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Influence of formulation methods on the in vitro controlled release of protein from poly(ester) microspheres

H.T. Wang, E. Schmitt, D.R. Flanagan and R.J. Linhardt

Division of Medicinal and Natural Products Chemistry and Division of Pharmaceutics, College of Pharmacy, University of Iowa City, Iowa, U.S.A.

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Poly(DL-lactide/glycolide, 50:50) microspheres containing bovine serum albumin (BSA) were prepared with and without Carbopol® 951 (a potential adjuvant agent) by o/o, o/w and (w/o)/w emulsion methods. The protein loading of the microspheres reached 50–70% of the theoretical amount of protein put into the formulation medium. The microsphere particle size was approximately 500 μm, 25–100 μm, 10–20 μm using o/o, o/w, or (w/o)/w emulsion techniques, respectively. The release of BSA was dependent on the preparation method. The greatest burst of release was found for vacuum-dried microspheres formulated using the (w/o)/w method. This burst effect could be eliminated by lyophilizing the microspheres following their preparation. BSA was released at a higher initial rate from microspheres prepared by the o/w emulsion method that contained Carbopol® 951 than from microspheres not containing Carbopol® 951. Release studies also suggested that the release of BSA could be sustained for 54, 36, or 34 days for microspheres prepared by o/o, o/w, or (w/o)/w methods, respectively.

Keywords: Biodegradable; Polyester; Microspheres; Protein; Vaccine

Introduction

Synthetic biodegradable polymers have been studied for various applications including controlled-release systems for drugs and biologicals [1–18]. Biodegradable polymers often have low toxicity, are not tissue reactive and do not require surgical removal from the host [1]. Poly(esters) were chosen for study because they are currently used in medical applications and they biodegrade to natural materials that have very low toxicity [19–24]. We recently described the preparation of BSA-containing polyester microspheres by a spray-drying method [25–28].

There is considerable interest in developing new methods to deliver both vaccines and adjuvants [28–31]. Our laboratory has shown that spray-dried microspheres incorporated with BSA or vaccines show sustained release over a period of 30 days [28]. Current methods of preparing microspheres also include o/o, o/w, and (w/o)/w emulsion methods [32–34]. Although there is no evidence that sustained release of low levels of immunogen will enhance vaccine efficacy, this represents the simplest type of release kinetics. The purpose of the present investigation was to explore methods that would produce a vaccine

Correspondence to: R.J. Linhardt, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242, U.S.A.

matrix formulation giving continuous release over the lifetime of the matrix. Poly(DL-lactide/glycolide, 50:50) was used as the polymer matrix because its rapid degradation should guarantee its complete elimination in a reasonable time period following administration [27].

Materials and Methods

Materials

Poly(DL-lactide/glycolide, 50:50) copolymers were obtained from Dupont (Wilmington, DE), Birmingham Polymers, Inc. (Birmingham, AL) and Henley Chemicals, Inc. (Montvale, NJ). The polymer from each supplier was of a single lot number. Carbopol® 951 was purchased from B.F. Goodrich (Cleveland, OH). Polyvinylalcohol (MW 9000–10 000, 80% hydrolyzed) was from Aldrich Chemical (Milwaukee, WI). Bovine serum albumin (BSA), sodium oleate and sorbitan trioleate (Span 85) were from Sigma (St. Louis, MO). Silicone oil (Silicone Fluid 500) was purchased from Spectrum Chemical Co. (Gardena, CA). Coomassie dye reagent was obtained from Biorad (Richmond, CA). Triethanolamine was obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were reagent grade.

Methods

Preparation of microspheres

Three methods of microsphere preparation were investigated:

(1) o/o Emulsion method. A TTA-60 titration assembly (Radiometer, Copenhagen, Denmark) was used in this method for efficient stirring. Poly(DL-lactide/glycolide, 50:50, Dupont) (0.5 g) was dissolved in methylene chloride (3.3 ml). Spray-dried BSA [28] (25 mg) was then dispersed in this solution by applying sonification for 30 s in an ultrasonic cleaner (Branson 3200, Branson Cleaning Company, Shelton, CT). This suspension was passed dropwise through a syringe with a 22-gauge needle into a well-stirred emulsion containing silicone oil (20–30 ml), CH_2Cl_2 (30–40 ml) and Span 85 (2 ml). Petroleum ether (30 ml) was then added dropwise into the above dispersion. Stirring was continued for 2 h. Microspheres were filtered, washed with petroleum ether and dried in a vacuum for 72 h.

(2) o/w Emulsion method. An ultrasonified suspension of spray-dried BSA (25 mg), poly(DL-lactide/glycolide, 50:50) (Dupont or Birmingham Polymers) (0.5 g) and CH_2Cl_2 (2 ml) was emulsified with an aqueous solution (50 ml) containing sodium oleate (0.2 g) in a TTA-60 titration assembly for 5 min. The methylene chloride was then removed with a rotary-evaporator (120 rpm) at 360 Torr (1 h at 22°C), 160 Torr (0.5 h at 22°C) and 160 Torr and 40°C (1 h). The microspheres obtained were filtered, washed with water and vacuum dried at room temperature.

A slightly modified procedure was used to prepare microspheres containing Carbopol® 951 as an adjuvant. BSA (25 mg) was dissolved in 0.5% Carbopol® 951 aqueous solution (500 μl). This solution was ultrasonified with poly(DL-lactide/glycolide, 50:50) and methylene chloride solution (3.33 ml, 15% (w/v), (Dupont polymer) or 2 ml, 25% (w/v), (Birmingham Polymers)). At the same time, triethanolamine (2.5 μl) was added to gel the droplets of Carbopol® 951 and BSA in the polymer solution. The suspension was then emulsified, the solvent removed on a rotary-evaporator, filtered and dried as described above.

(3) (w/o)/w Emulsion method. A solution of BSA (2.6 mg) in distilled water (100 μl) was emulsified with a methylene chloride solution (0.5 g/2 ml) of poly(DL-lactide/glycolide, 50:50 Henley Chemical, RG503) through the use of a probe sonicator (Branson, Danbury, CT) at 125 W and 40% duty cycle, pulsed mode. This emulsion (w/o) was then emulsified in an aqueous solution (50 ml, 35°C) containing 0.1% polyvinyl alcohol with a homogenizer (5000 rpm, ESGE Handmixer M122, Biospec Products, Bartlesville, OK) for 5 min. Methylene chloride was removed from the resulting (w/o)/w emulsion on a rotary-evaporator at 300 Torr and 34°C (120 rpm) for 1 h. The microspheres obtained

were filtered, washed with water and either vacuum dried at room temperature or lyophilized (Consol 4.5, Virtis Co., Gardiner, NY).

Characterization of polymer and microspheres

Molecular weight and particle size

Polymer molecular weights were determined by gel permeation chromatography (GPC) using a 7.8 mm ID × 30 cm column packed with Ultrastyragel® (< 10 μm, mixed bed resin) with methylene chloride as eluent. Polystyrene standards were used for calibration. Particle sizes of microspheres were determined by scanning electron microscopy (SEM, Hitachi S-570, Tokyo, Japan). The details of these measurements were published previously [27].

BSA content in microspheres

Microspheres (50–100 mg) were dissolved in CH_2Cl_2 (5 ml). BSA was retained using a solvent-resistant filter. The buffer washes from the filter were analyzed by the Bradford method to determine the BSA content [35]. Elemental analysis was also used to determine the BSA content (based on nitrogen content) of the microspheres. Detailed procedures were described previously [28].

In vitro protein release studies

In vitro protein release studies were performed in duplicate. Microspheres (200 mg) were suspended in pH 7.2 phosphate-buffered saline (PBS) (2.5 ml) and agitated at 37°C and 100 rpm in an environmental incubator shaker (G-24, New Brunswick Scientific Co., Edison, NJ). At specific sampling times (each day for the first 4 days and every other day for the remainder of the study) the buffer solution was completely removed and replaced with fresh PBS. The protein content of the PBS was measured using the Bradford method [35].

Results and Discussion

Characterization of polymers and microspheres

The number average molecular weights measured by GPC of poly(DL-lactide/glycolide, 50:50) were 60 000 (Dupont), 67 000 (Birmingham Polymers) and 31 000 (Henley Chemicals). Each dissolved completely without residue at the indicated concentrations in methylene chloride. Degradation studies on each of these polymers formulated by spray-drying as microspheres had been previously performed [27,28].

The theoretically and experimentally determined BSA loadings of the poly(DL-lactide/glycolide, 50:50) microspheres made by each of the 3 methods described are given in Table 1. The experimentally measured protein contents for microspheres prepared by the o/o emulsion method were closest to the theoretical protein loadings. In the o/w emulsion method, the experimental loadings were lowest (~20% of theoretical) for microspheres prepared from 15% (w/v) polymer (Dupont) in methylene chloride solution, while they were higher (54–70%) for microspheres prepared from 25% (w/v)

TABLE 1

Protein loading of poly(DL-lactide/glycolide, 50:50) microspheres

Manufacturer	Preparation method	Loading (%)	
		Theoretical	Experimental
1. Dupont	o/o emulsion[a]	5	5.06, 4.7[d]
2. Dupont	o/o emulsion[b]	5	5.12
3. Dupont	o/w emulsion	5	1.28
4. Birmingham Polymers	o/w emulsion	5	2.72
5. Dupont	o/w emulsion[c]	5	1.12
6. Birmingham Polymers	o/w emulsion[c]	5	3.52
7. Henley Chemicals	(w/o)/w emulsion	5	2.02

[a] CH_2Cl_2/silicone oil = 1:1.
[b] CH_2Cl_2/silicone oil = 2:1.
[c] Carbopol® 951 incorporated.
[d] Determined from elemental analysis.

polymer (Birmingham Polymers) in methylene chloride solution. Protein was incompletely entrapped in all microspheres that were prepared in the presence of water due to protein extraction into the aqueous phase. We have previously reported the formulation of BSA by a spray-drying method in the absence of water that results in 100% loading [28]. An increase in the concentration of polymer in methylene chloride increased BSA incorporation. Increasing polymer concentration to improve incorporation is limited by both polymer solubility in methylene chloride (15% for Dupont polymer and 25% for Birmingham and Henley polymers) and increased viscosity of the resulting concentrated polymer solution. The use of poly(D,L-lactide/glycolide, 50:50) of lower molecular weight (<25 000) having increased solubility and lower viscosity may improve experimental protein loadings.

For particles prepared by the o/o emulsion method, the Dupont polymer was employed. These had a large average particle size of approximately 500 μm with an irregular shape (Fig. 1a). Similar particle sizes have been reported using this method [32]. Microspheres prepared by

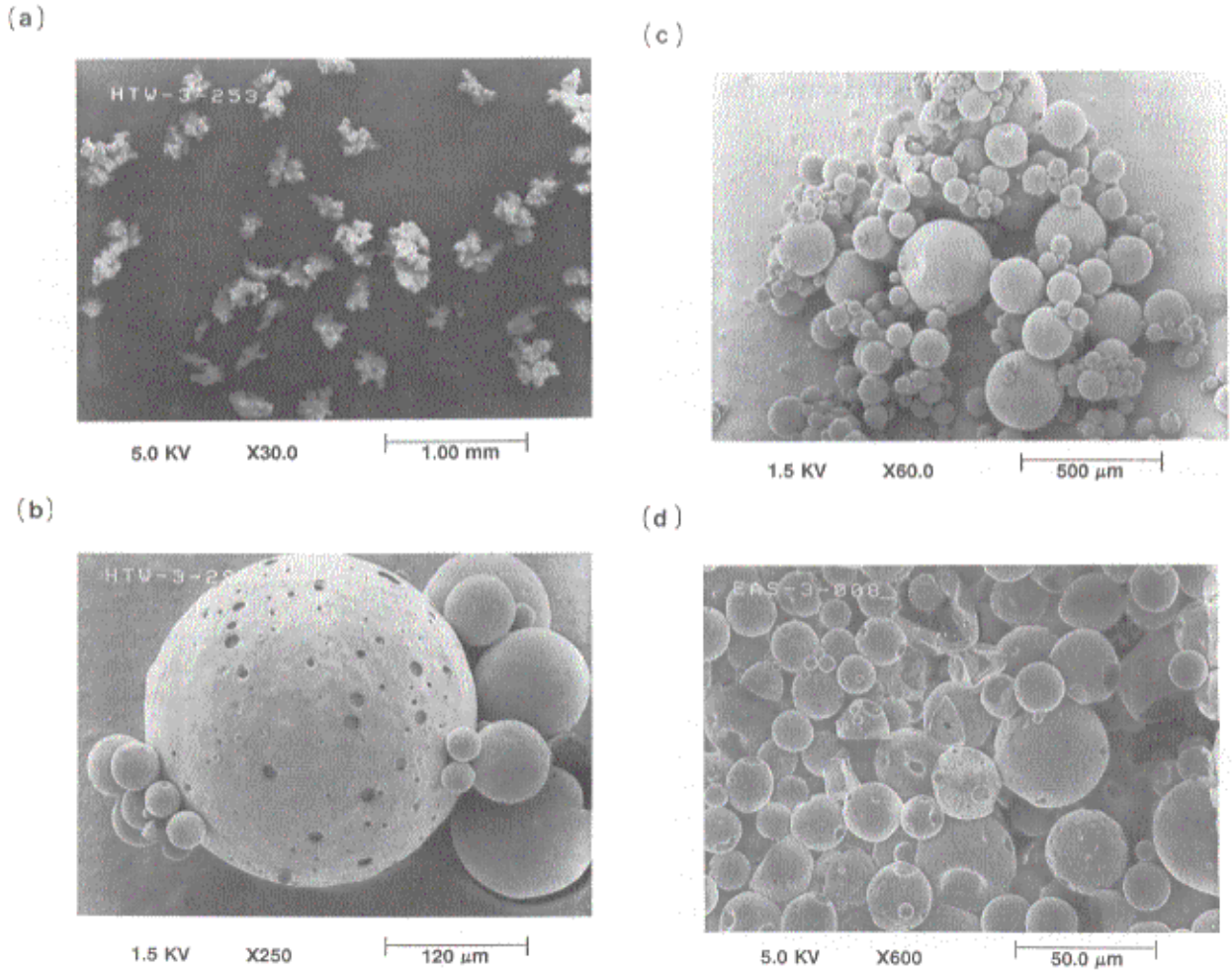

Fig. 1. SEM photographs of BSA incorporated poly(DL-lactide/glycolide, 50:50) microspheres prepared by different methods: (a) o/o emulsion, (b) o/w emulsion, (c) o/w emulsion with Carbopol® 951, (d) (w/o)/w emulsion.

o/w emulsion method, which employed polymers obtained from Dupont or Birmingham Polymers, had a wide distribution of particle sizes; $<25\ \mu m$ (32%), 25–50 μm (25%), 50–100 μm (25%), $>100\ \mu m$ (18%) (Fig. 1b). These microspheres showed a uniform exterior pore size as evaluated by SEM, possibly the result of solvent evaporating from the interior of the particle. Microspheres incorporating Carbopol® 951, prepared by a modified o/w emulsion procedure, gave a similar particle size distribution; $<25\ \mu m$ (23%), 25–50 μm (27%), 50–100 μm (35%), $>100\ \mu m$ (15%) (Fig. 1c). Microspheres prepared by the (w/o)/w emulsion method employed Henley polymer (RG503). These particles were much smaller with a narrower size distribution; $<10\ \mu m$ (25%), 10–20 μm (53%), 20–30 μm (17%) and $>30\ \mu m$ (5%) (Fig. 1d). This may be the result of using a probe sonicator and homogenizer when preparing the emulsion.

In vitro protein release studies

The release of BSA from polymer (Dupont) particles prepared by the o/o emulsion method (Fig. 2a) showed that the burst effect was higher (30%) for microspheres prepared with a 1:1 ratio of methylene chloride to silicone oil than for particles prepared using a 2:1 ratio. After the burst effect, however, both o/o emulsion methods gave particles that showed approximately the same release rate. The release of BSA could be sustained for a period of 54 days from these formulations. The cumulative release curve (Fig. 2a) also shows that less protein is released from particles formulated using a 2:1 ratio of methylene chloride to silicone oil although most of this difference is attributable to the different magnitudes of the burst effect. Not all the loaded protein could be accounted for in these release studies. The daily BSA release (Fig. 2b) was measured until day 54 after which both formulations released $<312.5\ \mu g/g/day$. At these release rates the protein assay method had insufficient sensitivity to determine BSA released. The increase in daily release of BSA (Fig. 2b), in the 1:1 methylene chloride to silicone oil formulation, after 14 days of hydrolysis, could probably be explained by the degradation of particles and creation of porous structure due to a random chain-scission process [27,28]. In both o/o formulations, polymer residue remained after 60 days in PBS. We previously reported that spray-dried microspheres (1–2 μm), prepared from the same polymer (with or without BSA), showed complete degradation (matrix dissolution) within 40 days in PBS at 37°C suggesting that the particle size plays a primary role in extending the release from this formulation. These particles did not aggregate during the release studies probably due to the presence of Span 85.

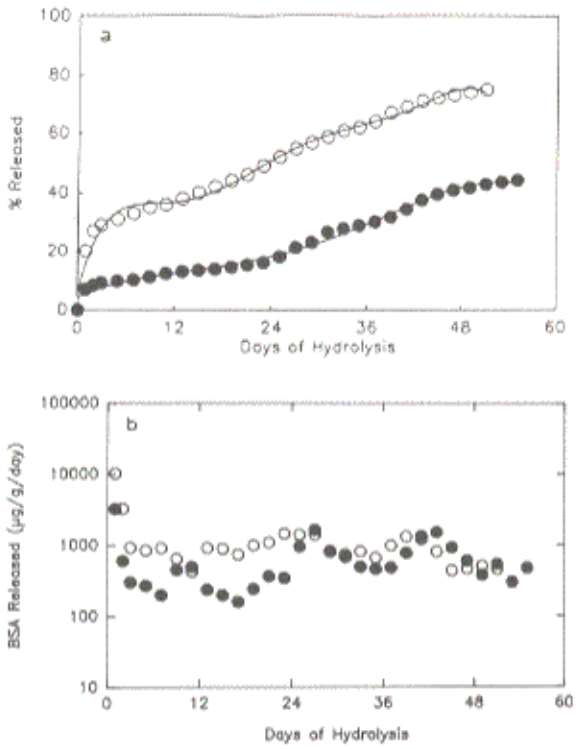

Fig. 2. BSA released from poly(DL-lactide/glycolide, 50:50, Dupont) microspheres prepared by the o/o emulsion method: (a) cumulative percent released from microspheres prepared with a ratio of methylene chloride to silicone oil of 1:1 (○) or 2:1 (●); (b) daily release from the same microspheres (μg released/g microspheres/day).

Microspheres used in the o/w emulsion method were prepared from polymers obtained from Dupont and Birmingham Polymers. The microspheres prepared from the polymer ob-

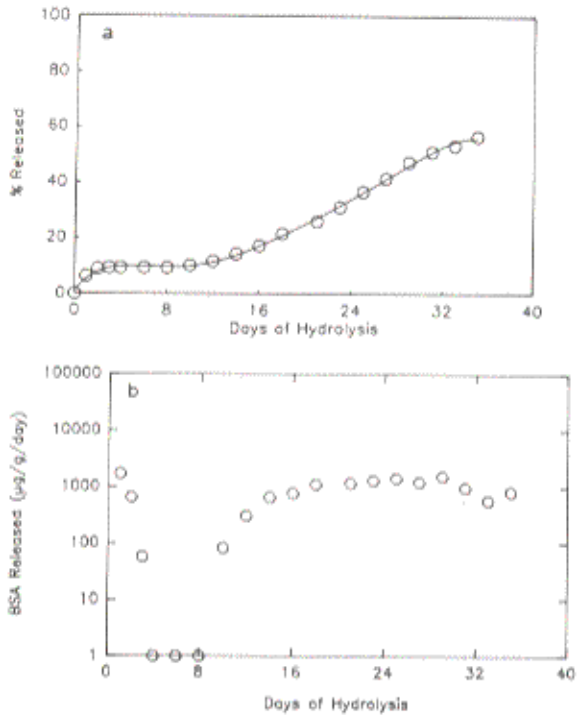

Fig. 3. BSA released from poly(DL-lactide/glycolide, 50:50, Birmingham Polymers) microspheres prepared by the o/w emulsion method: (a) cumulative percent released, (b) daily release of BSA into PBS (μg released/g microspheres/day) from microspheres.

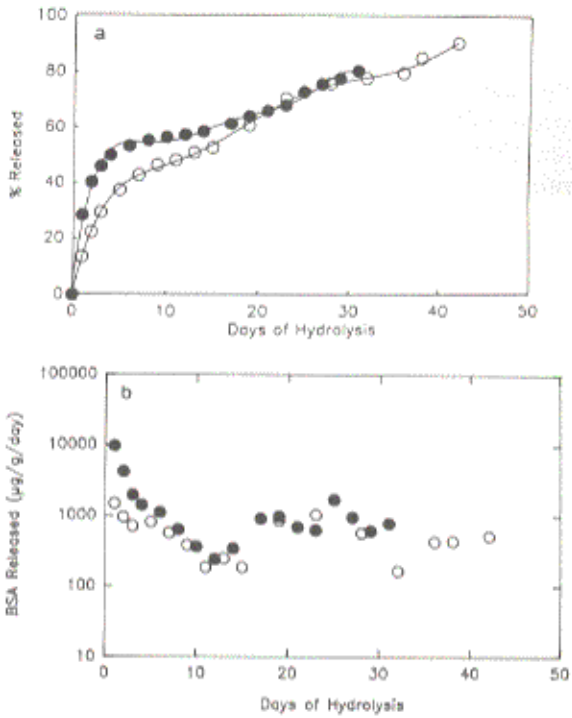

Fig. 4. BSA released from poly(DL-lactide/glycolide, 50:50), microspheres prepared by the o/w emulsion method and Carbopol® 951 incorporated: (a) cumulative percent released, (b) daily release of BSA into PBS (μg released/g microspheres/day) from microspheres prepared with polymer from Dupont, 1.12% (w/w) BSA loading (○); Birmingham Polymers, 3.52% (w/w) BSA loading (●).

tained from Birmingham Polymers showed a higher protein loading (possibly the result of the increased solubility of this polymer in methylene chloride). Similar release curves were obtained for microspheres prepared from both polymers. Thus the detailed release studies were performed using microspheres prepared with the polymer obtained from Birmingham Polymers having the higher protein loading. The release from microspheres prepared by the o/w emulsion method is shown in Fig. 3a. A small burst effect of $<10\%$ BSA release was observed during the first 8 days. About 50% of the BSA loaded was released in a period of 24 days. No protein release was measured between day 4 and 8 of the hydrolysis (Fig. 3b). A 1250 μg/g/day release rate was detected from day 16 through day 34. These particles had a tendency to aggregate during the release studies, which could be partially overcome by agitation. Although the SEM shows the presence of small pores on the surface of these microspheres, the small burst effect and the low but constant daily release of protein suggests that these are low-porosity microspheres.

Microspheres prepared by the o/w emulsion method in which a small amount of Carbopol® 951 was incorporated showed a high burst effect of 40–50% (Fig. 4a). The BSA released was 65–75% of the protein loaded (experimentally measured) over a 25-day period. The daily BSA release (Fig. 4b) was between 625 and 1250 μg/g/day level from days 5 through 30. Carbopol® 951 was incorporated into microspheres prepared by this emulsion method as a potential adjuvant agent [36] and also to enhance protein loading. Hydrophilic agents can diffuse out of the microspheres during emulsification and evaporation steps [34]. Ogawa et al. [34] demonstrated that

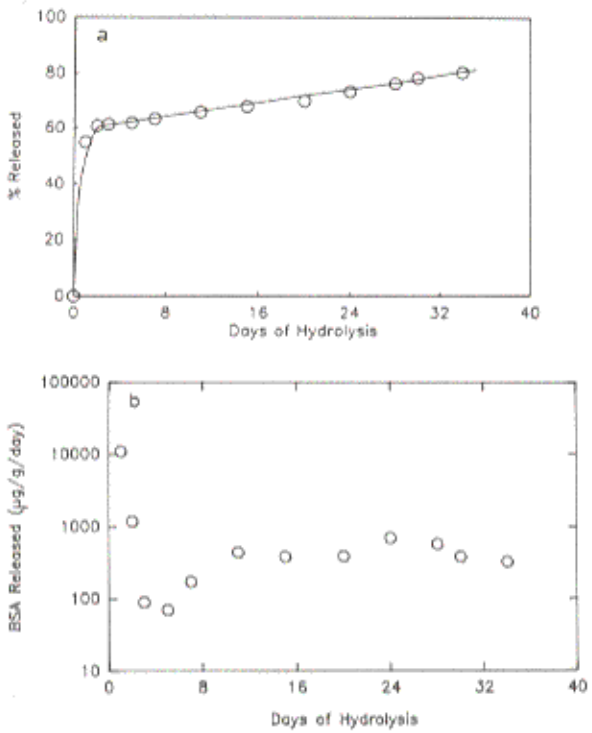

Fig. 5. BSA released from poly(DL-lactide/glycolide, 50:50, Henley Chemicals) microspheres prepared by the (w/o)/w emulsion method: (a) cumulative percent release, (b) daily release of BSA into PBS (µg released/g microspheres/day) from microspheres.

incorporation of gelatin into the internal aqueous phase gave higher entrapment. This study employed a variation on this approach using Carbopol® 951 at elevated pH to gel the inner phase. Microspheres containing Carbopol® formulated with different concentrations of polymers (from different sources but having similar molecular weight) gave different BSA loadings. The microspheres having a higher protein loading gave a greater burst effect (50% vs. 40%) as well as a slightly higher daily release of BSA. The microspheres with lower BSA loading showed a prolonged period of measurable protein release corresponding to the expected decreased porosity of these microspheres. When comparing the microspheres with and without added Carbopol® 951 (Figs. 3 and 4) prepared by the o/w emulsion method, the daily release was found to be higher from the microsphere formulated with Carbopol® 951. This difference could probably be attributed to one or more factors. The formulation without Carbopol® used solid spray-dried BSA microspheres while the Carbopol® formulation used dissolved spray-dried BSA to increase protein loading [34]. The Carbopol®-containing formulation might contain more entrained water resulting in a more porous microsphere. The Carbopol® also may act as a wetting agent or may promote the osmotic uptake of water into the microsphere's pore structure resulting in a large burst effect. It is unclear what effect if any the large size range observed in particles prepared by the o/w emulsion method had on release kinetics.

The Henley polymer was used to prepare microspheres formulated by the (w/o)/w emulsion method. This polymer was selected because its lower molecular weight resulted in increased methylene chloride solubility with reduced viscosity. This permits the use of the high polymer concentrations required to obtain high levels of protein loading. The release profile for microspheres formulated using a (w/o)/w emulsion method is shown in Fig. 5. As in the case of the microspheres prepared by o/w emulsion, some aggregation was observed during the release studies. Again this aggregation could be reduced by increased agitation of the release medium. The burst effect was very high (60%) for these microspheres. After a 2-day burst release, the remaining protein was released at a constant rate over a 30-day period. The BSA released during this period was between 125 and 625 µg/g/day. The burst effect could be entirely eliminated by lyophilizing the microspheres prepared by the (w/o)/w emulsion method. No measurable release of protein occurred for 15 days of hydrolysis. This method may result in more complete drying of the microspheres or may result in the formation of smaller pores by the evaporation of solvent during the sublimation process of freeze-drying. Further studies on drying methods will be required to determine whether it is possible to reduce the burst effect without markedly diminishing the normal desired protein release over the first 15 days of hydrolysis.

Conclusions

Poly(DL-lactide/glycolide, 50:50) microspheres have been prepared by o/o, o/w, and (w/o)/w emulsion methods, giving average particle sizes of 500, 25–500, and 10–20 μm, respectively. The microspheres prepared by o/w and (w/o)/w emulsion techniques were spherical particles while those prepared by the o/o emulsion method were irregularly shaped. The particles prepared by the o/o emulsion method did not aggregate during hydrolysis in PBS.

Complete entrapment of BSA was only achieved with the o/o emulsion technique. 50–75% of the BSA could be retained in microspheres formulated by the o/w emulsion method. Only 40% of the BSA was found in microspheres formulated by the (o/w)/w emulsion method. Increased polymer concentration in methylene chloride, from 15% (w/v) to 25% (w/v), resulted in improved BSA loading from 22% to 54–70%.

The period of sustained release of BSA was 54, 36, and 34 days for microspheres prepared by o/o, o/w, and (o/w)/w emulsion methods, respectively. The daily release of BSA was 625–1250, ~1250, 500–1250, and 125–625 μg/g/day for microspheres formulated using o/o, o/w, o/w with Carbopol® 951, and (w/o)/w emulsion methods, respectively. The reduced burst effect and extended release time for BSA from microspheres prepared by o/w emulsion without Carbopol® 951 may be the result of using solid BSA microspheres in the place of dissolved BSA during formulation. The particle size of the microspheres is also an important factor in protein release. For instance, the daily BSA release of large particles prepared by the o/o emulsion technique was considerably lower than from the small microspheres prepared by o/w emulsion.

The burst effect which was greatest for microspheres prepared by the (w/o)/w emulsion method could be eliminated by lyophilizing microspheres following their preparation. This reduced release to undetectable levels for at least 15 days and thus was probably not desirable for the control release of proteins.

In vivo testing of all of the formulations will be necessary to evaluate the efficacy of the sustained, continuous release of vaccine from microspheres for immunization. Alternative approaches including pulsatile release of vaccines from polymer matrices also warrant investigation.

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