resuspension of nanoparticles pellet, the formation of aggregates was observed. Evidence for the effect of the enzyme load on the aggregation of nanoparticles after ultracentrifugation are presented. Results of the size distribution of resuspended nanoparticles separated on different layers of a sucrose gradient are presented in Table 5, showing the indicated increase in particles size.

As the quantification of the retention of catalytic activity on disrupted nanoparticles requires a previous separation of nanoparticles by ultracentrifugation, the increase in the aggregation of nanoparticles, when the enzyme load is increased, can explain the lack of efficiency of the disruption process when the enzyme load of nanoparticles is increased (as shown in Fig. 3).

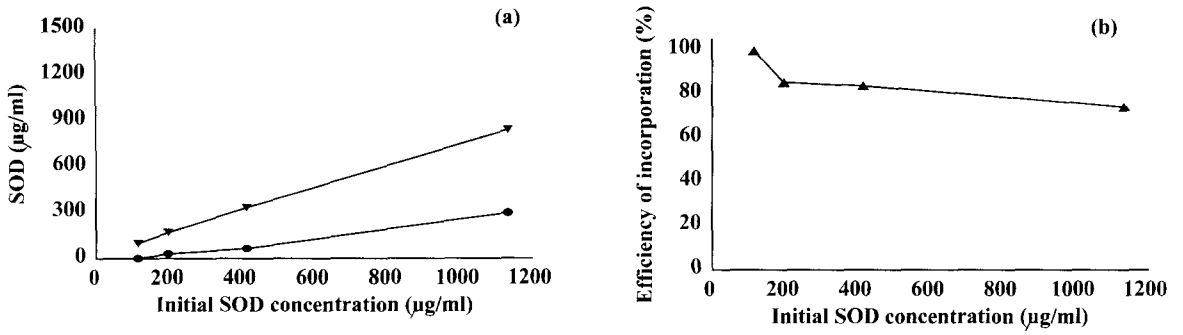

Fig. 1. Effect of the initial concentration of SOD on the: (a) distribution of the total enzyme between the immobilized (▼) and non-immobilized (●) portions; (b) efficiency of incorporation of enzyme (▲) in nanoparticles.

4. Discussion and conclusions

Some contradictory results observed in the literature with the incorporation of peptide drugs in PACA nanoparticles were considered to be related to the physico-chemical complexity of peptides (Grangier et al., 1991), which can be completely valid to the incorporation of enzymes in these types of colloidal carriers.

Due to the greater structural complexity of enzymes when compared with peptides, the analysis of results for incorporation of enzymes in nanoparticles must consider both the interaction of the enzyme with the components of the emulsion polymerization system and the effect of the process of polymerization on the characteristics of the enzyme. Some examples are pH-modulated intermolecular interactions, adsorption or interaction with other molecules as emulsifiers or unreacted monomer present in polymer particles, and the covalent linkage of the enzyme with the polymer.

In an emulsion polymerization initiated by water-soluble initiators (Heller, 1987), such as is used in this work, anionic polymerization takes place almost exclusively in the micelles which serve as the meeting place for the water-soluble initiator and water-insoluble monomer dispersed in the medium. Cyanoacrylates polymerization initiators are mainly the OH^- ions resulting from the dissociation of water, but other bases can be initiators. Basic groups of enzymes can act as polymerization starters, with the corresponding covalent linkage of the enzyme to the polymer.

As polymerization proceeds, the size of active micelles increases when monomer, from the monomer droplets, diffuse into the polymer parti-

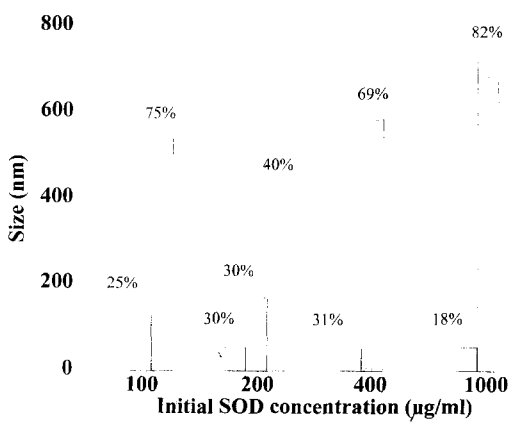

Fig. 2. Effect of initial SOD concentration on the size distribution of loaded nanoparticles. Polydispersity in the range 0.1–0.3.

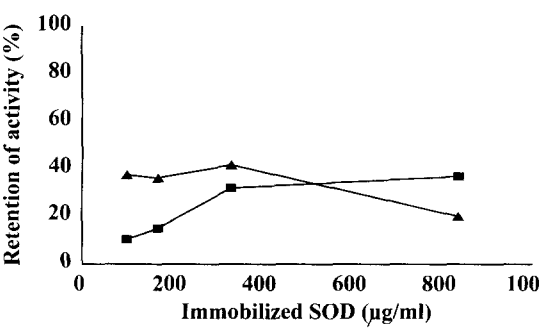

Fig. 3. Retention of enzyme activity on intact (■) and disrupted (▲) SOD-loaded nanoparticles.

Table 5
Size distribution of L-ASNase-loaded nanoparticles in a sucrose gradient

	Size of SOD-loaded nanoparticles (nm)	
	100 μg/ml	840 μg/ml
Before ultracentrifugation	130; 540	56; 723
After ultracentrifugation		
Layer 1	—	490; 3090; 5600
Layer 2	200	2200
Layer 3	600; 760	1440; 3870
Layer 4	—	1400

Layer 1: low sucrose concentration; layer 4: high sucrose concentration.

cles, the emulsifier molecules being absorbed from the solution (Heller, 1987). This mechanism is evidenced by the significant influence of emulsifiers on the characteristics of nanoparticles (Kreuter, 1994). Considering the presence of the enzyme in the aqueous phase, the extent of the capture of enzymes during the enlargement of the small oligomeric subunits that form the nanoparticles (Couvreur and Vauthier, 1991) can be dependent on interactions of the enzyme with the emulsifiers or with micellar structures. Micellar effects on enzymology are discussed in the literature (Fendler and Fendler, 1975; Bunton, 1984).

When the emulsifier concentration in solution falls below the critical micelle concentration, the micelles become unstable and dissolve; polymerization continues in polymer particles with emulsifier molecules adsorbed on them (Heller, 1987). Also in this step, the interactions between enzyme and emulsifier can interfere with the incorporation of the enzyme into the nanoparticles.

At a high degree of conversion (Heller, 1987), monomer droplets disappear completely and all the unreacted monomer or growing unterminated polymer molecules are contained in primary polymer particles. The termination frequency in this environment is reduced due to the reduced H^+ ions concentration, responsible for termination. The presence of unreacted monomer can affect the characteristics of the enzyme incorporated in nanoparticles.

The influence of the different parameters, including the presence of enzymes as new macromolecules in a polymerization process, is very complex (Kreuter, 1994). Additionally, a complete evaluation of the effects of the polymerization process on the characteristics of an enzyme incorporated in nanoparticles is very difficult to establish. As the preservation of the catalytic activity of an enzyme is closely related to the preservation of its structure, we discuss the incorporation of enzymes in nanoparticles of PIBCA, focusing mainly on the retention of activity of the enzyme after incorporation.

The retention of activity quantified on intact nanoparticles includes the contribution of unloaded enzyme that remains in the supernatant. This explains the high retention of activity observed on intact L-ASNase-loaded nanoparticles, where a retention of activity of 90% is explained by the 80% of initial enzyme remaining in the supernatant. The very low retention of activity observed on resuspended nanoparticles can also be explained by the aggregation of resuspended nanoparticles.

The retention of activity on intact SOD-loaded nanoparticles, where 12% of the enzyme remains in the supernatant, is consistent with the 15% retention of activity on intact nanoparticles.

For an increase of 100–800 μg of immobilised SOD per ml of nanoparticles suspension, containing the same amount of polymer, the small increase (from 10 to 40%) in the corresponding retention of activity on intact nanoparticles is also explained by the effect of the inaccessibility of substrate to the enzyme entrapped inside the structure of the nanoparticles.

A correlation between nanoparticles breakdown and enzyme release, quantified by the retention of activity on disrupted nanoparticles, was observed for enzyme load smaller than 170 μg/ml. At higher enzyme loads, no enzyme release was observed, which may be a consequence of an inefficient breakdown of nanoparticles. This interpretation is in accordance with the observed difficult disruption of high enzyme-load nanoparticles aggregates (results not shown).

The very small increase in retention of activity observed after disruption when the enzyme load is

330 μg/ml, and the large decrease observed when the enzyme load is 840 μg/ml, can be examples of an inefficient disruption. In effect, if no release of incorporated enzyme occurs, the retention of activity on disrupted nanoparticles is necessarily smaller than retention of activity on intact nanoparticles, due to the free enzyme removed before disruption. This can explain the results expressed in terms of retention of activity, when considering disrupted nanoparticles with the highest enzyme load.

In conclusion, considering the characteristics of L-ASNase and SOD, macromolecules with very different three-dimensional structures, the incorporation of enzymes in PIBCA nanoparticles must be strongly dependent on the enzyme structure. The incorporation of the L-ASNase in PIBCA nanoparticles must be improved. Although the performances of the incorporation of SOD in PIBCA nanoparticles can be compared with results from other systems, the efficiencies of incorporation observed are similar to results for this enzyme incorporated in liposomes (Tanswell and Freeman, 1987; Martins et al., 1992; Cruz et al., 1994). The retention of activity on disrupted nanoparticles of lower enzyme load (40% of initial enzyme) seems not to be restrictive for the utilization of this system as a drug delivery system for SOD (Jadot et al., 1995). A better characterization of enzyme-loaded nanoparticles can be achieved with improvement in separation and disruption processes.

The application of the method used for PIBCA nanoparticles in order to incorporate enzymes imposes a number of constraints in selecting the process parameters. The effect of pH on the incorporation of SOD, previously discussed (Martins et al., 1996a), and the effect of enzyme molecular weight, enzyme concentration and different molecules such as an enzyme substrate, discussed in this work, evidence the influence of the process parameters. Some other process parameters, such as pH, monomer concentration, stabilizers, or ionic strength, may also affect the physico-chemical properties of the nanoparticles formed.

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