ELSEVIER International Journal of Pharmaceutics 184 (1999) 73–84

international journal of pharmaceutics

Preparation and characterization of protein-loaded poly(ε-caprolactone) microparticles for oral vaccine delivery

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Received 12 November 1998; received in revised form 16 March 1999; accepted 19 March 1999

Abstract

This paper describes the conditions of preparation of poly(ε-caprolactone) (PCL) microparticles with a mean size between 5 and 10 μm, obtained by a double emulsion-solvent evaporation technique, suitable for oral vaccine delivery. Bovine serum albumin (BSA) was used as water-soluble model antigen for encapsulation. Different parameters influencing the microparticle size, the BSA loading and entrapment efficiency were investigated. Spherical, smooth and homogeneously distributed microparticles were produced with a BSA loading and entrapment efficiency reaching, respectively, 5% (w/w) and 30%. Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) analyses of BSA released from these particles confirmed that the entrapped protein seemed to remain unaltered by the protein encapsulation process. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Poly(ε-caprolactone); Biodegradable polymers; Protein delivery system; Protein microencapsulation; Oral vaccine; Emulsion-solvent evaporation method

1. Introduction

In studies over the past 20 years, the adjuvant effect achieved through the association of antigens with various polymeric microparticles has been repeatedly demonstrated (O'Hagan, 1994). The adjuvant effect of microparticles is thought to be directly related to their ability to be taken up by macrophages (Tabata and Ikada, 1990). Poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLG) are the primary polymeric candidates for the development of microparticles as vaccine because they are biodegradable and biocompatible polymers. Moreover, PLG polymers have been used in humans for many years as suture material and as controlled-release delivery system for peptide drugs (Wise et al., 1979).

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PII: S0378-5173(99)00109-X

The humoral adjuvant effect (production of antibodies from different isotypes) achieved by the entrapment of antigens in biodegradable PLG microparticles has been demonstrated only relatively recently (Eldridge et al., 1991; O'Hagan et al., 1991). Recent studies in mice have shown that microparticles also exert an adjuvant effect for cell-mediated immunity, including the induction of a cytotoxic T lymphocyte (CTL) response against ovalbumin or gp120 from HIV-1 following both systemic and mucosal administration (Maloy et al., 1994; Moore et al., 1995). A delayed-type hypersensitivity (DTH) response and potent T-cell proliferative responses against ovalbumin were also elicited following oral immunization with these particulate carriers (Maloy et al., 1994).

Even if microparticles with adsorbed antigens were also effective adjuvants (O'Hagan et al., 1993b), the entrapment of the antigen within the macroparticles should allow to use them as controlled-release delivery systems with the potential for the development of single-dose vaccines.

However, one major drawback of these polymers is that their degradation generates extreme acid environment (pH 2–3) in which many antigens are found to lose their structural integrity and antigenicity (Gander et al., 1993; Schwendeman et al., 1996).

Poly(ε-caprolactone) (PCL) is an other biocompatible and biodegradable polyester polymer that degrades slowly and does not generate an acid environment unlike the PLA/PLG polymers. Although the permeability of macromolecules in PCL is low, such low permeability may be sufficient enough for protein delivery (Pitt, 1990). Other advantages of PCL includes its hydrophobicity, its in vitro stability and its low cost. Moreover, this polymer has been under clinical evaluation for sustained delivery of levonorgestrel worldwide (Pitt, 1990), and a preliminary study performed by Jameela et al. (1996) has demonstrated that specific humoral immune responses (production of IgG antibodies) were elicited in rats following intramuscular administration of BSA-loaded microspheres suspended in Freund incomplete adjuvant.

The administration of vaccines via a mucosal route (oral, intranasal, intrarectal and inhalation) offers several significant advantages over the traditional approach to vaccine delivery. These advantages include easier administration, reduced side effects, the potential for frequent boosting without the need of trained personnel and the induction of mucosal immunity at the site of initial infection.

Particle size was shown to be an important parameter affecting immunogenicity, because smaller microparticles (< 10 μm) were significantly more immunogenic than larger particles (> 10 μm; Eldridge et al., 1991; O'Hagan et al., 1991). Although the factors controlling the particle uptake across the gastrointestinal tract are poorly defined, some studies have shown that, in order to be taken up by M cells of the Peyer's patches after oral administration, the microparticles must have a diameter less than 10 μm (Eldridge et al., 1990).

The objectives of the current investigations were to optimize a particle preparation process, firstly described by Jeffery et al., (1993) to produce PLG microparticles, based on a double emulsion-solvent evaporation method (water-in-oil-in-water method, w/o/w; Jeffery et al., 1991), to produce PCL particles with entrapped protein having a size and size distribution potentially suitable for oral delivery of antigens and particle uptake across the gastro-intestinal tract (i.e. between 5 and 10 μm).

We describe here the effect of processing conditions on the particle size, protein loading and efficiency of entrapment as well as the eventual modification on the release rate of a water-soluble model antigen (Bovine Serum Albumin) following incubation in gastric conditions. The effect of the microparticle preparative process on BSA integrity was also investigated by polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) to finally provide design and formulation guidelines for delivery systems for oral vaccines based on resorbable microparticles.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) (44 000 MW) and polyvinyl alcohol (PVA) (13–23 K, 87–89% hydrolyzed) were supplied by Aldrich (Bornem, Belgium). Dichloromethane, chloroform and acetone (reagent grade) were supplied by UCB (Braine L'Alleud, Belgium). Sodium dodecyl sulphate (SDS) was purchased from Sigma (St. Louis, MO, USA). Low molecular weight markers (range from 14 to 94 kDa) and isoelectric point markers (broad p*I* kit range from pH 3.50 to 9.50) were, respectively, supplied by Bio-Rad (Nazareth, Belgium) and Pharmacia Biotech (Uppsala, Sweden). Ampholine PAG plate pH range 3.5–9.5 was supplied by Pharmacia Biotech (Uppsala, Sweden). Albumin (from bovine serum, fraction V) (BSA), Folin–Ciolcateu's phenol reagent, β-mercaptoethanol and other materials (reagent grade) were supplied by Merck (Darmstadt, Germany). Other chemicals were reagent grade and used as supplied.

2.2. Formulation of PCL microparticles with entrapped protein

A solution of BSA (1 ml containing 188 mg) in ultra-pure water (internal aqueous phase) was emulsified with a 6% (w/v) solution of PCL (10 ml) in dichloromethane (DCM; oil phase) using an Ultraturrax model T25 (IKA Laboratory Technology, Staufen, Germany) at high speed (8000 rpm) and room temperature. The resulting water-in-oil (w/o) emulsion (2.5 ml) was then emulsified with a 5% (w/v) PVA solution (50 ml) in the same conditions that for the first emulsion to produce a water-in-oil-in-water (w/o/w) emulsion. The latter was then stirring magnetically at ± 800 rpm (magnetic stirrer EOA 9 basic IKA-MAG, IKA Laboratory Technology, Staufen, Germany) overnight at room temperature and pressure, to allow the evaporation of the organic solvent and the formation of microparticles. Once their wall was hardened, the microparticles were collected by centrifugation (10 min at $4000 \times g$), washed three times with 10 ml of ultra-pure water and freeze-dried.

In these studies, the effects of the following formulation variables on microparticle size were investigated:

1. Concentration of the polymer solution: this was investigated by variation in the weight of polymer dissolved in a fixed volume of DCM (200, 400, 500 or 600 mg PCL in 10 ml DCM).
2. Effect of protein/polymer ratio: microparticles were prepared with a range of BSA/PCL ratios (1/24, 1/6 or 1/3), by increasing the initial weight of BSA dissolved in the internal aqueous phase.
3. Nature and concentration of emulsion stabilizer in the external aqueous phase: while maintaining a constant volume for the external aqueous phase (50 ml), microparticles were produced using various stabilizers (PVA, SDS or Tween 80) at various concentrations (0.5, 1, 5 or 10%, w/v).
4. Viscosity of the internal aqueous phase: the viscosity was modified by adding PVA in various concentrations (1, 2 or 5%, w/v) in the BSA solution.
5. Volume of the external aqueous phase: this was studied by variation in the volume of the second aqueous phase (10, 20 or 50 ml) while the PVA concentration was maintained at 5% (w/v).
6. Duration of agitation during emulsifications (2, 5 or 15 min at 8000 rpm).
7. pH (3, 7 or 10) of the internal aqueous phase.
8. Presence of salts (NaCl) in the internal aqueous phase.

2.3. Microparticle characterization

2.3.1. Morphology

The shape and surface texture of microparticles were determined with a Hitashi S-570 scanning electron microscope. A small amount of microparticles was suspended in ultra-pure water. A drop of the suspension obtained was placed on the sample holder, dried and observed after coating with gold-palladium under an argon atmosphere.

2.3.2. Particle size distribution

The microparticles sonicated for 30 s at 50–70 Watts (Sonifier B-12, Branson Sonic Power Company) and dispersed in filtered (0.1 μm) saline solution of 0.9% (w/v) NaCl were sized by employing a Coulter Multisizer (Coulter Electronics Ltd., Luton, UK) equipped with a sieve of 100 μm aperture and under continuous stirring. The results obtained from measurements of at least three batches of microparticles are described in volumetric mean diameter of the microparticles (VMD) in micrometers ± SEM which is the diameter that divides the volume distribution curve of the sampled microparticles in two equal parts.

2.3.3. Determination of the BSA entrapment

Estimation of the BSA content was carried out after alkaline hydrolysis of the polymer using NaOH and extraction by SDS as previously described (Hora et al., 1990). Briefly, 30–50 mg of microparticles, accurately weighted, were shaken with 3.0 ml of 1 M NaOH containing 5% (w/v) SDS during 24 h at room temperature. The supernatant obtained by centrifugation ($4000 \times g$ for 10 min at room temperature), was analyzed by Lowry's method of protein assay after making background corrections for PVA (Lowry et al., 1951). The percentage (w/w) of protein entrapped per dry weight of microparticles was determined. The percentage of entrapment efficiency was expressed by relating the actual protein entrapment to the theoretical protein entrapment as previously described (Jeffery et al., 1993). Each sample was assayed in triplicate.

2.4. In vitro release of BSA

In vitro release of BSA from microparticles (500 mg) was measured after a preincubation step for 2 h at 37°C under gentle agitation in screw-capped vials containing 3 ml of phosphate buffer (0.01 M phosphate buffered saline; NaCl 0.138 M; KCl 0.0027 M, pH 7.4) or artificial gastric medium (7 ml HCl (36.5%, w/w), 3.2 g pepsin (0.6 U/mg) and 2.0 g NaCl for 1 l of water, pH 1.2) (USP XXII/NFXVIII). Each medium also contained 0.1% (w/v) sodium azide as bacteriostatic agent. After neutralization of the pepsin activity by addition of 0.1 N perchloric acid and elimination of the surrounding media by centrifugation ($4000 \times g$, 10 min), the microparticles were washed three times with 10 ml of PBS and incubated again in 5 ml of PBS to evaluate the BSA release. In order to overcome the interference due to PVA in protein estimations, blanks containing 500 mg of placebo microparticles were also incubated as controls. At definite time intervals, the content of vials (5 ml) was totally withdrawn by centrifugation ($4000 \times g$, 10 min) and replaced by the same volume of fresh medium. The amount of released BSA was estimated by Lowry's method (Lowry et al., 1951).

2.5. Molecular weight and isoelectric point of the entrapped BSA

PAGE analysis of untreated BSA and BSA released from PCL microparticles was undertaken following the method described by Laemmli (1970) by using the HOEFER electrophoresis Cell SE 600 Series (Pharmacia Biotech, Uppsala, Sweden). Samples of BSA released from PCL microparticles in ultra-pure water, untreated BSA and a molecular weight reference marker (molecular weight 14–94 kDa) were solubilized with sample buffer containing SDS and a reducing agent (β-mercaptoethanol), loaded onto a vertical slab gel (10%), and subjected to electrophoresis at 45 mA. The proteins separated by this way were fixed and stained with Coomassie brilliant blue R 250 (0.1%, w/v) in water:acetic acid:methanol (50:10:40). The gel was finally dried by using a gel dryer model 583 from Bio-Rad (Nazareth, Belgium).

Retention of the isoelectric point was assessed by IEF by using an Ampholine® PAG plate precast polyacrylamide gel (pH 3.5–9.5) with the Multiphor II electrophoresis unit from Pharmacia Biotech (Uppsala, Sweden). Samples of BSA released from PCL microparticles in ultra-pure water, unencapsulated BSA and isoelectric point markers (pI 3.50–9.30) were applied on the surface of the gel and focused at the following running conditions (1500 Volts, 50 mA, 30 W for 1.5 h at 10°C).

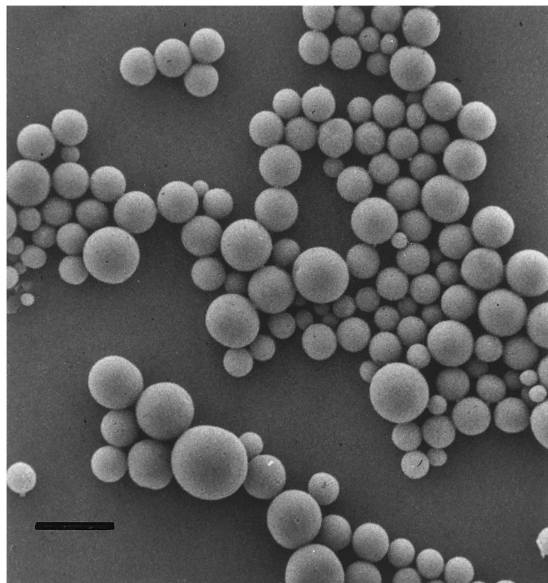

Fig. 1. Scanning electron micrograph of PCL microparticles entrapping BSA produced by the standard double emulsion-solvent evaporation method (Scale bar = 5 μm).

3. Results and discussion

3.1. Microparticles morphology

Fig. 1 displays a representative SEM micrograph of PCL microparticles 2.7 ± 0.07 μm in diameter containing $2.53 \pm 1.03\%$ (w/w) BSA obtained in standard conditions mentioned in Section 2. The particles appeared to be spherical, smooth and homogeneously distributed without evidence of collapsed particles.

3.2. Concentration of the polymer solution

BSA loaded microparticles were prepared using different concentrations of PCL (from 0.5 to 6%, w/v) by variation in the weight of polymer dissolved in dichloromethane to investigate the eventual modifications of the particle size, protein loading and efficiency of entrapment (Table 1).

Increasing the concentration (weight) of polymer in a fixed volume of organic solvent resulted in an increase in mean particle size by at least a factor of 2.7 and in an improvement of the protein entrapment efficiency and BSA loading, respectively, by a factor of 20 and 5 when a 5% (w/v) polymer solution was used. This is in agreement with the findings of Jeffery et al. (1991), suggesting that the higher concentration of polymer in the sample may have led to an increased frequency of collisions, resulting in fusion of semi-formed particles and producing finally an overall increase in the size of the microparticles. Nevertheless, a monomodal size distribution was maintain (data not shown). Moreover, the high concentration of polymer in the emulsion droplets led to an enhancement of the efficiency of protein entrapment because the high viscosity of the organic phase tends to restrict migration of the inner aqueous/protein phase to the external water phase (Rafati et al., 1997). Finally, at high polymer concentration (6%, w/v), the viscosity was so high that the efficiency of emulsion stirring was reduced and allowed the production of large particles (5.88 ± 0.74 μm in mean diameter) with a reduced entrapment efficiency ($3.96 \pm 0.31\%$).

Whatever the initial concentration of the polymer solution, the entrapment efficiency of BSA

Table 1
Effect of the polymer concentration

Polymer concentration (%)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
0.5	2.12 ± 1.02	12.24 ± 0.75	0.98 ± 0.09	1.56 ± 0.17
1	2.56 ± 1.24	14.95 ± 1.22	1.03 ± 0.14	3.54 ± 0.21
3	2.73 ± 0.62	9.75 ± 1.68	1.44 ± 0.24	4.25 ± 0.32
5	4.33 ± 0.52	12.45 ± 0.97	5.52 ± 1.12	30.29 ± 5.01
6	5.88 ± 0.74	10.92 ± 0.90	1.38 ± 0.11	3.96 ± 0.31

Table 2
Effect of protein/polymer ratio

Weight of BSA (mg; ratio BSA/polymer)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
25 (1/24)	6.05 ± 0.96	9.22 ± 1.90	1.50 ± 0.18	35.92 ± 4.24
100 (1/6)	5.26 ± 0.18	7.94 ± 0.32	1.09 ± 0.22	6.54 ± 1.30
188 (1/3)	5.88 ± 0.74	10.92 ± 0.90	1.38 ± 0.11	3.96 ± 0.31

Table 3
Effect of the emulsion stabilizer type and concentration in the external aqueous phase

Stabilizer	Concentration (%)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
PVA	0.5	8.09 ± 0.44	15.59 ± 1.28	0.38 ± 0.02	1.21 ± 0.10
	1	8.51 ± 0.62	16.54 ± 0.97	0.51 ± 0.02	1.61 ± 0.09
	5	5.88 ± 0.74	10.92 ± 0.90	1.38 ± 0.11	3.96 ± 0.31
	10	6.27 ± 1.74	18.34 ± 1.39	0.31 ± 0.02	0.99 ± 0.08
Tween 80	5	15.27 ± 0.40	34.13 ± 2.72	0.23 ± 0.01	0.73 ± 0.01
SDS	10	13.60 ± 1.14	19.62 ± 1.46	0.19 ± 0.02	0.71 ± 0.24

obtained in our experimental conditions was always lower than the results described by Jameela et al. (1998) (30 vs. 60%). This difference could be related to a difference in the crystallinity of the polymers used. Since the crystalline phase of PCL is essentially impermeable to water, the entrapment of hydrophilic drugs likely occurs in the amorphous regions of the polymer. Thermal analyses (data not shown) showed that the polymer selected for our studies is characterized by a high crystalline content (> 70%). Hence, the number of amorphous domains available for the encapsulation of hydrophilic compounds is reduced, allowing a low entrapment efficiency. On the contrary, the entrapment efficiency of BSA into microparticles from PCL (Tone polymer P-767 Union Carbide) characterized by a low crystalline content (< 50%) can reach 65% (data not shown).

3.3. *Effect of protein/polymer ratio*

The increase of the initial weight of BSA (25, 100 or 188 mg) dissolved in the internal aqueous phase produced a dramatic decrease in the entrapment efficiency (87% lower) without alteration in the microparticle size or in the BSA loading (Table 2). It was postulated that, at high BSA concentration (and thus at high protein/polymer ratio), the quantity of polymer present was insufficient to cover the BSA completely.

3.4. *Nature and concentration of emulsion stabilizer in the external aqueous phase*

Of the stabilizers studied (PVA, Tween 80 or SDS) all resulted in successful preparation of microparticles. Nevertheless, 5% (w/v) PVA was selected as stabilizer of choice for further studies, since it allowed the preparation of particles in the desired size range (1–10 μm) with an interesting BSA loading (1.38 ± 0.11%, w/w; Table 3).

Except when 5% (w/v) PVA is used, it could be shown that changes in PVA concentration were devoided of effect on protein entrapment, particle size and particle size distribution. The same trends were reported by Rafati et al. (1997) for BSA-loaded PLG microparticles when high polymer concentrations (6%, w/v) were used. Whatever the PVA concentration in our experimental conditions, the emulsion droplets formed during agitation seemed to be stable enough to harden after solvent evaporation and form the microparticles.

3.5. Viscosity of the internal aqueous phase

Increasing viscosity of the internal phase (Table 4) by addition of increasing concentrations of PVA (1, 2 and 5%, w/v) led to a slight increase in the particle size (from 6.44 ± 0.08 μm without PVA to 9.62 ± 0.29 μm with 5% PVA, w/v) with out alteration on the unimodal size distribution. Contrary to the results obtained by Ogawa et al. (1988) claiming that an increase in the internal aqueous phase viscosity, which was related to a reduction in the partitioning of the antigen into the external aqueous phase, produced a significant increase in ovalbumin entrapment into PLG microparticles, our results demonstrated no important effect of this parameter on BSA loading and entrapment efficiency when PVA solutions at 1 or 2% (w/v) were used as described by Jeffery et al. (1993). Nevertheless, the use of 5% (w/v) PVA in the inner aqueous phase was characterized by a significant decrease in BSA loading and entrapment efficiency (respectively, by a factor of 3.6 and 3.7) which could be due to a reduction of the stirring efficiency correlated to the diffusion of BSA from the inner aqueous phase to the external aqueous phase.

3.6. Volume of the external aqueous phase

An increase in the volume of the external aqueous phase (from 10 to 50 ml) resulted in an increase in both BSA entrapment (four times higher) and microparticle size (2.5 times higher; Table 5). A volume of 50 ml was selected as the used volume for further studies, since it resulted in a good entrapment efficiency with microparticle size around 5 μm. The increase in particle size was attributed to a reduction in agitation that occurred because of a decrease in mixing efficiency associated with larger volumes. A reduction in mixing efficiency probably produced an increase in the size of the emulsion droplets formed during the preparative process, which would result in the formation of large microparticles. As a result of increased particle size, there is an associated increase in particle volume, which enables more BSA to be incorporated into the microparticles (Jeffery et al., 1993). A linear relationship was shown to exist between the volume of the external aqueous phase and the amount of BSA entrapped. In addition, a linear relationship also existed between the volume of the external aqueous phase and the particle size of the microparticles produced (relationships not shown).

Table 4
Effect of PVA in the internal aqueous phase

PVA concentration (%)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
0	6.44 ± 0.08	15.61 ± 2.35	2.66 ± 0.28	8.51 ± 0.88
1	6.12 ± 0.40	11.54 ± 1.98	2.15 ± 0.06	6.88 ± 0.16
2	8.35 ± 0.55	14.24 ± 0.28	2.16 ± 0.18	6.93 ± 0.56
5	9.62 ± 0.29	17.35 ± 0.46	0.73 ± 0.14	2.32 ± 0.32

Table 5
Effect of the volume of the external aqueous phase

Volume (ml)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
10	3.26 ± 0.35	9.16 ± 1.53	0.69 ± 0.11	2.20 ± 0.35
20	3.09 ± 0.61	10.05 ± 1.94	1.39 ± 0.00	4.44 ± 0.00
50	7.17 ± 1.24	11.22 ± 2.98	2.67 ± 0.28	8.51 ± 0.88

Table 6
Effect of the agitation duration during emulsifications

Duration (min)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
2	4.40 ± 0.28	10.67 ± 0.35	0.69 ± 0.11	2.21 ± 0.35
5	3.52 ± 0.57	6.46 ± 0.19	1.57 ± 0.08	5.03 ± 0.28
15	3.26 ± 0.35	10.33 ± 0.90	0.47 ± 0.13	1.50 ± 0.40

3.7. Duration of agitation during emulsifications

For a constant speed of 8000 rpm, an increase of the stirring time from 2 to 15 min resulted in a 30% reduction in microparticle size (from 4.40 ± 0.28 to 3.26 ± 0.35 μm; Table 6). These observations could be explained by the increased shear stresses generated in the emulsions associated to the increase in the duration of agitation at high homogenization rates tending to divide the droplets of the emulsions and finally inducing a decrease in the mean particle size. A 5-min stirring time was chosen because the entrapment efficiency was higher (5.03 ± 0.28%) than after 15 min (1.50 ± 0.40%).

3.8. Effect of the salts concentration and pH of the internal aqueous phase

An increase in the internal aqueous phase pH (from 3 to 10) induced a slight increase in the particle size (5.54 ± 0.28 to 8.02 ± 0.90 μm) without any important alteration in BSA loading or entrapment efficiency (Table 7). Future studies will be conducted with the internal aqueous phase at pH 7.0.

If the presence of salts (NaCl 0.9%, w/v) in the inner aqueous phase failed to modify the particle size, it led to reduced BSA loading and entrapment (by a factor of 1.6; Table 8). Future studies will be conducted with a minimal concentration of salts by desalting the inner aqueous phase by using centricon concentrators (Amicon, Beverly, USA). Nevertheless, the eventual modifications in the release of entrapped protein following pH and salt concentration variations related to modifications in the interactions between the matrix and protein remains to be investigated.

3.9. In vitro release behavior

The protein release profile of BSA-loaded mi-

Table 7
Effect of the pH of the internal aqueous phase

pH	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
3	5.54 ± 0.28	10.36 ± 0.46	1.73 ± 0.07	6.28 ± 0.23
7	5.84 ± 0.38	10.47 ± 0.24	2.53 ± 0.30	8.58 ± 1.03
10	8.02 ± 0.90	12.36 ± 0.96	2.21 ± 0.44	8.02 ± 1.28

Table 8
Effect of salts (NaCl) in the internal aqueous phase

Salt concentration (%)	Mean size (μm)	90% of the volume ≤ (μm)	BSA loading (%, w/w)	Entrapment efficiency (%)
0	6.12 ± 0.40	11.54 ± 1.98	2.15 ± 0.06	6.88 ± 0.16
0.9	6.40 ± 0.65	11.86 ± 2.06	1.37 ± 0.13	4.39 ± 0.40

croparticles prepared using 5% (w/v) PCL solution and 5% (w/v) PVA was investigated after a preincubation step in gastric conditions or in PBS (Fig. 2) in order to evaluate the suitability of these microparticles as vaccine carriers devoted to oral route. For both conditions of preincubation, the release profiles exhibited a burst phase of protein release in the early stages of testing amounting to approximately 30% of the protein loading. This first release step was followed by a low and constant rate of BSA release occurring in our in vitro conditions for more than 3 months (data not shown) and probably linked to the low permeability of macromolecules in this polymer. This burst release of protein was normally considered to be due to the surface-located protein (Yan et al., 1994). This phenomenon seems to be more accentuated by the high hydrophobicity of the polymer, associated to high water-soluble properties of the entrapped molecule for which the surface situation of the entrapped protein was already demonstrated (Benoit et al., 1997). This release profile would be especially well-adapted for vaccine release requiring a high initial dose for the priming of immune responses and a slow continuous release to induce the booster doses after a delay-time.

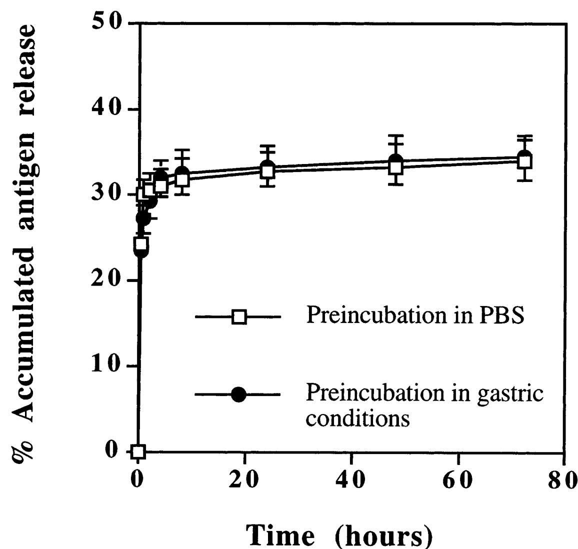

Fig. 2. Protein release from BSA-loaded microparticles prepared following the standard conditions and preincubated in gastric medium or in PBS before study.

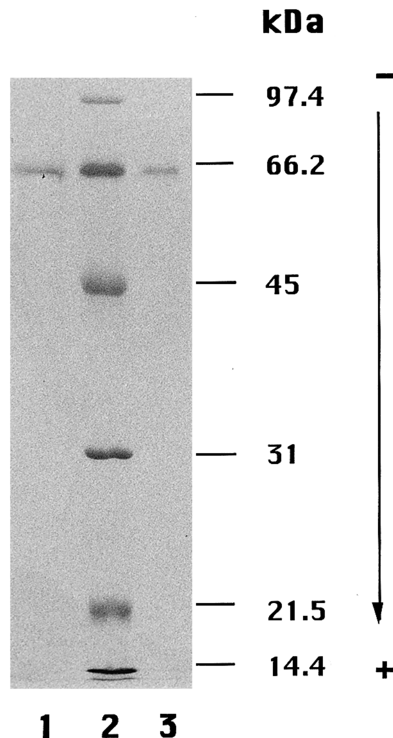

Fig. 3. Coomassie R 250-stained electrophoretic gel of BSA released from PCL microparticles produced by the double emulsion-solvent evaporation method. Lane 1: purified BSA (1 μg) untreated for encapsulation. Lane 2: molecular weight markers. Lane 3: BSA (1 μg) released from particles.

3.10. Study of the structural integrity of entrapped BSA

During microparticle preparation, the antigen was exposed to potentially harsh conditions, such as contact with organic solvents and mechanical agitation. These conditions may result in irreversible denaturation and loss of antigenicity of proteins. PAGE and IEF analyses followed by Coomassie blue staining revealed identical bands for the released and untreated BSA (Figs. 3 and 4). There were no additional bands to indicate the presence of aggregates (molecular weight $>$ 66

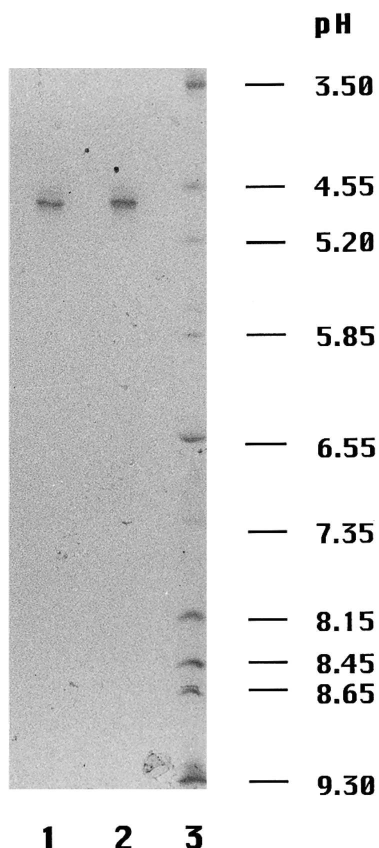

Fig. 4. Coomassie R 250-stained isoelectric focusing gel of BSA released from PCL microparticles produced by the double emulsion-solvent evaporation method. Lane 1: purified BSA (0.8 μg) untreated for encapsulation. Lane 2: BSA (1.0 μg) released from particles. Lane 3: isoelectric point markers.

kDa) or other fragments produced by proteolysis (molecular weight <66 kDa). Hence, the data suggest that the structural integrity of this protein was not significantly affected by the entrapment procedure. In previous studies, using SDS-PAGE and rarely IEF, it was demonstrated that the double emulsion-solvent evaporation technique did not affect the primary structure of microencapsulated antigens such as ovalbumin (Jeffery et al., 1993), human serum albumin (Hora et al., 1990), tetanus toxoid (Almeida et al., 1993) or cholera toxin B subunit (O'Hagan et al., 1993a).

4. Conclusion

This study has demonstrated that microparticles of poly(ε-caprolactone) loaded with protein may be produced by a modified w/o/w technique. These investigations have also provided an understanding of the effects of some process parameters on particle size, protein loading and entrapment efficiency.

Selection of the appropriate conditions has enabled the preparation of smooth, spherical and homogeneous PCL microparticles, with a mean size of 10 μm, and without significantly altering the entrapped protein. The parameters were selected to obtain high protein entrapment efficiency, with more than 30% of the initial BSA dissolved in the internal aqueous phase being entrapped into microparticles when a polymer concentration of 5% (w/v) and a BSA initial weight of 25 mg were used. High entrapment efficiency is important when entrapping antigens in relatively short supply. These formulations would be produced with a desalted internal aqueous phase and the volumes of the internal and external aqueous phases, respectively, of 1 and 50 ml, with 5% (w/v) PVA as emulsion stabilizer in the external aqueous phase. The two emulsifications would be performed by stirring emulsions 5 min at 8000 rpm.

The biodegradable property of the polymer, its hydrophobicity and its resistance to acidic pH make this delivery system a potential carrier for mucosal vaccines. Within this framework, the potentialities of this kind of microparticle containing an antigen from *Schistosoma mansoni* as an oral vector, and inducing a long lasting immunity following a single administration, were clearly demonstrated (Benoit et al., 1998). Comparatively to poly(lactide-co-glycolide) microparticles loaded with the same antigen, this hydrophobic polymer (PCL) was shown to be capable of delaying the systemic immune responses after oral or nasal administration (Baras et al., 1999).

Acknowledgements

This work is supported by a grant No. BIO4-

CT96-0374 from EEC. B. Baras was supported by a fellowship from the F.R.I.A. (Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture). The size analyses of microparticles were kindly carried out by P. Rombaut at the Laboratorium voor Galenische en Klinische Farmacie (Professor R. Kinget, Katholieke Universiteit Leuven, Belgium). We are grateful to A. Nguyen at the Unité de Chimie et Physique des Hauts Polymères (Professor R. Legras, Université Catholique de Louvain, Belgium) for SEM analyses. We wish to thank Professors P. Devos at the Laboratoire de Biochimie et Physiologie Comparées and J. Remacle at the Laboratoire de Biochimie Cellulaire (FUNDP, Namur) for the use of their electrophoresis materials.

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