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Parameters influencing the antigen release from spray-dried poly(DL-lactide) microparticles

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Abstract

Microparticles were produced by spray-drying from a high molecular weight polylactide (PLA R207) for the development of long-lasting controlled release systems of vaccines, which may be designed to obviate the need for booster doses. The current investigation considered the effect of both technological parameters (inlet air temperature and spray rate of feed) and polymeric solutions (polymer concentration and nature of organic solvents) on characteristics of microparticles (morphology, size and antigen loading) containing a water-soluble model antigen (bovine serum albumin, BSA). Following parameters chosen, microparticles were characterized by a mean size from 3.08 ± 0.06 to 9.43 ± 0.26 μm and a BSA loading from 2.45 ± 0.13 to $18.20 \pm 2.25\%$ (w/w). The BSA release rate from microparticles varied from 11.17 ± 2.20 to $92.60 \pm 3.46\%$ in 24 h. The modification of the inlet temperature, the spray-rate of feed or the use of a mixture of dichloromethane/chloroform (DCM/CFM) instead of DCM alone resulted in the modification of the BSA burst release. This burst release was followed by a BSA release rate slower for microparticles with a low BSA loading. Moreover, the increase of the R207 concentration resulted in a decrease of the BSA release rate while the burst release was not modified. SDS-PAGE electrophoresis and isoelectric focusing analyses of the BSA released from microparticles confirmed the preservation of its physicochemical characteristics. Together, results showed that the spray-dried microparticles loaded with hydrophilic antigen could be used as a potential delivery system for the long-lasting controlled release of vaccines.

Keywords: Microparticles; Polylactide; Spray-drying; Vaccine; Controlled release

1. Introduction

In conventional protein-based vaccines, multiple-dose administration of an antigen is usually required to induce a long-lasting immunity. The preparation of formulations capable of releasing the antigen in a controlled manner for a time that

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is sufficient to induce this immunity may be a promising improvement in immunization coverage and individual compliance (Bittner et al., 1998). The continuous release of antigens from polymeric delivery systems can provide a prolonged immune response while avoiding the need for multiple administration. To design these systems, it is important to choose a polymer that is biodegradable, biocompatible, and safe for use in humans. Polylactide (PLA) was chosen as polymer to prepare microparticles. These polyester polymers have several advantages (Okada et al., 1991). PLA degrade to molecules normally found in the body as metabolic by-products and hence have no unwanted side effects. In addition to their proven safety record, these polyesters can be tailored to fit many delivery applications by altering some aspects of their manufacture, such as composition and particle size. Polymeric microparticles have several advantages including a reduction of the number of inoculations required as well as the total amount of immunogen needed to generate a good immune response (Iguarta et al., 1998). Moreover, in case the antigen is administered by the oral route, the microencapsulation procedure may protect the antigen from the gastric acidic environment and proteolytic enzymes (Benoit et al., 1998, 1999; Baras et al., 1999).

The production technology mainly used in the manufacture of microparticles has been solvent evaporation or solvent extraction from emulsion systems. Problems encountered with these methods of production include the use of high shearing forces, long exposure time of the antigen to organic solvents and the necessity of lyophilization to obtain a stable powder. These problems may result in the degradation of a sensitive antigen. The spray-drying technique is another technique which can be used for the microencapsulation of antigens. The use of this technique appears to be attractive for the preparation of microparticles and it seems to come close to the properties desired: simple, reproducible, rapid and easy to scale-up (Bodmeier and Chen, 1988). It consists of spraying an emulsion of polymer and drug through the nozzle of a spray dryer apparatus; the solvent evaporates very quickly, leaving solid microparticles (Pavanetto et al., 1992). The spray-drying process involves the following four sequential stages: atomization of the product into a spray nozzle, spray–air contact, drying of the sprayed droplets and collection of the solid product (spray-dried microparticles) obtained (Broadhead et al., 1992). A stable powder is rapidly obtained without lyophilization. In a single operation a drug can be entrapped into a polymer either as a solution, a dispersion or an emulsion (Bittner et al., 1998). Due to the rapid evaporation of the solvent, the temperature of the droplets can be kept below the drying air temperature, and for this reason spray-drying can be applied to heat-sensitive materials (Broadhead et al., 1992). Hydrophobic as well as hydrophilic drugs can be incorporated into a biodegradable polymer by spray drying. In this study, the spray-dryer is a Mini Büchi 190 which is a laboratory scale spray-dryer. The main components of the equipment of a standard spray-dryer include the air heater, the nozzle/atomizer, the desiccation chamber (spraying cylinder), the fan, the cyclone (for the separation between the product and the air flow) and a final vessel which collects the spray-dried product.

Until now, process parameters such as concentration of the polymeric solution, inlet air temperature and spray-rate of feed (pump setting) have been principally investigated for the preparation of microparticles containing a lipophilic drug (Conte et al., 1994). As polypeptides usually used for vaccines are water soluble, while the selected polymers are soluble only in organic solvent, preparation methods of microparticles that combine both organic and water phases have been studied. The initial stages in the microencapsulation of proteins involved emulsification of aqueous solutions of proteins with polymers dissolved in an organic solvent. Indeed, the addition of antigen as a powder to the organic phase could result in an immediate release of the protein in the buffered medium from microparticles. The insolubility of the antigen in methylene chloride (dichloromethane) or chloroform could give rise to a nonuniform distribution of large protein islands in the polymer matrix, presumably closer to the microparticle surface, and thus result in the fast release of the antigen in the external aqueous medium (Cohen et al., 1991). Uncontrolled distribution of proteins may produce variable and non-

reproducible release kinetics, making the design of controlled-release systems a difficult task. To overcome these problems, polyvinyl alcohol (PVA) has been associated with the antigen in the aqueous phase. Indeed, we have recently observed that PVA was required to stabilize the emulsion (Baras et al., 2000a). This stabilizer acts as a protective polymer by being adsorbed at the oil/water interface of droplets to produce a steric barrier which protects the antigen against the external organic solvent. PVA also decreased the coalescence of the microparticles, increase the antigen loading within microparticles, decrease the microparticle size and decrease the porosity of microparticles to allow a slow antigen release from microparticles (Baras et al., 2000a). Finally, Lee et al. (1999) recently showed that PVA binding affects the hydrophobicity and thus the digestibility of the microparticle surface, leading to the decrease of the polymer degradation rate and the drug release rate.

Thus, our study tried to produce microparticles containing a hydrophilic model antigen by a specific microencapsulation process to obtain a long-lasting continuous antigen release which could provide a prolonged immune response. For this aim, we have chosen to use a high molecular weight PLA polymer (R207). Here, we describe studies that were undertaken to optimize the spray-drying technique to produce microparticles of approximately 5–10 μm in mean diameter and entrapping a model antigen (Bovine Serum Albumin, BSA, 66 kDa). This study considered how various common process parameters (concentration of the polymeric solution and nature of the organic solvents) could affect the microparticles characteristics (shape and size, BSA loading and in vitro BSA release behaviour). The current investigation also investigated the effect of other critical technological parameters such as inlet air temperature and spray rate of feed on the preparation and the characteristics of the microparticles obtained by the spray drying method. Finally, the structural integrity of the entrapped protein was investigated by polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing. Indeed, during the spray-drying process, the protein is exposed to physical stresses (organic solvents, aqueous/organic interface and relatively adverse temperature and emulsion conditions) that may produce structural changes of the protein and thus may result in partial or total loss of enzymatic activity and antigenicity (Gander et al., 1995).

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial suppliers and used as received: poly-D,L-lactide R207 (molecular weight, 209 000) (Boehringer, Ingelheim, Germany); polyvinyl alcohol (PVA) (molecular weight, 13 000–23 000; 87–89% hydrolyzed) (Aldrich, Bornem, Belgium); dichloromethane (DCM) and chloroform (CFM) (UCB, Braine L'Alleud, Belgium); sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO); low molecular weight standards (molecular weight from 14 400 to 97 400) and isoelectric focusing (IEF) standards (broad pI kit range from pH 3.5 to 9.3) were respectively supplied by Bio-Rad (Nazareth, Belgium) and Pharmacia Biotech (Uppsala, Sweden); Ampholine® PAG plate pH range 3.5–9.5 (Pharmacia Biotech, Uppsala, Sweden); albumin (from bovine serum, fraction V) (BSA), Folin-Ciolcateu's phenol reagent and other materials (reagent grade) (Merck, Darmstadt, Germany).

2.2. Preparation of microparticles with an entrapped model antigen

An aqueous phase solution (1 ml of Ultrapure water), containing BSA (50 mg/ml) and polyvinyl alcohol (PVA, 10 mg/ml) as stabilizer, was emulsified with a solution (100 ml) of polymer (0.5, 1 or 3.0%, w/v) in various organic solvents (DCM or a mixture DCM/CFM) using an Ultraturrax model T25 (IKA Laboratory Technology, Staufen, Germany) at 8000 rev./min for 5 min and room temperature.

Microparticles were obtained by spraying this emulsion through the nozzle (0.5 mm) of a Büchi mini Spray Dryer Model 190 (Büchi Laboratoriums-Technik AG, Flawil, Switzerland). The pro-

cess parameters were set following the process conditions listed in Table 1. Air flow rate (500 l/h, volume of the air input), spray flow (6 bar) and aspirator setting (15) were maintained constant. The inlet temperature was varied between 44 and 54°C whereas the spray-rate of feed was varied from 3.0 to 5.0 ml/min.

2.3. Microparticle characterization

2.3.1. Morphology

A Hitachi S-570 scanning electron microscope was used to assess the shape and surface morphology. A small amount of microparticles was suspended in Ultrapure water, sonicated for 30 s at 50–70 W (Sonifier B-12, Branson Sonic Power Company). A drop of this suspension was placed on the sample holder, dried and observed with an electron microscope after coating with gold–palladium under an argon atmosphere.

2.3.2. Particulate size analysis

The microparticles dispersed in filtered (0.1 μm) saline solution of 0.9% (w/v) NaCl were sized with a Coulter Multisizer (Coulter Electronics, Luton, UK) equipped with a sieve of 100 μm aperture and under continuous stirring. Particle size was expressed as volume mean diameter (VMD) in micrometers $\pm$ S.E.M. ($n = 3$).

2.3.3. BSA loading in microparticles

Thirty to fifty milligrams of spray-dried microparticles, accurately weighted, were dissolved in 3.0 ml of 1 M NaOH containing 5.0% (w/v) SDS during 24 h at room temperature (Hora et al., 1990). After centrifugation ($4000 \times g$ for 10 min at room temperature), the supernatant was assayed following the method of Lowry et al. (1951) to determine the BSA concentration. The percentage of BSA entrapped per dry weight of spray-dried microparticles (w/w) was determined. The percentage entrapment efficiency was expressed by relating the actual BSA loading to the theoretical BSA loading as previously described (Jeffery et al., 1993). Each sample was assayed in triplicate.

2.4. In vitro release study

Microparticles (40–60 mg) were suspended in 3 ml of phosphate buffered saline (PBS, pH 7.4) (0.01 M phosphate buffered saline, 0.138 M NaCl and 0.0027 M KCl) containing 0.1% (w/v) sodium azide as bacteriostatic agent. Release studies were conducted at 37°C under continuous stirring. Each assay was replicated three time. At each time interval the medium (accurately measured) was withdrawn and albumin release was determined by the analytical method describe above (Lowry et al., 1951), the suspension being refilled with the same volume of fresh medium.

2.5. Study of the structural integrity of the entrapped antigen

The assessment of the integrity of the BSA released from microparticles into phosphate buffered saline (PBS, pH 7.4) and the non-entrapped BSA in PBS was investigated using both polyacrylamide gel electrophoresis (SDS-PAGE) and IEF (isoelectric focusing) techniques. For SDS-PAGE, protein samples were analysed by using a 12% gel and the Hoefer system (Hoefer Scientific Instruments, SE 600 Series Electrophoresis Unit, San Francisco, CA) according to the method described by Laemmli (1970). Low molecular weight standards (range from 14.4 to 94 kDa) were used. The gel was fixed and stained with Coomassie brilliant blue R-250.

Retention of the structural integrity of the entrapped antigen (BSA) was also assessed by isoelectric focusing (IEF). BSA released from microparticles into Ultrapure water, non-entrapped BSA in Ultrapure water, and IEF standards for a range of isoelectric points (pI) from 3.50 to 9.30, were loaded onto a precast polyacrylamide gel (Ampholine® PAG plate) and focused using a Multiphor II electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden). The gel was fixed, stained with Coomassie brilliant blue R-250 and destained as described in the Pharmacia Biotech protocol (Ampholine PAG plate-instructions, Pharmacia Biotech, Uppsala, Sweden).

Table 1
Effect of various technological parameters and organic solvents on characteristics of R207 microparticles

Batch #	Organic solvent	Inlet/outlet temperature (°C)	Spray-rate of feed (ml/min)	Mean size (VMD ± S.E.M.) (μm)	90% of the volume ≤ to (μm)	BSA loading (%, w/w)
1	DCM	44–46/34–35	3.0–3.5	3.62 ± 0.36	5.36 ± 0.58	12.63 ± 0.35
2	DCM/CFM	44–46/35–36	3.0–3.5	3.26 ± 0.09	5.38 ± 0.22	8.84 ± 0.74
3	DCM	44–46/33–34	4.5–5.0	4.10 ± 0.41	5.84 ± 0.46	18.20 ± 2.25
4	DCM/CFM	44–46/35–36	4.5–5.0	3.08 ± 0.06	4.51 ± 0.08	8.75 ± 0.98
5	DCM	52–54/39–40	4.5–5.0	3.32 ± 0.17	4.74 ± 0.12	13.97 ± 1.55
6	DCM/CFM	52–54/38–39	4.5–5.0	3.13 ± 0.09	4.43 ± 0.19	7.18 ± 0.40

2.6. Statistical analyses

Statistical evaluation of the data used either Mann–Whitney's test (for comparisons of the effect of various formulation parameters on microparticle characteristics) or the ANOVA test (for comparisons of the % BSA released from various formulations). P-values of 0.05 or less were considered significant.

3. Results

3.1. Microparticle characterization

The shape and surface texture of R207 microparticles were investigated using scanning electron microscopy (Fig. 1). Photomicrographs of microparticles obtained from R207 (Fig. 1) showed irregular and incompletely formed microparticles. Microparticles produced from 0.5% (w/v) of R207 (Fig. 1A) were characterized by a porous crumpled surface whereas microparticles produced from 3.0% (w/v) of R207 (Fig. 1B) were clearly less porous. Microparticles were characterized by a size smaller than 13.92 ± 0.09 μm and, according to the parameter selected, a mean size of about 3.08 ± 0.06 to 9.43 ± 0.26 μm and a BSA loading ranging from 2.45 ± 0.13 to 18.20 ± 2.25% (w/w) (Table 1).

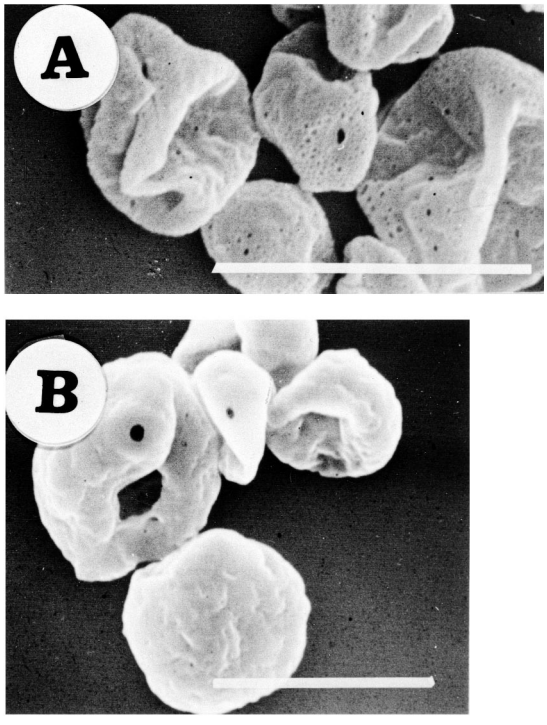

Fig. 1. Scanning electron photomicrographs at different magnifications of microparticles produced from 0.5% (w/v) (A) or 3.0% (w/v) (B) of R207 microparticles. Horizontal bar, 10 μm.

3.2. Effect of the spray-rate of feed, the inlet temperature and the nature of the organic solvent

When DCM was used as organic solvent (Table 1), an increase of the spray-rate of feed (from 3.0–3.5 to 4.5–5.0 ml/min) during the microparticle preparation from R207 (0.5%, w/w) was without effect ($P > 0.05$) on the mean size of microparticles (from 3.62 ± 0.36 to 4.10 ± 0.41 μm at an inlet temperature of 44–46°C) but induced a significant increase ($P < 0.05$) of the BSA loading (from 12.63 ± 0.35 to 18.20 ± 2.25%, w/w, at an inlet temperature of 44–46°C). An increase of the inlet temperature (from 44–46 to 52–54°C) was without effect ($P > 0.05$) on the mean size of microparticles and resulted in a significant decrease ($P < 0.05$) of the BSA loading (from 18.20 ± 2.25 to 13.97 ± 1.55%, w/w, for a spray-rate of feed of 4.5–5.0 ml/min) (Table 1).

When compared to the results obtained for those prepared with DCM alone, the use of a DCM/CFM mixture as organic solvent always allowed a lower BSA loading ($P < 0.05$) (e.g. from 18.20 ± 2.25 to 8.75 ± 0.98%, w/w, for an inlet temperature of 44–46°C and a spray-rate of feed of 4.5–5.0 ml/min) without modification of the microparticle size ($P > 0.05$) (Table 1).

3.3. Effect of the concentration of the polymer solution

When inlet air temperature (44–46°C) and spray-rate of feed (4.5–5.0 ml/min) were maintained constant, an increase in the concentration of the polymer solution (from 0.5 to 3.0%, w/v) in DCM induced a significant increase ($P < 0.05$) of the mean size of microparticles (from 4.10 ± 0.41 to 9.43 ± 0.26 μm) and a significant decrease ($P <$

Table 2
Effect of polymer concentration on characteristics of R207 microparticles[a]

Batch #	Polymer concentration (%, w/v)	Mean size (VMD ± S.E.M.) (μm)	90% of the volume ≤ to (μm)	BSA loading (%, w/w)
3	0.5	4.10 ± 0.41	5.84 ± 0.46	18.20 ± 2.25
7	1.0	4.86 ± 0.13	8.84 ± 0.41	4.07 ± 0.52
8	3.0	9.43 ± 0.26	13.92 ± 0.09	2.45 ± 0.13

[a] The technological parameters were fixed with an inlet temperature of 44–46°C (outlet temperature of 33–34°C), a spray-rate of feed of 4.5–5.0 ml/min and the organic solvent was DCM alone.

0.05) of the BSA loading (from 18.20 ± 2.25 to 2.45 ± 0.13%, w/w) (Table 2).

3.4. In vitro release behaviour

The effect of inlet temperature, spray-rate of feed and nature of organic solvent on the release profiles of BSA from microparticles prepared with a solution of 0.5% (w/v) R207 is shown in Fig. 2. When compared to the batch # 3 (24.79 ± 4.41% of BSA released in 24 h), a decrease of the spray-rate of feed (from 4.5–5.0 to 3.0–3.5 ml/min, batch # 1), the use of a DCM/CFM (batch # 4) instead of DCM alone and an increase of the inlet temperature (from 44–46 to 52–54°C, batch # 5) always resulted in a higher BSA burst release (76.34 ± 5.95, 92.60 ± 3.46% and 63.15 ± 4.01 of BSA released in 24 h, respectively from batches # 1, # 4 and # 5, $P < 0.05$) followed by a slightly slower BSA release rate which was sustained for 240 h.

The in vitro BSA release profile from microparticles produced with R207 characterized by various concentration (0.5, 1.0 and 3.0%, w/v) is presented in Fig. 3. The dissolution profiles always showed a gradual release of BSA within 340 h after approximately the same burst release at the beginning. The increase of the polymer concentration (from 0.5 to 3.0%, w/v) was characterized by a significant decrease ($P < 0.05$) of the BSA release rate (from 61.77 ± 5.62 to 19.73 ± 3.57% of BSA released in 360 h).

3.5. Conservation of the structural integrity of the entrapped antigen

The effect of the preparative process on the structural conformation of BSA has been investi-

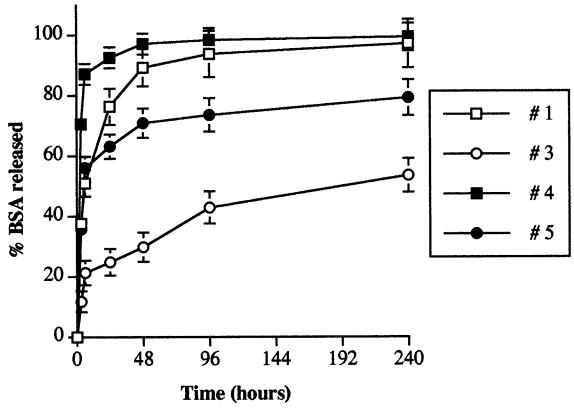

Fig. 2. In vitro release profile of BSA from 0.5% (w/v) R207 microparticles prepared as follows: inlet temperature of 44–46°C (batches # 1, 3 and 4) or 52–54°C (batch # 5); spray-rate of feed of 4.5–5.0 ml/min (batches # 3, 4 and 5) or 3.0–3.5 ml/min (batch # 1) and DCM (batches # 1, 3 and 5) or a DCM/CFM mixture (batch # 4) as organic solvent.

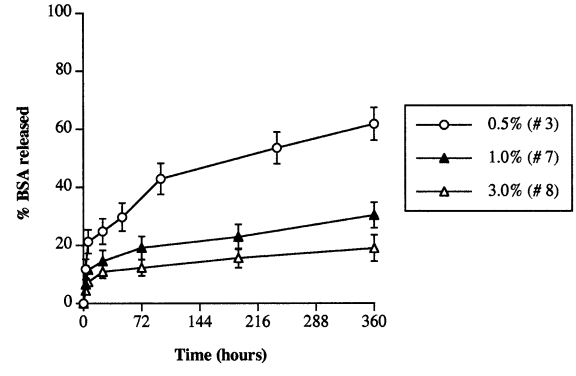

Fig. 3. In vitro release profile of BSA from R207 microparticles at various concentrations (from 0.5 to 3.0%, w/w).

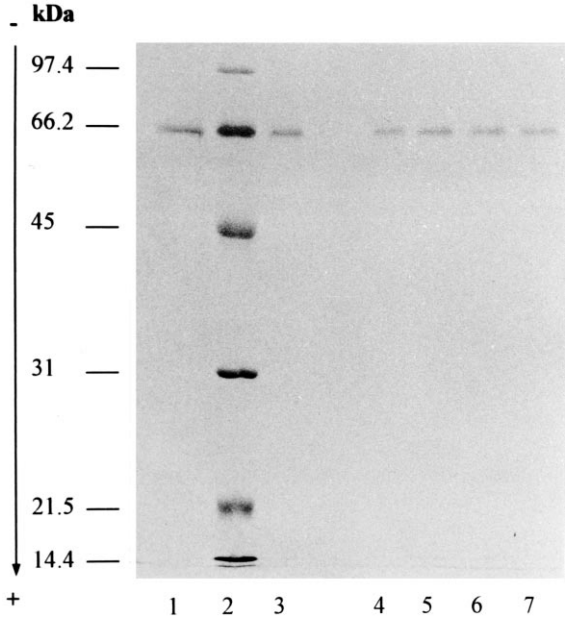

Fig. 4. Coomassie R250-stained SDS-PAGE electrophoretic gel of BSA (1.5 μg) released from microparticles (batches # 1, 3, 4, 5 and 8, respectively lines 3 to 7). Lane 1, non-entrapped purified BSA. Lane 2, molecular weight markers.

gated by using SDS-PAGE (Fig. 4) and IEF (Fig. 5). SDS-PAGE analysis of the entrapped BSA released from microparticles (Fig. 4, lanes 3–7), prepared in different conditions of polymer concentration, temperature and organic solvents showed that no larger or smaller molecular weight fragments of BSA were observed, when compared with non-entrapped BSA (Fig. 4, lane 1). The released and non-entrapped BSA migrate to a molecular weight of 66.2 kDa as compared with the molecular weight markers (Fig. 4, lane 2). Isoelectric Focusing (IEF) indicated identical bands for the non-entrapped BSA (Fig. 5, lane 2) and the BSA released from microparticles (Fig. 5, lanes 3 and 7). The isoelectric point of BSA was pI 4.8, as compared with the isoelectric point markers (Fig. 5, lane 1).

4. Discussion

This study confirmed that spray drying is a convenient technique to prepare microparticles containing a model antigen as vaccine carrier. This one-step fast method would allow large batches of product to be obtained which suggests that spray-drying is a convenient method for microparticle technology. For the first time, a high molecular weight polymer (R207) was used to produce spray-dried microparticles containing a water soluble protein. This polymer was presumed to degrade very slowly when compared to other polymers of lower molecular weight and, thus, was designed to obviate the need for booster doses.

In this study, all preparations resulted in incompletely formed microparticles. It was found

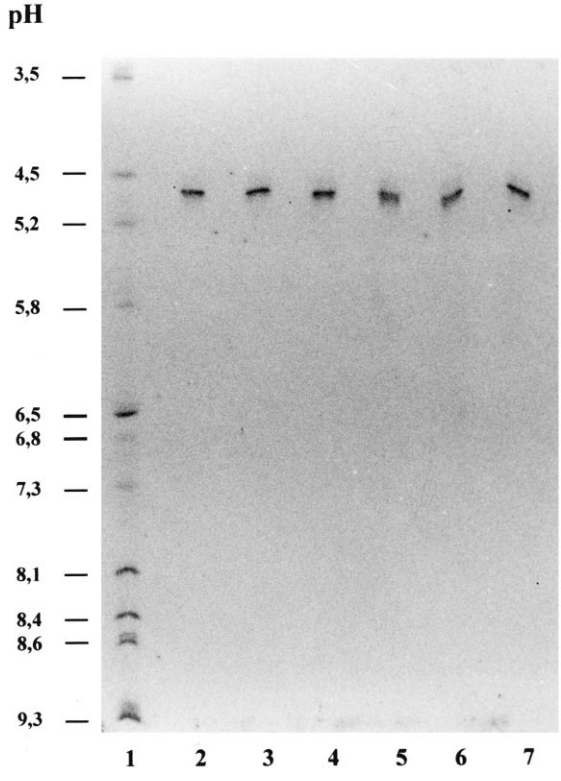

Fig. 5. Coomassie R250-stained isoelectric focusing gel of BSA (1.0 μg) released from microparticles (batches # 1, 3, 4, 5 and 8, respectively lines 3 to 7). Lane 1, isoelectric point markers. Lane 2, non-entrapped purified BSA.

that the achievement of a spherical product from PLA microparticles could not occur because the main problem encountered in the spray-drying of polymeric solutions was the formation of fibres owing to insufficient forces to break up the liquid into droplets (Bodmeier and Chen, 1988). The filament formation strongly depends on the type of polymer used and to a lesser degree on the viscosity of the spray solution. Bodmeier and Chen (1988) demonstrated that PLA organic solutions in dichloromethane resulted in fibres even at as low as 1% concentrations. Intermolecular bonds of the polymeric chains can be responsible for very strong chain interactions, and thus for a high degree of fibre formation. However, the polymer concentration was a limiting parameter to obtain the best results in terms of microparticle shape. Indeed, this study showed that the increase in polymer concentration resulted in microparticles characterized by a lesser porous surface than microparticles produced from a very dilute solution of polymer.

As previously described, microparticle characteristics are dependent on different process parameters such as inlet temperature, spray-rate of feed, polymer concentration in the organic solvent (Conte et al., 1994) and the nature of the organic solvent (Gander et al., 1995). We have studied the effect of these parameters on the preparation of microparticles produced from a high molecular weight PLA. Two technological parameters have been evaluated. The inlet temperature, which is the temperature of air at the entrance to the drying chamber, was sufficient to allow solvent evaporation (the boiling point of dichloromethane and chloroform is respectively, 40 and 61°C) but was not too high to prevent the destruction of the polymer which was characterized by a low glass transition temperature ($<$ 60°C). The spray-rate of feed, which represents the rate at which the polymer and drug solution were sprayed through the nozzle by a peristaltic pump (Conte et al., 1994), was varied from 3.0 to 5.5 ml/min. It corresponded to the flow rate of product through the nozzle when the concentration of polymer in the solution to be sprayed was kept constant. When the concentration of polymer and drug were varied, the peristaltic pump speed was modified to obtain a constant spray-rate of feed. In our study, the modification of these two parameters allowed modulation of protein loading without an effect on microparticle size. An increase of BSA loading was obtained when a high spray-rate of feed or a low inlet temperature was selected.

The selection of organic solvent was also investigated. Gander et al. (1995) showed that to increase the entrapment efficiency and reduce the burst release of entrapped protein, the polymer–drug interaction must be dominant, and the polymer–solvent and drug–solvent interactions must be reduced. To obtain these conditions, dichloromethane proved to be an appropriate solvent to produce microparticles. However, Pavanetto et al. (1993) studied the encapsulation of vitamin D3 (lipophylic drug) in microparticles from different molecular weight polymers (R203, 16 000 M.W.; R206, 109 000 M.W. and R207, 209 000 M.W.). The best results obtained by this group, in terms of microparticle morphology and entrapment efficiency for each polymer, were always correlated with the use of a DCM/CFM mixture (1:1). In our study, where a water-soluble protein must be entrapped, the use of DCM always resulted in the preparation of microparticles characterized by a higher BSA loading than microparticles produced from polymer dissolved in a DCM/CFM mixture without modification of the microparticle size.

Finally, this work also investigated the influence of polymer concentration on microparticle characteristics. A high concentration of the polymer solution resulted in an increased frequency of collisions, resulting in fusion of semi-formed particles. This would produce an overall increase in the microparticles size. Moreover, the increase in the polymer concentration seemed to be related to a higher viscosity of the emulsion which might reduce the efficiency of stirring of the emulsion. These results could explain the significant increase of the microparticle size and the significant decrease of the BSA loading in microparticles.

Whatever the polymer concentration and technological parameters, all preparation were characterized by a size distribution between 1 and 10 μm. These results were in accordance with those

obtained by Pavanetto et al. (1992) who produced spray-dried microparticles containing a lipophylic drug. Therefore, the mean size of spray-dried microparticles obtained in this work would allow their uptake by the M cells of Peyer's patches of the gut and thus permit their use for future oral immunization (Eldridge et al., 1990).

In the second part of this study, the release profile of the model antigen from microparticles has been studied. The protein release from bioerodible polymer matrix can occur by two main routes: by diffusion through a tortuous, water-filled path in the polymer matrix and by matrix bioerosion (Langer, 1990). The latter occurs when the release of the protein from the matrix follows the erosion of the polymer surface and/or bulk matrix rather than by simple diffusion (Cohen et al., 1991). Theoretically, the release pattern often exhibits several phases (Coombes et al., 1998). A significant 'burst release' of protein from the surface of microparticles is generally observed in the first 24 h of testing. This may be followed by a lag phase when little or no release of the entrapped protein occurs due to slow matrix resorption. The release of the entrapped protein from non-porous biodegradable microparticles is preceded by hydrolysis of the particle matrix and diffusion of water soluble, degraded chain fragments into the external medium. Porosity development subsequently permits extraction of the entrapped proteins. The release rate of proteins from microparticles is dependent on the physico-chemical properties of the polymer used (e.g. molecular weight, crystallinity, hydrophobicity), the protein loading level, the microparticle size, the protein distribution into the matrix and on the porosity of the microparticles (O'Hagan et al., 1994). The purpose of this study was to characterize the release profile of each preparation to allow their selection for future vaccine carrier. Indeed, the continuous release of antigens from polymeric delivery systems could provide a prolonged immune response while avoiding the need for multiple administration. However, in this study, the model antigen used was a water-soluble protein and an emulsion step was necessary before spray-drying. Thus, it was important to stabilize the model antigen in microparticles to enhance its prolonged delivery. The stabilization of our model antigen during the process could be carried out by the presence of PVA associated with the antigen in the aqueous phase (Baras et al., 2000a).

Our study on the in vitro BSA release profile showed that the modification of the inlet temperature, the spray-rate of feed or the use of a DCM/CFM instead of DCM alone resulted in the modification of the protein burst release from the surface of microparticles. Indeed, the modification of technological parameters could result either in an incomplete solvent evaporation (residual solvent) or a faster solvent evaporation and thus the formation of pores increasing the burst release. In addition, the boiling point of CFM was always higher than the inlet temperature used in our experiments, which could also result in an incomplete evaporation and the formation of pores. Moreover, the DCM/CFM mixture could interact more intensely with the protein than the DCM alone, thereby transporting the protein towards the microparticle surface during the solvent evaporation in the manufacturing process (Gander et al., 1996). If the protein concentration near the surface of microparticles is high, a more pronounced burst release will be observed. Finally, our results confirmed the results obtained by Gander et al. (1996) who observed that the burst release is inversely proportional to protein loading. The solvent systems giving the highest incorporation rate tend to give the lowest burst release. After this burst release, a sustained release profile was observed. In this second release step, the protein release rate was faster for microparticles characterized by higher protein loading. Indeed, O'Hagan et al. (1994) showed that microparticles with high protein loading resulted in a fast protein release which was facilitated by diffusion through channels of microparticles.

The release profiles of BSA from microparticles produced with various polymer concentrations showed that the increase of these concentrations always resulted in a significant decrease of the protein release rate while the burst release was not modified. This behaviour was consistent with the decrease in protein loading and increase in microparticle size. Moreover, the increase in polymer concentration was always related to the

decrease in porosity, but also to the decrease in protein diffusion and polymer degradation, which resulted in the decrease in the protein release rate (O'Hagan et al., 1994).

In the tested conditions, the slower release profile of protein from microparticles was obtained by employing a polymer concentration of 3.0% (w/v), a spray-rate of feed of 4.5–5.5 ml/min, an inlet temperature of 44–45°C and DCM as organic solvent.

During the microencapsulation process, proteins were subjected to potentially damaging conditions, including exposure to organic solvents, high shear during the emulsification and, with the spray-drying technique, relatively high inlet temperature or spray-rate of feed. Although it is believed that the protein solution is uniformly dispersed throughout the organic phase and consequently that the protein is not actually in contact with the organic solvent, the possibility of denaturation at the interface surrounding the aqueous phase cannot be discounted. These relatively adverse chemical and physical stresses could degrade the antigen entrapped within the microparticles (Gander et al., 1995). In addition to the potentially damaging conditions listed above, a major problem in the spray-drying of proteins is the risk of heat-induced degradation caused by exposure of the molecule to high temperatures. Indeed, the inlet temperature must be sufficient to evaporate the solvent without denaturation of the entrapped protein. However, when spray-dried in a hot air stream that flows in the same direction as the descending spray droplets, the product can reach a theoretical maximum temperature no higher than the temperature of the air stream at the dryer's outlet. In practice, it can generally be assumed that the actual maximum temperature is approximately 15–25°C below the outlet air temperature (Mumenthaler et al., 1994). Moreover, typically during spray-drying, the period of exposure of the drying droplets to elevated temperature is approximately 5–30 s (Mumenthaler et al., 1994). Theoretically, these conditions would fail to degrade the protein but confirmation was important for the use of this technique for the antigen encapsulation by spray-drying. SDS-PAGE and IEF analyses showed that the physiochemical characteristics of the BSA released from microparticles was not significantly affected by the entrapment procedure. These results confirmed that the method of BSA encapsulation within polyester microparticles using a spray-drying technique, which involved an organic solvent such as DCM or a DCM/CFM mixture and an inlet temperature until 62°C and various spray-rate of feed, did not lead either to a significant irreversible aggregation or degradation or to the formation of any new charged species of the entrapped model antigen. This work confirmed that spray-drying is a process capable of achieving a high encapsulation efficiency of a model antigen in microparticles produced from R207 while maintaining its structural integrity.

In conclusion, this study showed that spray-drying is a well-adapted technique to produce microparticles as vaccine carriers. Recently, we have shown that a single administration of polylactide and polylactide-co-glycolide microparticles produced by the spray-drying technique also resulted in a long-lasting humoral immune response (Baras et al., 1997a,b, 2000b). This technique is easy to scale up, simpler and quicker than the double emulsion-solvent evaporation technique, the most common technique used to encapsulate antigens. Finally, this work is the first approach to overcome the key obstacle that represents the scaling-up of the manufacturing process to produce sufficient quantities of vaccine for clinical trials and, ultimately, commercialization.

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