Study of the Influence of Several Stabilizing Agents on the Entrapment and *In Vitro* Release of pBC 264 from Poly(Lactide-Co-Glycolide) Microspheres Prepared by a W/O/W Solvent Evaporation Method

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INTRODUCTION

Over the last few years, microspheres prepared from biodegradable polylactides (PLA) and co-polymers of lactic/glycolic acids (PLG) have been widely used for the controlled delivery of peptides and proteins. In addition, biodegradable microspheres were able to protect these molecules against rapid enzymatic degradation (1–3). However, one remaining drawback associated to the use of microspheres for peptide and protein delivery is the drug release process which is very often characterized by a strong initial burst effect (4). We have recently developed a microsphere formulation prepared by the W/O/W solvent evaporation method for the encapsulation of a seven amino-acid peptide, derivative of cholecystokinin (pBC 264). In this study, it was shown that, although OVA used as stabilizing agent of the inner emulsion increased significantly the encapsulation efficiency of pBC 264 in microspheres, a strong burst release of the peptide was observed. In order to reduce this burst effect, we have investigated, in this report, the influence of different stabilizing agents added to the internal aqueous phase as well as the concentration of the polymer in the organic phase, on the encapsulation efficiency and the *in vitro* release of pBC 264.

MATERIALS AND METHODS

Materials

Poly(dl-lactide-co-glycolide) acid 75/25 (PLG) (Mw 128000) was supplied by Birmingham Polymers, USA. Methylene chloride (MC) (Prolabo, France) was used without further purification. Poly(vinyl alcohol) (Mowiol®) (PVA) (Aldrich chemicals, France) was used as surfactant in the inner and outer emulsions. Pluronic® F 68, Span 80 and ovalbumin (OVA, grade V) were purchased from Sigma (France) and used as stabilizing agents in the inner emulsion. Tris(hydroxymethyl)aminomethane (TRIS) was obtained from Bio-rad laboratories (France). Perylen was obtained from Aldrich chemicals, (France). The peptide, pBC 264 (Mw 1105) (Propionyl-Tyr-(SO_3H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH_2), was synthesized as described by Charpentier *et al.* (5).

Microspheres Preparation and Characterization

Peptide-containing PLG microspheres were prepared by a W/O/W solvent evaporation technique as described previously (6). Briefly, the peptide (1 mg) was dissolved in 500 μl of distilled water, together with one of the following surfactants: PVA (2%), OVA (2%), OVA/Span 80 (1:1) (2%) or Pluronic® F68 (3%). This aqueous solution was mixed with a 8 ml methylene chloride solution containing PLG (10%). This mixture was emulsified using an Ultraturax® at a speed of 13500 rpm for 2 minutes, to form the inner emulsion (W/O). This emulsion was then added to 80 ml of a 2% aqueous PVA solution and mixed with an Ultraturax® to form a double W/O/W emulsion. This resulting emulsion was stirred at 1000 rpm at room temperature to evaporate off the methylene chloride. Since pBC 264 is a sulfate salt that is soluble at alkaline pH, the internal aqueous phase pH was adjusted to 8 and the external water phase to 2.5. Peptide-loaded microspheres were collected by centrifugation at 3000 rpm for 5 minutes, washed three times with distilled water and freeze-dried.

Microspheres diameter and size distribution were measured using a Coulter Counter® (Coultronics, France). Levels of pBC 264 entrapped in microspheres were determined using a procedure previously described by Hora et al. (7). A weighed amount of freeze-dried microspheres were dispersed in 2 ml of 0.1 N NaOH and shaken for 18 hours. Samples were centrifuged and the peptide concentration in the supernatant was determined by HPLC as described previously (6). The encapsulation efficency was calculated by comparing the amount of encapsulated peptide to the total amount of peptide introduced in the internal aqueous phase (actual loading × 100/theoretical loading). Microspheres containing ovalbumin and pluronic® F68 were observed by electron microscopy. A small drop of an aqueous suspension of microspheres was deposited on a copper planchett and rapidly frozen into liquid propane. Fracturing, etching and shadowing, using Pt-C, were performed in a Balzers BAF freeze-etch unit. The replicas were observed in a Philips 301 electron microscope.

Stability of the Inner Emulsion

Stability of the inner emulsion was determined using the method described by Schugens et al. (8). Briefly, the inner emulsion, prepared as reported above, was stored in a 15 ml glass tubes fitted with a rubber septum. The time required for initial macroscopic phase separation to occur was measured at room temperature. Perylen (10 μg/ml) was dissolved in the organic phase to facilitate the macroscopic examination of coalescence, coagulation or creaming of the emulsion.

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In Vitro Release Studies

Several samples of microspheres (10 mg) were placed in 5 ml glass tubes and incubated in 2 ml phosphate-buffered saline (PBS), pH 7.4, under stirring at 37°C. At different intervals of time, one sample was collected, centrifuged for 10 minutes at 4500 rpm and the peptide was assayed in the supernatant by HPLC.

RESULTS AND DISCUSSION

Microspheres were prepared with different surfactants introduced in the internal aqueous phase. The influence of the surfactant on the stability of the first emulsion as well as the size of the microspheres and the encapsulation efficiency of pBC 264 were examined (Table I). As far as the size of the microspheres was concerned, the inclusion of ovalbumin in the internal aqueous phase led to the largest microspheres (mean diameter = 7.20 μm). In contrast when OVA was associated with Span 80, the mean diameter decreased to 1.20 μm (Table I). The use of Span 80 reduced the diameter of the internal globules (data not shown). The mean diameter of the microspheres is generally smaller when internal globules have a low mean diameter (3).

The encapsulation efficiency was very low for the formulation containing PVA and, on the contrary, very high for the one containing OVA or for the formulation containing the mixture of OVA/Span 80 (Table I). Pluronic® F 68-containing microspheres displayed an average encapsulation efficiency (Table I). The most important factor for the successful encapsulation of hydrophilic compounds in microspheres prepared by the W/O/W solvent evaporation method is the stability of the first inner emulsion (9). From the stability studies (Table I), it was shown that, when PVA was used as a stabilizer, the internal emulsion was unstable and the droplets coalesced. Thus, it was assumed that the peptide leak out to the external aqueous phase. A similar phenomenon occured with Pluronic® F 68 but to a lesser exent since the stability of the emulsion was improved. Pluronic® F 68 and PVA are hydrophilic surfactants that are not suitable for stabilizing W/O emulsions. On the contrary, proteins have a tendency to localize at the interfaces, increasing the stability of emulsions, therefore reducing the leakage of the peptide to the external aqueous phase. The association of Span 80 to OVA was also tested. This association was previously shown to allow the formation of a rigid interfacial complex (10) that is able to contribute to a large extent to the stability of the first emulsion.

Release of pBC 264 from microspheres prepared with Span/OVA, OVA and Pluronic® F 68 was studied in PBS at pH 7.4. Since the encapsulation efficiency was very low when PVA was used as stabilizer in the internal emulsion, release from this formulation was not studied. Release profiles of pBC 264 are shown on figure 1. An initial burst release took place during the first 30 min of the experiment. 80% of the total peptide content was released from microspheres prepared in the presence of OVA and 67% from those prepared with a mixture OVA/Span. By contrast, the burst was much smaller when the microspheres were prepared in the presence of Pluronic® F 68 (Figure 1). Thus, the release profile and the extent of initial peptide burst was significantly influenced by the structure of microspheres. The microspheres prepared with OVA had a porous structure and, thus, provide a fast release profile with a large peptide burst (Figure 2, A). By contrast microspheres prepared in the presence of Pluronic® F 68, did not have pores and were characterized by a smooth surface (figure 2, B). Therefore, the differences in the initial burst from the tested formulations seems to result from morphological differences.

The effect of the polymer concentration on the release of the peptide was also studied *in vitro* with microspheres prepared in the presence of Pluronic® F 68. The increase in PLG concentration gave a highly viscous polymer solution. The higher the viscosity of the polymer solution was, the more difficult was the formation of small emulsion droplets, resulting in PLG microspheres with larger size (data not shown). The initial burst release of pBC 264 decreased as the concentration of PLG in the organic phase was increased (Table II). When concentration of polymer used for preparing microspheres was 10%, 28% of peptide was released after 48 hours. A further increase in polymer concentration to 30% resulted in particles with a smaller degree of burst release (17% of peptide was released in 2 days) (Table II). This may be explained by the fact that an increase in the amount of polymer could produce microspheres with a more compact polymer phase, and a dense core, thus decreasing the initial peptide burst. These results are in agreement with

Table I. Effect of Different Stabilizing Agents, Added to the Inner Water Phase, on the Encapsulation Efficiency, the Mean Diameter of PLG Microspheres and on the Stability of the Inner Emulsion. Loaded Amount of pBC 264 Was 1 mg. The Number of Replicates Was 3

Stabilizing agent	Encapsulation efficiency (%)	μg pBC/100 mg microspheres	Mean diameter (μm)	Phase separation time (min)
PVA, (2%)	9.6 ± 2.5	12	5.8 ± 0.2	15
OVA, (2%)	95 ± 4.0	118.8	7.20 ± 0.4	>300
OVA/Span 80, (2%)	61.5 ± 3.9	76.3	1.2 ± 0.05	>300
Pluronic® F68, (3%)	36 ± 5.0	45	6.0 ± 0.35	45

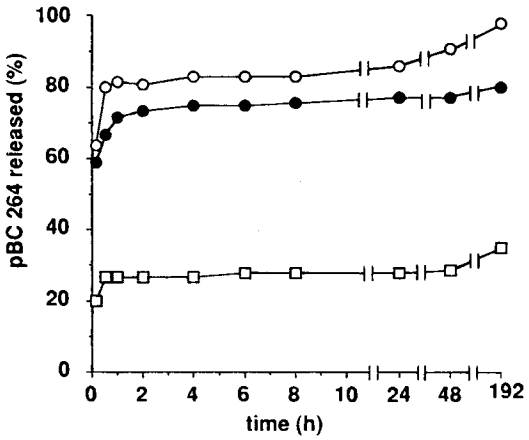

Fig. 1. Release Kinetics of pBC 264 from PLG microspheres, prepared with differents stabilizing agent: OVA (○); OVA/Span 80 (●); Pluronic® F 68 (□), in PBS, pH 7.4. The results are from single experiments that were replicated three times. The error ranged from 3% to 7%.

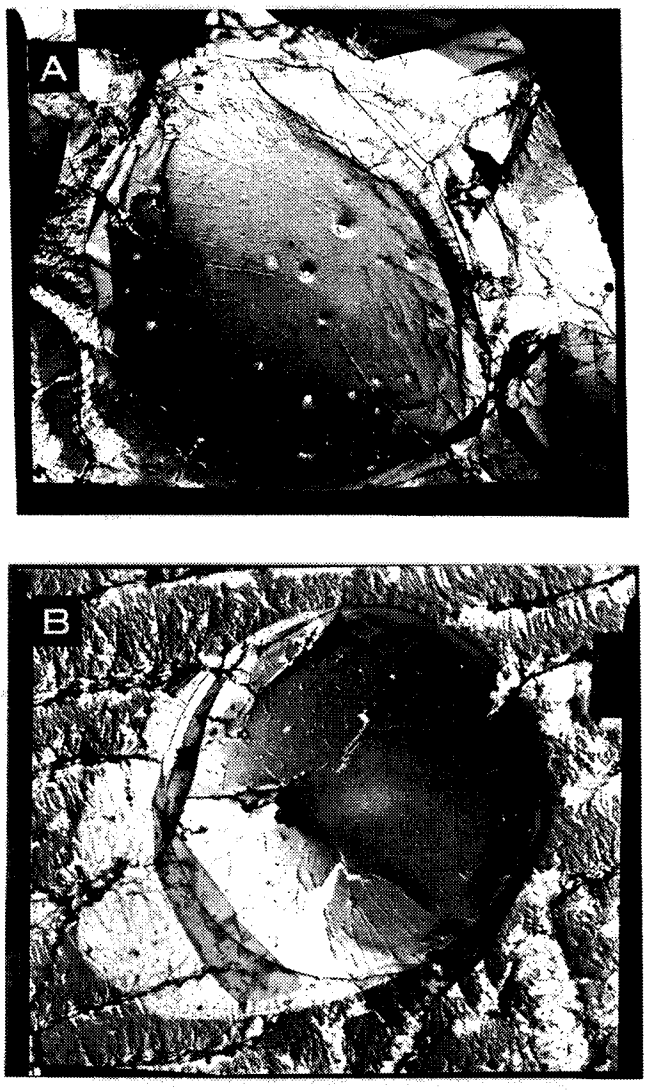

Fig. 2. Electron micrograph of microspheres prepared in the presence of Ovalbumin (A) and Pluronic® F 68 (B).

Table II. Effect of Polymer Concentration on Peptide Burst Release from PLG Microspheres. The Number of Replicates Was 3

	Peptide released (%)	
PLG (%)	(1 h)	(48 h)
10	20.7 ± 5.0	28 ± 4.1
20	15 ± 4.7	23 ± 3.7
30	8 ± 2.5	17 ± 2.8

many other studies that were able to evidence the role of the polymer concentration in reducing the burst effect (11,12).

In conclusion, the encapsulation efficiency of pBC264 was influenced by the nature of surfactant used to stabilize the inner emulsion. Furthermore, the release of the peptide from microspheres, prepared by a W/O/W solvent evaporation method, was found to be influenced by the stabilizing agent added to the internal aqueous phase and by the amount of polymer added to the organic phase.

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