The Preparation and Characterization of Poly(lactide-co-glycolide) Microparticles. II. The Entrapment of a Model Protein Using a (Water-in-Oil)-in-Water Emulsion Solvent Evaporation Technique

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Received May 15, 1992; accepted September 9, 1992

Poly(lactide-co-glycolide) (PLG) microparticles with entrapped antigens have recently been investigated as controlled-release vaccines. This paper describes the preparation of PLG microparticles with an entrapped model antigen, ovalbumin (OVA), using a (water-in-oil)-in-water emulsion solvent evaporation technique. In a series of experiments, the effects of process parameters on particle size and OVA entrapment were investigated. It was found that smooth, spherical microparticles 1–2 μm in diameter containing up to 10% (w/w) OVA could be produced using a small volume of external aqueous phase containing a high concentration of emulsion stabilizer and a 1:5 antigen:polymer ratio. PAGE analysis, isoelectric focusing, and Western blotting of OVA released from the microparticles *in vitro* confirmed that the molecular weight and antigenicity of the protein remained largely unaltered by the entrapment procedure.

KEY WORDS: poly(lactide-co-glycolide); biodegradable microparticles; (water-in-oil)-in-water emulsion; solvent evaporation; controlled-release vaccine; ovalbumin.

INTRODUCTION

In recent years, there have been various attempts to improve the efficacy of the currently available vaccines, often by using novel adjuvants or antigen delivery systems (1). One approach of considerable interest is the use of poly(lactide-co-glycolide) (PLG) microparticles with entrapped antigens as controlled-release vaccines (2,3). Controlled-release vaccines may be designed to release entrapped antigens at predetermined intervals following a single immunization, thereby eliminating the need for booster doses. Single-dose vaccines would be particularly advantageous in the developing countries, where the dropout rate from vaccination programs is high and many individuals do not return for the required booster doses (3).

The biodegradable and biocompatible nature of PLG makes it a suitable candidate for the development of controlled-release vaccines. The degradation rate of PLG is controlled by factors including polymer molecular weight, polymer crystallinity, and lactide:glycolide ratio (4,5). Hence, microparticles prepared with a range of different PLG polymers may be utilized to develop vaccines with multiple dosing properties but which require only a single immunization.

Preliminary studies in mice (2) and rats (6) have demonstrated the induction of enhanced systemic antibody responses following parenteral administration of antigens entrapped in microparticles. The potent immune responses induced by the microparticles could be attributed to efficient antigen presentation achieved, due to phagocytosis of the microparticles by antigen presenting cells, e.g., macrophages. Since particle size greatly influences phagocytosis (7), it would be expected to have a significant effect on the efficacy of microparticulate vaccines.

Ideally, vaccines would be administered orally. Oral vaccines generally have fewer side effects, are cheaper to manufacture, do not require trained personnel for administration, and are more acceptable to the patient than parenteral vaccines. In addition, vaccines administered by a noninvasive route would be preferred in the developing world, where needles are in short supply and are occasionally reused, a practice which increases the risks of infection with HIV and hepatitis. Since it has been shown that orally administered particles are taken up from the intestine into the Peyers' patches (PP) (8), studies have been undertaken to investigate the potential of microparticles as oral vaccines. O'Hagan *et al.* and Challacombe *et al.* have demonstrated the induction of enhanced serum and secretory antibody responses in rats (9) and mice (10) following oral administration of antigens entrapped in microparticles.

Although the parameters controlling particle uptake into the PP are poorly understood, several studies have shown the importance of particle size on uptake (8). Jani *et al.* (11) claimed that optimal particle uptake was observed with particles 1 μm in diameter and below. If PLG microparticles are to be developed as controlled-release vaccines, then the importance of the control of particle size in both oral and parenteral formulations must be appreciated.

Here we describe studies that were undertaken to prepare PLG microparticles approximately 1 μm in diameter, entrapping a high molecular weight model antigen, ovalbumin (OVA; 45K). A previous report from this group described the preparation of PLG microparticles 1–2 μm in diameter using an oil-in-water (o/w) emulsion solvent evaporation technique (12). Although o/w emulsion solvent evaporation has been used successfully by several groups to entrap hydrophobic materials into PLG microparticles, poor levels of entrapment have normally been observed with hydrophilic agents (13,14). Poor entrapment results from partitioning of the water-soluble material from the inner oil phase to the outer aqueous phase. Consequently, Ogawa *et al.* (15) developed a (water-in-oil)-in-water (w/o/w) solvent evaporation technique to entrap a water-soluble peptide into PLG microparticles of 20–125 μm. This paper describes adaptations to Ogawa's method to prepare PLG particles with entrapped OVA with a mean diameter of 1–2 μm. The effects of process parameters on particle characteristics such as particle size, surface morphology, and OVA entrapment efficiency were investigated. The effect of the microparticle preparative process on OVA structural conformation and antigenicity was also investigated by polyacrylamide gel

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0724-8741/93/0300-0362$07.00/0

electrophoresis (PAGE), Western blotting, and isoelectric focusing (IEF).

MATERIALS AND METHODS

Materials

Poly(lactide-co-glycolide):DL lactide-co-glycolide polymers, composition 50:50 (molecular weight 22K) and 85:15 (molecular weight 53K), were kindly donated by Alpha Chemicals, Preston. Polyvinyl alcohol (13-23K, 87–89% hydrolyzed) was supplied by Aldrich Chemical Co. Inc., Dorset. Dichloromethane (HPLC grade), methanol (reagent grade), and acetic acid (Reagent grade) were supplied by May and Baker Ltd, Essex. Rabbit anti-rat IgG conjugated to horseradish peroxidase was supplied by Dako Immunoglobins a/s, Denmark. Isoelectric markers (p*I* 4.7–10.6) were supplied by BDH Chemical Ltd, Dorset. Ovalbumin (chicken egg, grade V), bicinchoninic acid assay reagents, glycine, bromophenol blue (0.005%, w/v), sodium dodecyl sulfate (SDS), tris[hydroxymethyl]aminomethane, Coomassie brilliant blue, polyoxyethylene sorbitan monolaurate, 4-chloro-1-naphthol, hydrogen peroxide (30%, v/v), molecular weight markers (16–66K), isoelectric focusing ampholyte (p*I* 3–10), and phosphate-buffered saline tablets were supplied by Sigma Chemical Co., Dorset. All materials were used as supplied.

Preparation of PLG Microparticles with Entrapped Ovalbumin

A solution of OVA in distilled water (internal aqueous phase) was emulsified with a 6% (w/v) solution of polymer in dichloromethane (DCM) (oil phase) using a Silverson homogenizer (Silverson Machines, Chesham, Bucks) at high speed (approximately 12,000 rpm). The resulting w/o emulsion was then emulsified at high speed with a polyvinyl alcohol solution (PVA) (external aqueous phase) to produce a w/o/w emulsion. The w/o/w emulsion was then stirred magnetically for 12–18 hr at room temperature and pressure, to allow solvent evaporation and microparticle formation. The microparticles were isolated by centrifugation, washed three times in water, and freeze-dried. The final product was stored in a desiccator at below 25°C.

The study was designed to assess the effect of formulation variables on microparticle characteristics such as particle size, surface morphology, and OVA entrapment. The following variables were investigated.

Antigen:Polymer Ratio. Particles were prepared with a range of OVA:PLG ratios (O:P), by increasing the initial weight of OVA dissolved in the internal aqueous phase. The following O:P ratios were investigated: 1:10, 1:5, 1:3, 1:2, and 1:1.

Concentration of Emulsion Stabilizer in the External Aqueous Phase. While maintaining a constant volume for the external aqueous phase, the concentration of PVA was varied. Microparticles were prepared using concentrations of PVA in the outer aqueous phase at 1, 2, 5, and 10% (w/v).

Volume of the External Aqueous Phase. The volume of the external aqueous phase was varied, while the PVA concentration was maintained at 10% (w/v). Microparticles were prepared from outer phase volumes of 10, 20, 50, and 100 mL.

Volume of the Internal Aqueous Phase. The volume of the internal aqueous phase was varied, while the initial weight of OVA dissolved in this phase was maintained at 60 mg. Microparticles were prepared from internal aqueous phase volumes of 0.5, 1.0, 1.5, and 2.0 mL.

Viscosity of the Internal Aqueous Phase. The viscosity of the internal aqueous phase was varied by dissolving OVA in an internal aqueous phase which also contained dissolved PVA at various concentrations. Microparticles were prepared with the following concentrations of PVA in the internal aqueous phase: 1.0, 2.0, and 5.0% (w/v).

Unless otherwise stated, microparticles were prepared using 5 mL polymer solution (6%, w/v) and 20 mL PVA solution (10%, w/v).

Although particle size, surface morphology, and antigen entrapment efficiency are important considerations in the formulation of microparticles as vaccines, an essential consideration is the possible effects of the formulation process on the structural integrity of the entrapped antigen. Therefore, the antigenicity of entrapped OVA was assessed by Western blotting and the structural integrity of the protein was assessed by PAGE and IEF.

Particle Characterization

Particle Size. The freeze-dried particles were redispersed in double-distilled water and sized by laser diffractometry using a Malvern 2600D laser sizer. Particle size is expressed as volume mean diameter (vmd) in micrometers.

Surface Morphology of Microparticles. The freeze-fried microparticles were dispersed in water and air-dried onto metal stubs. Each sample was coated with gold and viewed under a scanning electron microscope (Joel 35C) to assess the surface morphology.

Ovalbumin Entrapment in Microparticles. The method used to determine antigen entrapment has been used previously by Hora *et al.* (16). Ten to fifteen milligrams of freeze-dried microparticles, accurately weighed, was dissolved in 5.0 mL of 0.1 *M* NaOH containing 5% (w/v) SDS (pH 7.4). The sample was centrifuged and a bichinchoninic acid (BCA) protein microassay was used to determine the OVA concentration in the supernatant. From this result, the percentage (w/w) of OVA entrapped per dry weight of microparticles was determined. Each sample was assayed in triplicate. The percentage entrapment efficiency was expressed by relating the actual OVA entrapment to the theoretical OVA entrapment,

$$\frac{\text{Actual OVA entrapment (\%, w/w)}}{\text{Theoretical OVA entrapment (\%, w/w)}} \times 100$$

Confirmation of the Structural Integrity of Ovalbumin. Polyacrylamide gel electrophoretic (PAGE) analysis of native OVA and OVA released from PLG microparticles was undertaken using a method previously described by Lugtenberg *et al.* (17). Samples of OVA released from PLG microparticles in phosphate-buffered saline (PBS), native OVA, and a molecular weight reference marker (molecular weight

16–66K) were solubilized with sample buffer, loaded onto a vertical slab gel (10%), and subjected to electrophoresis at 200 mV. Following electrophoresis, the gels were either stained with Coomassie brilliant blue (0.1%, w/v) in water: methanol:acetic acid (45:45:10) to visualize the protein or Western blotted as described below.

The antigenicity of the entrapped ovalbumin was assessed by Western blotting using a rat antiserum raised against the native protein. The antiserum was raised by a series of intraperitoneal injections of OVA emulsified in Freund's adjuvant. The OVA samples were transferred from the gel onto a nitrocellulose membrane by electroblotting and the membrane was blocked with Tris-buffered saline containing 0.3% (w/v) sorbitan monolaurate (TBS/T20). The blot was incubated for 2 hr at room temperature, with the rat antiserum, diluted 1:100 in TBS/T20. After washing with Tris-buffered saline (TBS), the blot was incubated with rabbit anti-rat IgG conjugated to horseradish peroxidase diluted (1:100) in TBS/T20. The ability of the rat antiserum to recognize OVA released from microparticles was demonstrated colourimetrically using 4-chloro-1-naphthol [15 mg in methanol:Tris (pH 7.4):hydrogen peroxide (30%, v/v), 5:40: 0.05].

Retention of the structural integrity of the entrapped OVA was further assessed by isoelectric focusing (IEF). IEF separates proteins on the basis of surface charge as a function of pH, and since a protein contains both positive and negative charge-bearing groups, the net charge of the protein will vary with pH. A significant alteration to the conformation of OVA following entrapment into PLG microparticles could result in a variation in the surface charge, this could be detected by IEF. OVA released from microparticles into PBS, native OVA in PBS, and IEF standards of a range of isoelectric points (p*I*), 4.7–10.6, were loaded onto a polyacrylamide gel and focused using a Bio-Rad mini IEF cell, Model 111 (Bio-Rad Laboratories, Richmond, CA). The gel was fixed, Coomassie blue stained, and destained as described in the Bio-Rad protocol for the mini IEF cell.

RESULTS AND DISCUSSION

For each variable studied, batches of microparticles were prepared in duplicate. The results of particle characterization obtained for each bath showed limited batch-to-batch variation.

Antigen:Polymer Ratio

As the OVA/PLG (O/P) ratio was increased by increasing the initial weight of OVA dissolved in the internal aqueous phase, a dramatic increase in OVA entrapment in microparticles was observed (Table I). There was also a small increase in mean particle size. However, scanning electron microscopy (SEM) revealed that while spherical particles were produced at all O/P ratios, the surface of the particles prepared at higher ratios (1:2 and 1:1) appeared pitted and some of the particles had collapsed. The surface of particles prepared at lower O/P ratios (1:10, 1:5, and 1:3) appeared to be smooth and there was no evidence of collapsed particles (Fig. 1). It was postulated that at high O/P ratios, the quantity of polymer present was insufficient to cover the OVA completely. This resulted in relatively high surface concentrations of OVA, which readily dissolved in the surrounding external phase, leaving a pitted microparticle surface. In some cases, this resulted in the collapse of the microparticles.

Table I. The Effect of Ovalbumin/Polymer Ratio on Particle Size and Ovalbumin Entrapment[a]

Weight OVA (mg)	Ratio OVA/ PLG	Particle size, vmd (μm)	OVA entrapment (%, w/w)	Entrapment efficiency (%)
30	1:10	1.8	3.9	42.9
60	1:5	2.4	9.9	59.4
100	1:3	3.1	13.9	55.6
150	1:2	3.8	23.5	70.5
300	1:1	3.9	32.3	65.0

[a] Particles were prepared using PLG (50:50, 22K).

The small increase in particle size associated with the increased O/P ratio was attributed to the increase in OVA entrapment. A linear relationship was observed between particle size and initial weight of OVA dissolved in the internal phase.

There was no obvious relationship between the O/P ratio and the OVA entrapment efficiency. However, for all the O/P ratios studied, the OVA entrapment efficiency was high, ranging from 40 to 70%.

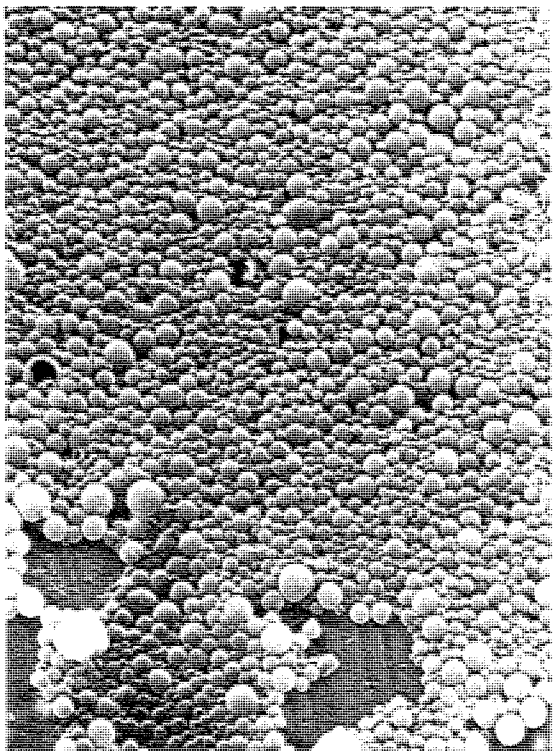

Fig. 1. Scanning electron micrograph of PLG microparticles entrapping OVA (1 in. = 5 μm).

Concentration of Emulsion Stabilizer in the External Aqueous Phase

Although a significant reduction in particle size was achieved as the concentration of the PVA was increased, OVA entrapment was not affected (Table II). At high PVA concentrations the rate at which the emulsion stabilizer molecules diffuse to the emulsion droplet/aqueous phase interface may increase, resulting in a greater presence of stabilizer at the surface of the emulsion droplet formed during the preparative process. This would provide an improvement in the protection of droplets against coalescence, resulting in the formation of smaller emulsion droplets at higher PVA concentrations. As the solvent evaporates from the system, these droplets harden to form the microparticles. Therefore, the size of the microparticles is dependent upon the size and stability of the emulsion droplets formed during agitation. At low PVA concentrations, small emulsion droplets are not stable, and the resulting microparticles are larger in size than those prepared with higher PVA concentrations.

The levels of entrapment for OVA in microparticles shown in Table II are significantly lower than those shown in Tables I, III, and IV. This is due to the use of PLG polymer with a different copolymer composition (85/15, 53K for batches recorded in Table II and 50/50, 22K for the other batches). The 85/15, 53K PLG is more hydrophobic than the 50/50, 22K PLG, and this resulted in lower entrapment levels for the hydrophilic OVA.

Volumes of the External Aqueous Phase

An increase in the volume of the external aqueous phase resulted in an increase in both OVA entrapment and particle size (Table III). However, the particle appearance was not affected by the external aqueous phase volume. 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 larger microparticles. As a result of increased particle size, there is an associated increase in particle volume, which enables more OVA to be incorporated into the microparticles. Linear relationships were shown to exist between the volume of the external aqueous phase and the amount of OVA 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 III. The Effect of External Aqueous Phase Volume on Particle Size and Ovalbumin Entrapment[a]

Volume PVA solution (ml)	Particle size, vmd (μm)	OVA entrapment (%, w/w)	Entrapment efficiency (%)
10	2.8	7.1	42.6
20	3.4	8.4	50.4
50	3.7	12.3	73.8
100	6.5	16.4	98.4

[a] Particles were prepared using PLG (50:50, 22K).

Volume of the Internal Aqueous Phase

An increase in both particle size and OVA entrapment was observed following an increase in the internal aqueous phase volume (Table IV). A linear relationship was shown to exist between the internal phase volume and the particle size. In addition, a logarithmic relationship is apparent between the internal phase volume and OVA entrapment (relationships not shown).

Viscosity of the Internal Aqueous Phase

Ogawa *et al.* (15) reported an increase in the entrapment efficiency of the hydrophilic peptide leuprolide acetate following the addition of gelatin to the peptide solution prior to emulsification. In the present work, the addition of gelatin to the internal aqueous phase produced a significant increase in OVA entrapment (1.3%, w/w, without gelatin was increased to 6.7%, w/w, with gelatin; results not shown). However, there was also a considerable increase in particle size (8.1 μm without gelatin to 42 μm with gelatin). Such an increase in particle size would limit the use of these microparticles as vaccines, particularly for oral administration. In addition, the use of gelatin should also be avoided due to the possibility of undesirable immunological reactions following administration to humans.

The effect of increasing the viscosity of the internal phase was studied by preparing microparticles using an increasing concentration of PVA dissolved in the internal aqueous phase (PVA at 1, 2, and 5%, w/v, was used). Ogawa *et al.* (15) claimed that an increase in internal aqueous phase viscosity led to a reduction in the partitioning of the antigen into the external aqueous phase. This was claimed to result in an increase in peptide entrapment. However, in the present work, no apparent effect on OVA entrapment or

Table II. The Effect of PVA Concentration on Particle Size and Ovalbumin Entrapment[a]

PVA concentration (%, w/v)	Particle size, vmd (μm)	OVA entrapment (%, w/w)	Entrapment efficiency (%)
1	8.3	2.5	15.0
2	6.0	2.9	17.4
5	4.6	3.3	19.8
10	3.7	1.9	11.4

[a] Particles were prepared using PLG (85:15, 53K).

Table IV. The Effect of the Internal Aqueous Phase Volume on Particle Size and OVA Entrapment

Inner aqueous-phase volume (ml)	Particle size, vmd (μm)	OVA entrapment (%, w/w)	Entrapment efficiency (%)
0.5	2.5	4.1	24.6
1.0	3.0	6.4	38.4
1.5	3.3	8.3	49.8
2.0	4.1	8.7	52.2

[a] Particles were prepared using PLG (50:50, 22K).

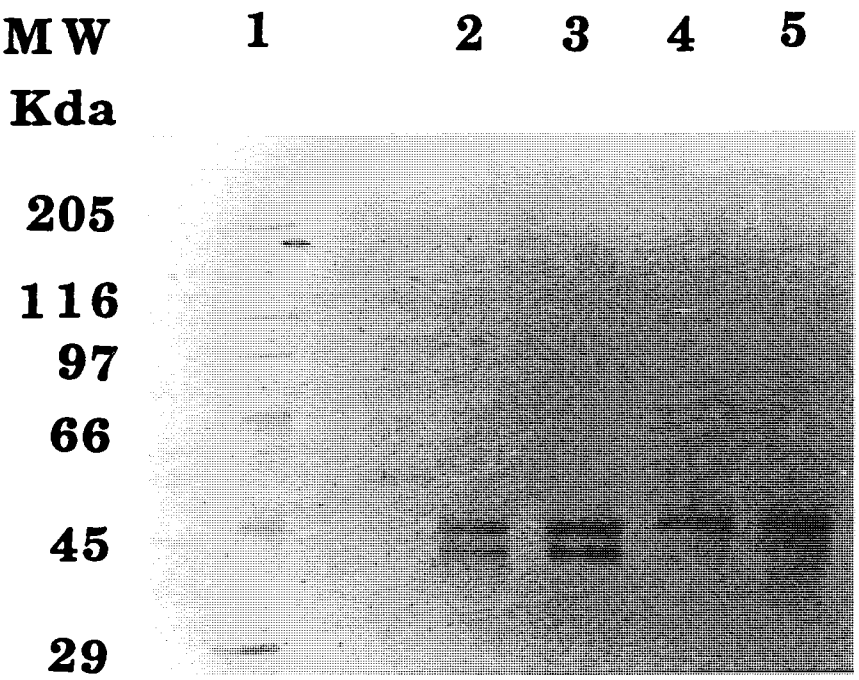

Fig. 2. PAGE behavior of native OVA and OVA released from PLG microparticles. Lanes represent the molecular weight marker (1), native OVA (2 and 3), and OVA released from PLG microparticles (4 and 5).

particle size was observed when the viscosity of the internal phase was increased by using increasing concentrations of PVA solution (results not shown).

Confirmation of the Structural Integrity of Ovalbumin

During microparticle preparation, the antigen was exposed to potentially harsh conditions, such as contact with DCM and mechanical agitation. These conditions may result in irreversible denaturation and loss of antigenicity of proteins. PAGE analysis followed by Coomassie blue staining revealed identical bands for the entrapped and native OVA (Fig. 2) and there were no additional bands to indicate the presence of molecular weight aggregates or fragments greater or less than 45K (the molecular weight marker actually contains OVA). Hence, the data suggest that the structural integrity of OVA was not significantly affected by the entrapment procedure.

The Western blot confirmed that the antigenicity of OVA was not significantly altered following entrapment in microparticles, since antiserum raised to the native protein still recognized the formulated antigen in the gel (Fig. 3).

IEF (Fig. 4) showed identical bands for the native OVA and the OVA released from microparticles. By comparing these bands with the isoelectric point marker, it can be seen that the isoelectric point of both the native OVA and the OVA released from microparticles is p*I* 4.5. This observation provides further evidence to support the claim that OVA was entrapped into PLG microparticles by w/o/w emulsion solvent evaporation without significantly altering the antigenic structure or conformation.

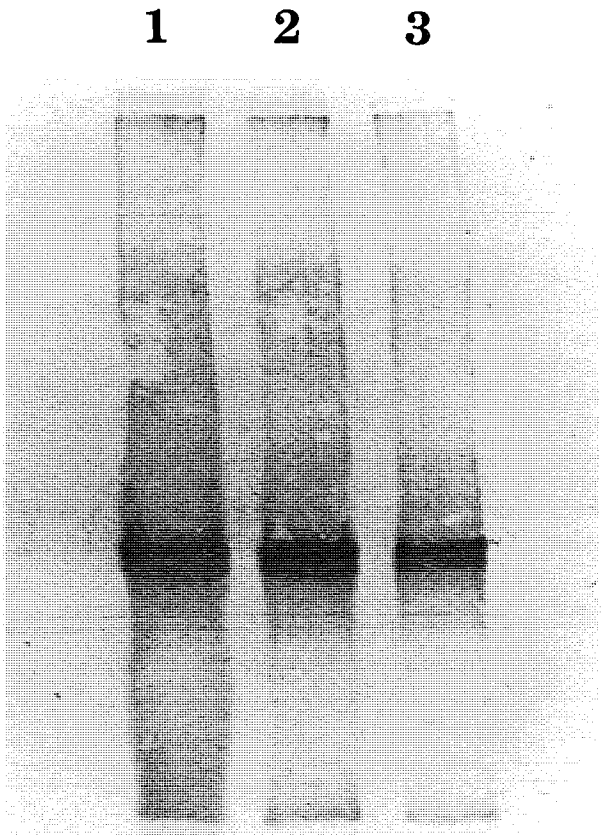

Fig. 3. Western immunoblot of OVA released from PLG microparticles. Lanes represent native OVA (1) and OVA released from PLG microparticles (2 and 3).

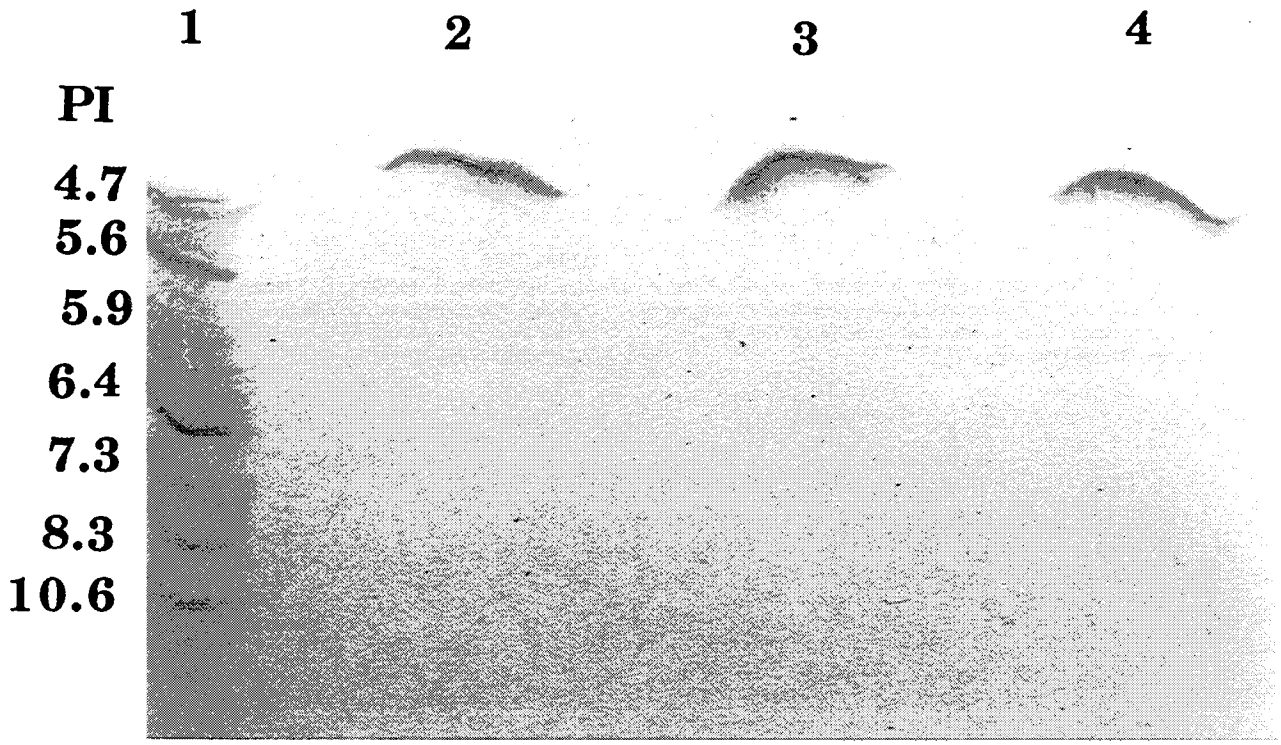

Fig. 4. Isoelectric focusing of native OVA and OVA released from PLG microparticles. Lanes represent isoelectric markers (1), native OVA (2), and OVA released from PLG (3 and 4).

CONCLUSIONS

Although high O/P ratios resulted in entrapment levels of up to 32% (w/w), the particles are probably too large for optimal uptake into PP (mean diameter, 3.9 μm) and the surface characteristics were poor, with pitted and collapsed particles present. When low O/P ratios were employed, smooth particles with a mean diameter of 1.8 μm were produced, but the entrapment levels were significantly lower (3.9%, w/w). The concentration of the emulsion stabilizer and the volume of the external aqueous phase also affected particle size. If small particles are to be produced, a low volume of aqueous phase containing a high concentration of PVA is required. However, a reduction in OVA entrapment levels was observed for the microparticles prepared from low aqueous-phase volumes.

The results described in the present study showed an obvious relationship between particle size and OVA entrapment levels, with both being influenced by several process parameters. When producing microparticles by w/o/w emulsion solvent evaporation using the technique described, it is essential that the appropriate formulation parameters are selected to enable the production of microparticles of the desired particle size with the required levels of entrapped antigens.

In the present studies high OVA entrapment efficiencies have been achieved, with approximately 50–70% of the initial OVA dissolved in the internal aqueous phase being entrapped into microparticles. High entrapment efficiency is important when entrapping antigens in relatively short supply.

ACKNOWLEDGMENTS

Miss Jeffery is the recipient of an SERC studentship. This work was partly funded by the Medical Research Council AIDS Directed Programme and The World Health Organisation.

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