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The preparation and characterization of poly(lactide-co-glycolide) microparticles: III. Microparticle/polymer degradation rates and the in vitro release of a model protein

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Summary

Poly(lactide-co-glycolide) (PLG) polymers may be used to entrap antigens for the development of controlled release vaccines, which may be designed to obviate the need for booster doses. Four commercially available PLG polymers (co-polymer compositions and molecular masses: 50:50, 33 kDa; 50:50, 58 kDa; 50:50, 84 kDa and 75:25, 83 kDa) were used to prepare microparticles and their in vitro degradation rates were assessed by three methods: (1) assessment of surface morphology by scanning electron microscopy, (2) weight loss and (3) molecular mass determinations by gel permeation chromatography. Each method confirmed that degradation was more rapid for polymers with a lower molecular mass and a higher glycolide content. Some parameters affecting the rate of release of a model protein (ovalbumin) from microparticles prepared with the polymers were assessed in vitro. The rate of release of the protein was shown to depend on at least three parameters: (1) the molecular mass of the polymer, (2) the co-polymer composition and (3) the protein loading of the microparticles. Generally, the rate of polymer degradation showed good correlation with the rate of protein release and it was shown that the polymers investigated may be appropriate for the development of controlled release vaccines.

Introduction

The effectiveness of disease prevention through vaccination is dependent to a large extent on the numbers of individuals immunised in the population at risk. Currently, one of the main objectives of the World Health Organisation (WHO) and other agencies is to increase vaccination coverage. However, several problems exist, not least of which is the requirement for repeated contact between health-care workers and the population needing to be immunised. Multiple contacts are necessary to fully implement the WHO recommended schedule for vaccination and this is difficult and costly to achieve. The 'drop out' rate of those individuals receiving the first dose of vaccine, but not the final dose necessary for full protection is high in many parts of the world (Bloom, 1989). This problem may be overcome through the development of controlled release vaccines, which will be designed to mimic the

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effect of booster doses of vaccine following a single immunisation.

Controlled release vaccines may be prepared by entrapping antigens in microparticles prepared from biodegradable polymers. The primary candidates for the development of polymeric controlled release vaccines are the poly(lactide-co-glycolides) (PLG). The biocompatability of microparticles prepared from PLG has been demonstrated by Visscher et al. (1987). As a result of their biodegradability and excellent tissue compatability, PLG and related polymers have been used for many years as surgical sutures (Wise et al., 1987) and in the preparation of drug delivery systems for administration to humans (Maulding, 1987). Recent reports have also highlighted the potential of PLG microparticles for the development of controlled release vaccines. For example, ovalbumin (OVA) entrapped in microparticles induced serum antibody responses that were comparable to those induced by Freund's adjuvant (O'Hagan et al., 1991a,b). In addition, microparticles have also been shown to be capable of inducing potent immune responses to entrapped OVA following oral immunisation (Challacombe et al., 1992). Therefore, the use of PLG microparticles as controlled release vaccines offers considerable promise for the future development of new and improved vaccines against a wide range of infectious diseases. Although polymeric microparticles may also be used for the delivery of protein drugs, the small particle size of the microparticles in the present study (< 5 μm) makes them much more appropriate for vaccine development. Small microparticles have been shown to be much more immunogenic than large microparticles (Eldridge et al., 1991; O'Hagan et al., 1993). The currently recommended schedule for the Expanded Programme of Immunisation in the developing world requires vaccination with the triple diphtheria, pertussis and tetanus (DPT) vaccine at 6, 10 and 14 weeks after birth (Hall et al., 1990). Since the rate of release of large proteins from PLG polymers is mainly controlled by polymer degradation (Hutchinson and Furr, 1986; Maulding, 1987), it is clear that relatively low molecular mass PLG polymers may be appropriate for the preparation of microparticles with entrapped DPT to obtain the required release profile in vivo. The current report describes the assessment of the degradation rates of several candidate PLG polymers for possible use in the preparation of controlled release vaccines. In addition, selective studies were undertaken to identify some of the parameters controlling the rate of release of an entrapped model protein from microparticles in vitro.

Materials and Methods

Materials

Poly(DL-lactide-co-glycolides) (PLG) with copolymer compositions and molecular mass in kilodaltons (kDa): 50:50, 33 kDa (Resomer RG503); 50:50, 84 kDa (Resomer RG506) and 75:25, 83 kDa (Resomer RG755) were supplied by Alpha Chemicals, Preston. PLG 50:50, 58 kDa was donated by Medisorb, Wilmington, U.S.A. The molecular mass of PLG was determined by gel permeation chromatography (GPC) as described below. Polyvinyl alcohol (PVA, 13–23 kDa, 87–89% hydrolysed) was supplied by Aldrich Chemical Co., Dorset. Bichinchoninic acid assay (BCA) reagents and OVA (chicken egg, grade V) were supplied by Sigma Chemical Co., Dorset and dichloromethane (DCM, HPLC grade) was supplied by May and Baker, Essex.

Preparation of microparticles

Microparticles were prepared using an oil-in-water (o/w) emulsion solvent evaporation technique as previously described by Jeffery et al. (1991). Briefly, the polymer was dissolved in DCM to give a 6% w/v polymer solution. The DCM solution was emulsified with an aqueous phase containing 10% w/v PVA for 5 min using a Silverson homogeniser (Silverson Machines Ltd, Chesham, Bucks) and the resulting o/w emulsion was stirred under ambient conditions to allow solvent evaporation and microparticle formation. The microparticles were washed and freeze-dried, and the final product was stored in a desiccator at 25°C. Microparticles without entrapped OVA will be referred to as 'blank' microparticles.

Preparation of microparticles with entrapped OVA

A water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique was used to prepare microparticles with entrapped OVA as previously described by Jeffery et al. (1993). Briefly, OVA was dissolved in water and emulsified with a 6% w/v solution of polymer in DCM. The resulting w/o emulsion was then emulsified with 10% w/v PVA to produce a w/o/w emulsion and stirred under ambient conditions to allow solvent evaporation and microparticle formation. The microparticles were isolated by centrifugation, washed and freeze-dried, and stored in a desiccator at 25°C.

Determination of microparticle 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 μm.

Determination of the levels of entrapped OVA

The levels of OVA entrapped in microparticles were determined using a procedure previously described by Hora et al. (1990) and Cohen et al. (1991). Approx. 10 mg of freeze-dried microparticles were acccurately weighed, dispersed in 5 ml of 0.1 M NaOH containing 5% w/v sodium dodecyl sulphate (SDS) and shaken for 12–18 h. The sample was centrifuged and a BCA microassay was used to determine the OVA concentration in the supernatant against a series of OVA standards prepared in 0.1 M NaOH containing 5% w/v SDS. Assays were performed in triplicate.

Determination of the residual levels of solvent in the microparticles

The residual levels of DCM in both blank microparticles and microparticles containing OVA were determined by gas chromatography. Approx. 10 mg of microparticles was accurately weighed and dissolved in 1-methyl-2-pyrrolidinone (MP), before being sealed in a headspace vial and allowed to stand at 60°C for 15 min before injection into the gas chromatograph (Perkin Elmer, CT, U.S.A.). During gas chromatography, helium was used as the mobile phase and DCM was detected by a flame ionisation detector. Standards of DCM in MP were used to construct a calibration curve for the instrument. (The gas chromatography was kindly performed by Professor M. Vert at the University of Montpelier, France.)

Degradation rate of microparticles / polymers

Blank microparticles were used to assess the rate of microparticle/polymer degradation during incubation in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4). 10 vials, each containing 40 mg microparticles dispersed in 40 ml PBS, were rotated in a shaking water bath at 37°C. At various time intervals, one vial was removed, the microparticles were isolated by centrifugation, washed three times in double distilled water and freeze-dried. The rate of degradation was assessed in three ways and each data point represents a single vial.

Deterioration in microparticle surface morphology An aliquot of the microparticles from each vial was air-dried onto a metal stub, coated with gold using a splutter coater (Emscope SC 500, Emscope, Kent) and assessed by scanning electron microscopy (SEM; Joel 6400, Tokyo, Japan).

Reduction in the weight of microparticles At each sample point, the total weight of microparticles in the vial was determined and the percentage weight of microparticles remaining was calculated.

Reduction in polymer molecular mass GPC was used to determine the molecular mass of the polymers at each sample point. This work was undertaken by Rapra Technology Ltd, Shrewsbury, Shropshire. The microparticles were dissolved in tetrahydrofuran, before being passed through a column previously calibrated with polystyrene. The results are expressed as 'polystyrene equivalent' molecular mass.

Rate of release of OVA from microparticles

The following microparticle/polymer characteristics were investigated to identify the parameters that may be used to control the rate of release of proteins from microparticles.

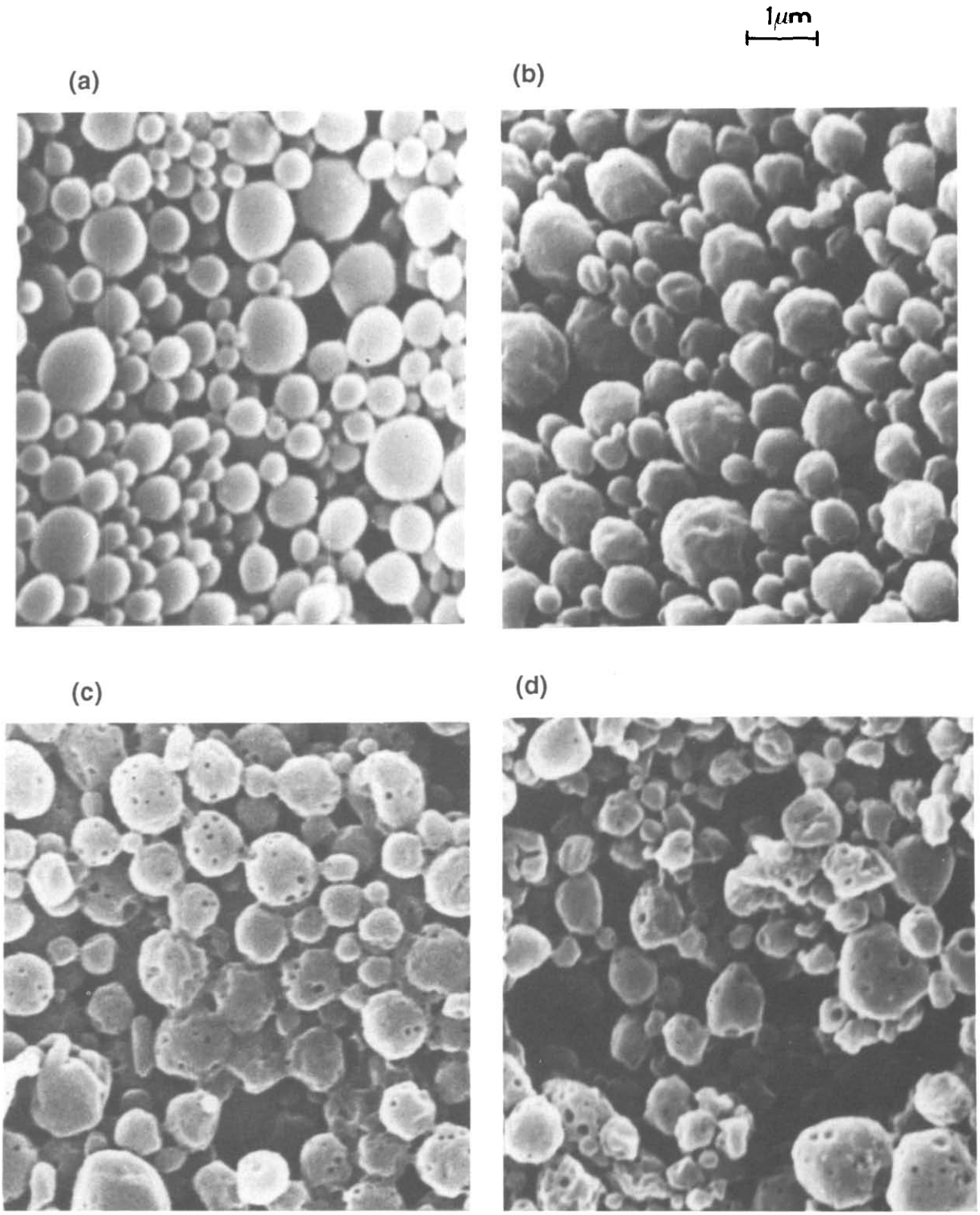

Fig. 1. The change in surface morphology of microparticles prepared from a PLG polymer with a lactide/glycolide ratio of 50:50 and a molecular mass of 84 kDa, following in vitro degradation. (a) Day 0, (b) day 10, (c) day 28 and (d) day 56.

The molecular mass of the polymer The molecular mass of the polymer was varied for a fixed ratio of lactide/glycolide of 50:50. Three batches of microparticles were prepared which contained similiar levels of entrapped OVA as follows; 50:50, 33 kDa polymer (8.5% w/w), 50:50, 84 kDa (9.1% w/w) and 50:50, 58 kDa (10.2% w/w). The mean sizes of the batches of microparticles were 2.6, 1.7 and 1.0 μm, respectively.

The co-polymer composition The lactide/glycolide ratio was varied for two polymers with a similiar molecular mass. The two batches of microparticles were prepared with the 50:50, 84 kDa polymer and the 75:25, 83 kDa polymer and contained 9.1 and 7.9% w/w OVA, respectively. The mean particle sizes were 1.7 and 1.5 μm.

The levels of entrapped OVA The 50:50, 84 kDa polymer was used to prepare microparticles with three different amounts of entrapped OVA. Three batches of microparticles were prepared containing 5.0, 9.1 and 16.2% w/w OVA. The mean sizes of the three batches of microparticles were 1.3, 1.7 and 3.3 μm, respectively.

A series of vials, each containing 20 mg of microparticles dispersed in 5 ml PBS, were rotated in a shaking water bath at 37°C. At various time points, one vial was removed, the contents were filtered through a 0.2 μm polyamide membrane filter (Sartorious, Gottingen, Germany) to remove the microparticles and the filtrate was stored at −40°C. The OVA contents of the filtrates were determined in a BCA assay and from these data, a release profile was calculated. Each data point represents one vial.

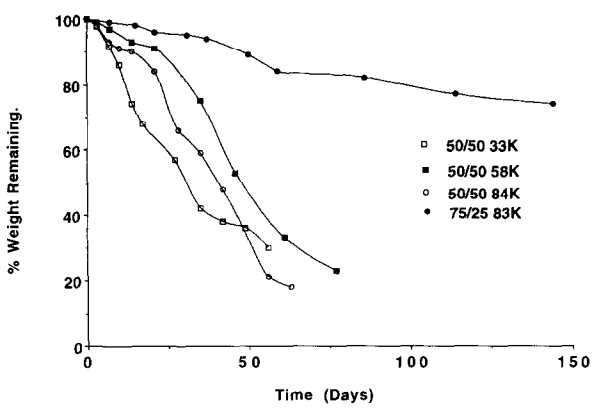

Fig. 2. The reduction in weight of different batches of freeze-dried microparticles with time as a result of polymer degradation.

Results and Discussion

Determination of the residual levels of solvent in the microparticles

The residual levels of DCM in both blank microparticles and microparticles containing OVA were < 250 ppm, which was the lowest level of detection of the instrument used.

Degradation rate of microparticles / polymers

Deterioration in microparticle surface morphology With increasing incubation times, deterioration in the microparticle surface morphology became apparent under SEM (Fig. 1). At the beginning of the study, the microparticles appeared smooth and spherical, with a non-porous surface (Fig. 1a). However, as the study progressed, the particles became less spherical and the surface became more porous (Fig. 2b–d). The rate of deterioration in the appearance of microparticles was dependent on the rate of polymer degradation, since microparticles prepared with higher molecular mass polymers showed surface deterioration at a much slower rate (data not shown).

Reduction in the weight of microparticles These results showed that more rapid weight loss occurred for the microparticles prepared from polymers with a higher content of glycolide. Thus, the polymers with a 50:50 ratio of lactide/glycolide degraded more rapidly than the polymer with a 75:25 ratio (Fig. 2). For the polymers with the same lactide/glycolide ratio (50:50), the polymer with the lowest molecular mass (33 kDa) degraded most rapidly. However, the 84 kDa polymer degraded more rapidly than the 58 kDa polymer (Fig. 2). These findings were generally consistent with earlier reports concerning the rates of degradation of PLG polymers (e.g., Pitt et al., 1981; Vert et al., 1991). Although the 50:50 84 kDa polymer degraded more rapidly than the 50:50 58 kDa polymer, these polymers were from alternative commercial sources. Vari-

ability in apparently similiar polymers from alternative sources has recently been reported by Schmitt et al. (1993).

To compare directly the degradation rates for each polymer, the time taken for a 50% reduction in microparticle weight ($t_{50\%}$) was calculated. The $t_{50\%}$ values for the polymers studied were as follows, 50:50, 33 kDa polymer (25 days); 50:50, 84 kDa (40 days); 50:50, 58 kDa (50 days) and 75:25, 83 kDa (270 days). The $t_{50\%}$ for the 75:25, 83 kDa polymer can only be estimated since at the end of the study at 150 days there was only a 26% reduction in microparticle weight. Thus, it can be concluded that for the polymers studied, the lactide/glycolide ratio has a greater influence on the rate of polymer degradation than the polymer molecular mass.

It has been suggested previously that during the early stages of the degradation of high molecular mass PLG polymers, there was a period of little or no weight loss (Sanders et al., 1986). During this period, it was suggested that the high molecular mass polymers were cleaved into lower molecular mass fractions. However, all the polymers used in the present study already had a relatively low molecular mass. This may explain why loss in sample weight was observed throughout the degradation study, although there did appear to be an initial period of negligible weight loss for the 75:25, 83 kDa polymer.

Reduction in polymer molecular mass The results from the GPC analysis showed a steady decrease in the weight average molecular mass of all the polymers as degradation occurred (Fig. 3). The most rapid decrease in molecular mass was observed for the polymers with a 50:50 ratio of lactide/glycolide. This observation confirmed that more rapid degradation occurs in polymers with a higher glycolide content. The glycolide units, which are more hydrophilic than the lactide units, are thought to promote water uptake into the polymer, which encourages hydrolytic degradation (Dunn et al., 1988). For the polymers with a 50:50 ratio of lactide/glycolide, a more rapid decrease in molecular mass was observed for the higher molecular mass polymers. This represents cleavage of the larger molecular mass polymers into smaller molecular mass fractions. For the lower molecular mass polymers, the cleavage of low molecular mass fractions into smaller fractions did not result in such a dramatic initial fall in molecular mass. Since the rate of PLG degradation in vitro has been claimed to show good correlation with in vivo degradation profiles (Pitt et al., 1981), the data from these studies may be used to predict the in vivo degradation profiles of PLG microparticles.

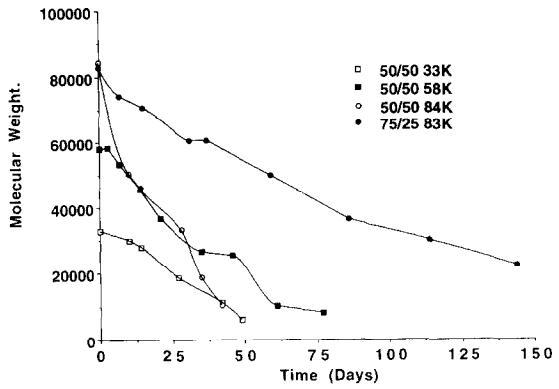

Fig. 3. The reduction in the molecular mass of different polymers with time as a result of polymer degradation.

Rate of release of OVA from microparticles

The molecular mass of the polymer Due to the large molecular size of proteins and their insolubility in the polymers, the rate of protein release from PLG microparticles is thought to depend largely on the rate of polymer degradation (Hutchinson and Furr, 1986). However, for all of the batches of microparticles studied, there was an initial 'burst' release of OVA during the first few hours of the study. This burst release probably represented the release of poorly entrapped and surface-associated OVA. For the microparticles prepared from the 50:50, 33 kDa polymer, virtually all the entrapped OVA was released within the first couple of hours of the study. Nevertheless, the rate of release of OVA from the 50:50, 33 kDa microparticles was not as rapid as that from blank microparticles with equivalent amounts of OVA adsorbed to their surface (data not shown).

The initial release of OVA from microparticles prepared with higher molecular mass polymers,

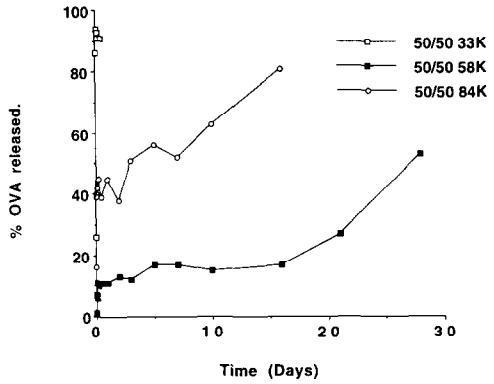

Fig. 4. The effect of the molecular mass of the polymer on the rate of release of OVA entrapped in microparticles.

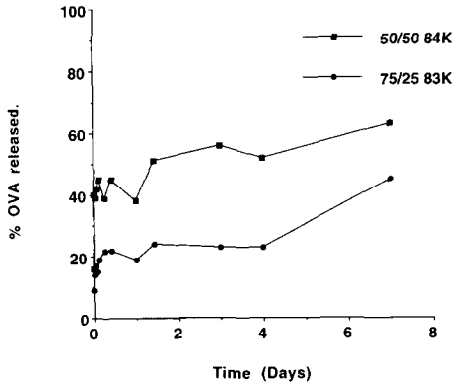

Fig. 5. The effect of the co-polymer composition on the rate of release of OVA entrapped in microparticles.

i.e., 58 and 84 kDa, was considerably lower than that for the 33 kDa polymer (Fig. 4). For the microparticles prepared from the 50:50, 58 kDa polymer, a typical triphasic release profile was observed. Following the initial burst release, there was a lag phase of little or no release, followed by a phase of constant OVA release (Fig. 4). The period of constant release did not begin until study day 16 and presumably would have continued until the microparticles were depleted had the study not been terminated. Previous reports have described a similiar triphasic release profile for macromolecules entrapped in PLG microparticles (Sanders et al., 1984; Hora et al., 1990; Singh et al., 1990). Hence, PLG microparticles with entrapped proteins inherently produce a release profile which appears more suitable for vaccine delivery rather than drug delivery. The slower rate of release of entrapped OVA from the 50:50 58 kDa polymer, in comparison to the 50:50 84 kDa polymer, can be explained by the slower degradation of the 50:50 58 kDa polymer (Figs 2 and 3).

The co-polymer composition The co-polymer composition was also shown to be important in controlling the rate of release of OVA from microparticles. The initial release of OVA was greater from the polymer with the higher glycolide content (50:50), which presumably takes up water more readily than the 75:25 polymer (Fig. 5).

It has been suggested that the length of the lag phase during the release profile of macromolecules from PLG microparticles is dependent on the rate of polymer degradation (Sanders et al., 1984). Therefore, the release profiles of macromolecules from microparticles prepared from polymers with a high molecular mass and a low glycolide content should show a prolonged lag phase and an extended duration of release. In the present study, the length of the lag phase and the duration of OVA release increased with higher molecular mass polymers (Fig. 4).

The levels of entrapped OVA The effect of the levels of entrapped protein on the release rates of OVA from the microparticles showed good agreement with previously published data (Burns et al., 1990; Hora et al., 1990). It has been sug-

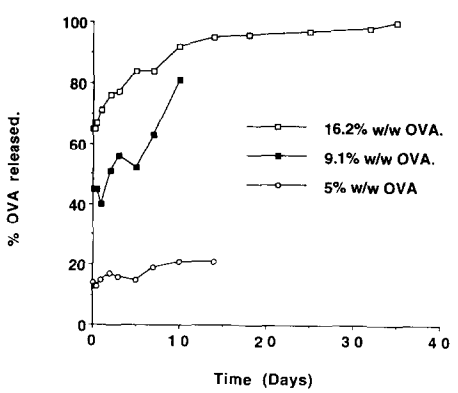

Fig. 6. The effect of protein loading on the rate of release of OVA entrapped in microparticles.

gested that for microparticles with high protein loading, protein release is facilitated by diffusion through channels, which are created by the elution of surface located protein (Hora et al., 1990). The data from the present study confirmed that the initial extent of OVA released was enhanced by increasing the levels of OVA entrapped in the microparticles (Fig. 6). Therefore, if the release of antigens is to be delayed for 4 and 8 weeks following injection at 6 weeks after birth, it is clear that low loading levels of antigen are required in the microparticles.

Conclusions

The results from the degradation studies confirmed that the rate of PLG polymer degradation is dependent on the lactide/glycolide ratio and the molecular mass of the polymer. More rapid degradation was observed for PLG polymers with a high glycolide content and a low molecular mass. The commercially available polymers used in the current studies clearly displayed degradation rates that make them suitable for the formulation of controlled release vaccines that could be designed to deliver booster doses of antigen at 10 and 14 weeks, following primary immunisation at 6 weeks after birth. However, the formulation parameters must be strictly controlled to allow the preparation of microparticles with appropriate loading levels of antigen in order to obtain the desired release profiles. A previous publication has described how formulation variables may be manipulated to control the levels of antigen incorporation into microparticles (Jeffery et al., 1993).

From the results of the in vitro release studies several conclusions can be drawn:
(1) PLG microparticles can be used to control the release of an entrapped protein and release tends to occur in a three stage profile. This profile consists of an initial 'burst' release of surface-located material, followed by a 'lag phase' of minimal release, while polymer degradation occurs and a third phase of continuous release, which is initiated when sufficient polymer degradation has occurred.
(2) By altering the co-polymer composition, the polymer molecular mass and the loading level, it is possible to vary the rate of protein release.
(3) Generally, the the rate of protein release correlated with the rate of polymer degradation.

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