CONTROLLED RELEASE OF PROTEIN AND VACCINES FROM POLY(ESTER) MICROSPHERES *IN VITRO*

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Biodegradable microspheres of poly(L-lactide) and copolymers of lactide and glycolide have been prepared by spray drying. Degradation studies of these microspheres using residual mass measurements, viscometry and gel permeation chromatography indicated the entire mass of polyester matrices was maintained for 10, 30 days and 6-10 months for 50:50 and 85:15 copolymers and poly(L-lactide), respectively. The continuous drop in polymer intrinsic viscosity and molecular weight during hydrolysis suggested that matrix degradation began as soon as these microspheres were placed in the buffer and that their degradation proceeded through random-chain scission. Protein release, using bovine serum albumin microspheres, showed that release from 50:50 copolymer was independent of polymer molecular weight over a range from 31,000 to 93,000. The release was, however, dependent on the polymer composition and BSA loading in the microspheres. A burst-effect was found in the release study for microspheres prepared from copolymers. The identity and integrity of the released protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on the release products of polymeric microspheres containing BSA, FPL-K and EcoBac^R-Plus vaccines. These results suggested that the release BSA (or vaccine) from polymeric microspheres could be sustained for up to one month.

INTRODUCTION

Biodegradable polymers have been used in delivery of drugs[1-9] and biologicals.[10-18] The major advantages of biodegradable polymers used in controlled release applications are that they do not require surgical removal from the host after delivery of bioactive agent is complete. Additionally, adverse tissue reactions from implanted polymer may be ameliorated.[19-20]

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There has been considerable interest in developing new methods to deliver both vaccines and adjuvants.[21-23] A polymeric delivery device incorporating an immunogen in the presence or absence of an adjuvant could sustain the release of a small amount of antigen over a prolonged period of time eliminating the need for boosters. Appropriate presentation of the vaccine to the immune system may also be required, particularly for highly purified antigens prepared by recombinant technology.

Poly(esters) were chosen for study as a vaccine delivery vehicle because they are currently used in suture materials and have demonstrated low toxicity.[24-27] In recent studies we have prepared polyester microspheres by a spray-drying method.[28-30] These microspheres undergo chemical hydrolysis to soluble monomeric or oligomeric units and degrade in four major stages: (1) Polymer hydration, (2) Strength loss caused by breakage of backbone bonds in polymer, (3) Loss of mass integrity, and (4) Solubilization.[30] Since poly(ester) matrix biodegradation generally involves bulk erosion, release takes place prior to matrix biodegradation under diffusion-control. This has created problems in the delivery of drugs where zero-order release kinetics is desirable.[1,4] Precise control of release kinetics, however, may not be as important as delayed or sustained release in the application of these polymers to vaccine delivery.

This research focuses on microspheres prepared from three biodegradable poly(esters) as potential carriers of vaccines. The matrix characteristics during degradation show matrix lifetimes compatible with vaccine applications. *In vitro* bovine serum albumin and vaccine release studies demonstrate sustained release is possible, making these formulations potentially useful for vaccine applications.

EXPERIMENTAL

1. Materials

Poly(DL-lactide:glycolide, 50:50 and 85:15) (Medisorb®, bioresorbable polymers) were obtained from Dupont (Wilmington, DE). Other poly(DL-lactide:glycolide, 50:50) copolymers were obtained from Birmingham Polymers, Inc. (Birmingham, AL) and Henley Chemicals, Inc. (Montvale, NJ). Poly(L-lactide) polymers were obtained from Birmingham Polymers, Inc. and Polysciences, Inc. (Warrington, PA), respectively. Bovine serum albumin (BSA) and Coomassie Brilliant Blue R were from Sigma (St. Louis, MO). Acrylamide was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). N,N'-Methylenebisacrylamide (BIS) was from Fisher Chemical Company (Pittsburgh, PA). Sodium dodecyl sulfate (SDS) was from BDH Chemicals Ltd (Poole, England). *Escherichia coli* Bacterin (EcoBac®-Plus) and killed panleukopenia virus (FPL-K) were used as vaccines and were obtained from Solvay Animal Health, Inc. (Mendota Heights, MN). All other chemicals and solvents were reagent grade and used without further purification.

2. Preparation of Microspheres

Polymeric microspheres, including poly(DL-lactide:glycolide, 50:50 or 85:15), were prepared by dissolving 10 g of polymer in 500 mL of methylene chloride, and were spray-dried at 37°C according to the methods described previously.[30] Spray-dried BSA (100-1000 mg), with a particle size distribution; <0.7 µm (69%), 0.7-1.0 µm (12%), 1.0-2.0 µm (4%), 2.0-3.0 µm (4%), 3.0-4.0 µm (4%) and >4 µm (7%), was suspended in a methylene

chloride solution of polymer (2%, w/v), 500 mL) using sonification, and this BSA suspension was subsequently spray-dried at 37°C. Vaccine containing polymer microspheres were prepared by dissolving poly(DL-lactide: glycolide, 85:15, 4.5 g) in methylene chloride (250 mL). Lyophilized vaccine (0.5 g) was then added and homogenized using a handmixer (ESGE Handmixer M122, Biospec Products, Bartlesville, OK) for 5 minutes and then spray-dried at 37°C. The FPL-K vaccine was prepared for formulation by dialyzing (molecular weight cut-off, 10-15,000) against 5 mM Na_2HPO_4 buffer (pH = 7) to remove most of the salt before lyophilization. The EcoBac®-Plus vaccine was lyophilized in the presence of L-lactose (5 mg/mL).

3. Characterization of Polymers and Microspheres

3A. Molecular Weight and Intrinsic Viscosity

Polymer molecular weights were determined by gel permeation chromatography (GPC) with 0.5% w/v solution of polymer in methylene chloride. The intrinsic viscosities of polymer methylene chloride solution, at 25°C, were measured by a Cannon-Ubbelode viscometer (Cannon, State College, PA). The details of these measurements were published previously.[30]

3B. BSA Content in Microspheres

Microspheres (50-100 mg) were dissolved in CH_2Cl_2 (5 mL) and passed through a glass syringe equipped with a solvent resistant filter (2.5 cm in diameter, 0.45 μm in pore size, Millex®-HV, Millipore Products Division, Bedford, MA). Three 5 mL volumes of CH_2Cl_2 were used to wash the residual polymer from the filter. Four 5 mL volumes of phosphate buffered saline (PBS, containing 150 mM Na_2HPO_4 and 0.9 w/v% NaCl) were passed through the filter to dissolve and remove the retained BSA particles. The buffer washes were collected and the BSA content was determined by Bradford's method.[31] Elemental analysis was also used to determine the BSA or vaccine content of the microspheres. The nitrogen content of BSA (or vaccine), polymer microspheres and protein loaded microspheres were each determined and used to calculate protein loading.

4. Degradation of Microspheres

Twelve to fifteen screw-cap test tubes containing microspheres (200 mg) dispersed in PBS (2.5 mL) were prepared and agitated at 37°C and 100 rpm in a G-24 environmental incubator shaker (New Brunswick Scientific Co., Edison, NJ). At various time intervals, one sample tube was removed, and the residual mass was separated, washed with double distilled water and dried under vacuum. The residual mass, polymer molecular weight and intrinsic viscosity were determined.

5. *In Vitro* Release Studies

Protein release studies were conducted using poly(DL-lactide: glycolide, 50:50 or 85:15) or poly(L-lactide) microspheres containing BSA, EcoBac®-Plus. FPL-K vaccine was prepared using poly(DL-lactide: glycolide, 85:15). Microspheres (200 mg) were placed in 2.5 mL of PBS (pH = 7.2). Each day the buffer solution was completely removed and the protein

content was measured using Bradford's method.[31] Fresh buffer (2.5 mL) was added to replenish that which had been removed.

The protein recovered in the release studies was identified using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The recovered buffer (1 mL) in the release studies was dialyzed (molecular weight cut-off, 10-15,000) against double distilled water and then frozen and freeze-dried. The sample was dissolved in 50 µL SDS reducing buffer,[32] and heated at 95°C for 5 minutes. A sample of the above buffer (10 µL) was loaded into a SDS-PAGE system, containing stacking gel (4% acrylamide, 0.1% bisacrylamide, 0.01% SDS and 0.05% ammonium persulfate) and separating gel (7.5% acrylamide, 0.2% bisacrylamide, 0.01% SDS, and 0.05% ammonium persulfate).[32] Electrophoresis was performed for 40 minutes at 200 volts in running buffer.[32] The gel was, then, removed from glass plates and stained with a solution of 0.1% Coomassie blue R in fixative (40% methanol/10% acetic acid). Destaining was carried out twice with 50 mL of 40% methanol and 10% acetic acid in water.

RESULTS AND DISCUSSION

1. Characterization of Polymers and Microspheres

The molecular weights and the intrinsic viscosities of a number of poly(DL-lactide-co-glycolide) and poly(L-lactide) polymers obtained from differently commercial sources are shown in Table 1. The poly(esters) used in this study had molecular weights between 31,000 and 93,000.

Microspheres of poly(esters) prepared by spray-drying gave a particle size around 1-2 µm and had a tendency to aggregate.[30] The BSA loadings of microspheres prepared by spray-drying were determined by the Coomassie dye binding assay.[31] The experimentally measured loadings (Table 2) were comparable to the theoretically calculated loadings based on the amount of BSA put into the methylene chloride solution of poly(ester). Nitrogen composition by elemental analysis of BSA loaded microspheres confirmed

Table 1. Properties of various biodegradable polymers

Polymer[a]	Composition and Manufacturer	Molecular Weight M_N, Da	Intrinsic Viscosity (dL/g)
1. P(DL-LA/GA)	(50/50) Dupont	60,000	0.34[c]
2. P(DL-LA/GA)	(50/50) Birmingham	67,000	0.46[c]
3. P(DL-LA/GA)	(50/50) Henley RG503[b]	31,000	0.4[d]
4. P(DL-LA/GA)	(50/50) Henley RG504[b]	42,000	0.5[d]
5. P(DL-LA/GA)	(50/50) Henley RG505[b]	74,000	0.7[d]
6. P(DL-LA/GA)	(50/50) Henley RG506[b]	93,000	0.8[d]
7. P(DL-LA/GA)	(85/15) Dupont	63,000	0.43[c]
8. P(L-LA)	Polysciences	88,000	0.51[c]
9. P(L-LA)	Birmingham	95,000	1.28[d]

(a) LA, Lactide; GA, Glycolide.
(b) Resomer®.
(c) Determined in CH_2Cl_2.
(d) Determined in $CHCl_3$ and specified by manufacturer.

Table 2. Protein loading of spray-dried microspheres

Spray-dried Microspheres	Composition and Manufacturer	Loading (%) Theoretical	Loading (%) Experimental
1. P(DL-LA/GA)	(50/50) Dupont	10	10.37, 8.3[a]
2. P(DL-LA/GA)	(50/50) Dupont	5	5.50
3. P(DL-LA/GA)	(50/50) Dupont	1	1.02
4. P(DL-LA/GA)	(50/50) Henley RG503[b]	5	5.44
5. P(DL-LA/GA)	(50/50) Henley RG504[b]	5	5.44
6. P(DL-LA/GA)	(50/50) Henley RG505[b]	5	5.50
7. P(DL-LA/GA)	(50/50) Henley RG506[b]	5	5.18
8. P(DL-LA/GA)	(50/50) Birmingham	5	6.08
9. P(DL-LA/GA)	(85/15) Dupont	10	10.75
10. P(DL-LA/GA)	(85/15) Dupont	5	5.18
11. P(DL-LA/GA)	(85/15) Dupont	1	1.60
12. P(L-LA)	Polysciences	5	6.08, 5.7[a]
13. P(L-LA)	Birmingham	5	5.95

(a) Determined from elemental analysis.
(b) Resomer®.

these theoretical and experimental loadings as shown for two systems in Table 2.

2. Degradation of Microspheres

Degradation studies *in vitro* were carried out with three types of polymer microspheres, poly(DL-lactide:glycolide, 50:50, (Birmingham Polymers), poly(DL-lactide:glycolide, 85:15, (Dupont) and poly(L-lactide, (Polysciences) possessing intrinsic viscosities of 0.46, 0.43 and 0.51 dl/g corresponding to number average molecular weights of 67, 63 and 88 x 10^3 Daltons, respectively. Poly(ester) microsphere degradation in PBS was followed by measuring residual mass, polymer intrinsic viscosity and polymer molecular weight (Figures 1-3).

No mass loss could be detected from the 50:50 and 85:15 copolymer microspheres for 10 and 30 days, respectively. However, polymer intrinsic viscosities and molecular weights decreased continuously during this period. The relative molecular weight ratio (Mn/Mn_o) dropped faster than the relative intrinsic viscosity ratio($[\eta]/[\eta]_o$). For example, it took 27 days to decrease the intrinsic viscosity of the 85:15 copolymer to half of its initial value, but it only took 17 days to decrease its initial molecular weight by half. After a lag period during which residual mass remained constant, weight loss began and then accelerated. Only 40 and 90 days were required for nearly complete mass loss of the 50:50 and 85:15 copolymers, respectively. In addition, the degradation profiles of residual mass, intrinsic viscosity and molecular weight for poly(DL-lactide: glycolide, 50:50) (Birmingham Polymers) microspheres (number average molecular weight 67,000) were similar to those profiles for microspheres of 50:50 copolymer (Dupont, with a number average molecular weight of 60,000 Daltons).[30]

It should be noted that even though no mass loss was observed in poly(L-lactide) over the time frame of these experiments (Figure 3a), significant changes in both polymer viscosity and molecular weight were

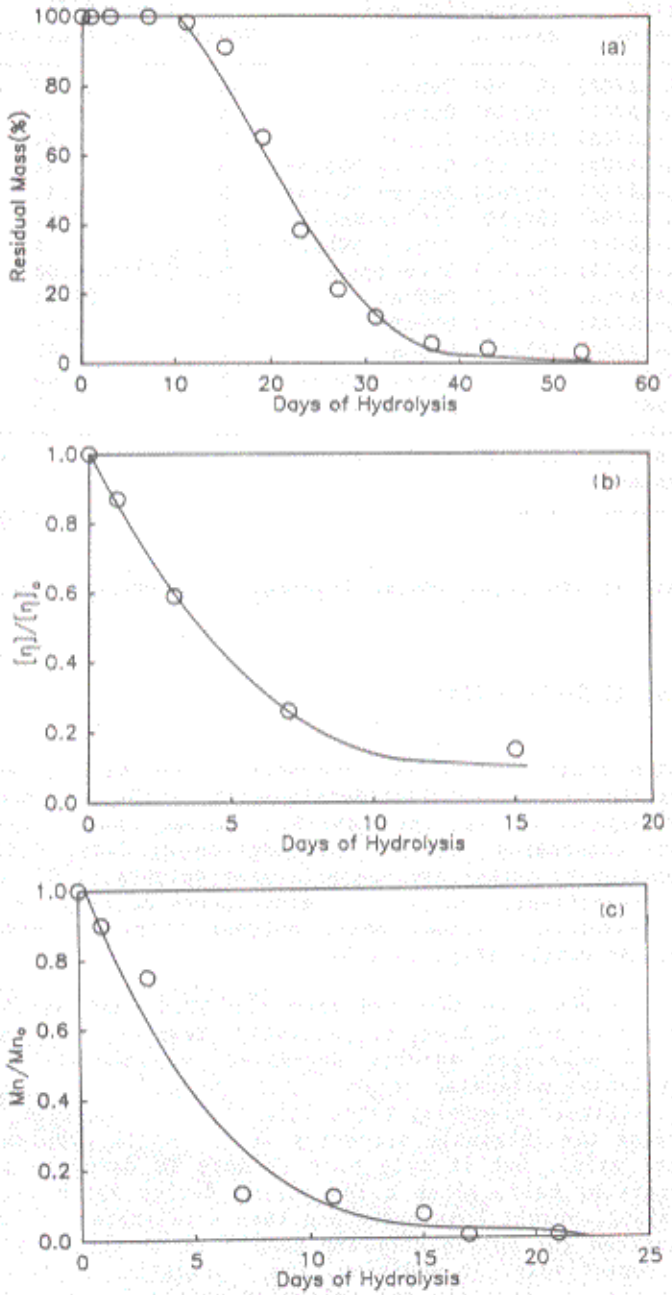

Figure 1. Degradation studies on poly(DL-lactide:glycolide, 50:50), (Birmingham Polymers) microspheres by various methods: (a) Residual mass (%); (b) ratio of intrinsic viscosity; (c) molecular weight ratio (degraded vs. initial) are shown as a function of time.

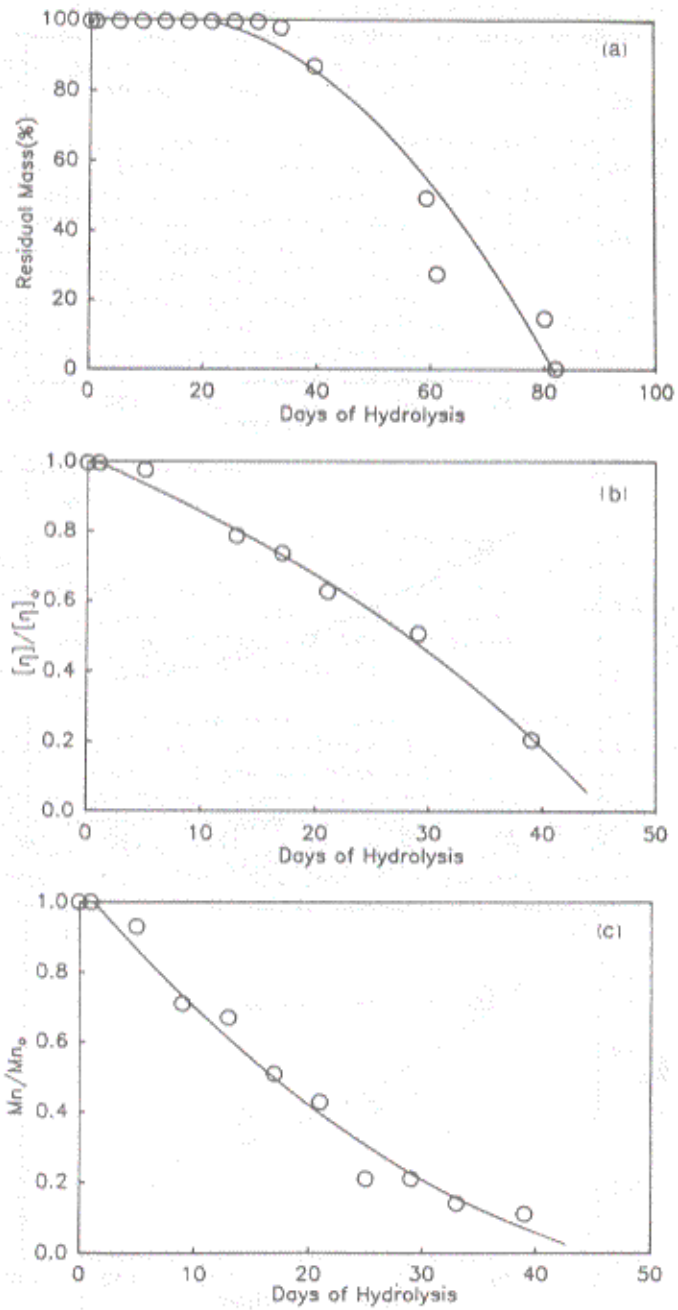

Figure 2. Degradation studies on poly(DL-lactide:glycolide, 85:15), (Dupont) microspheres by various methods: (a) Residual mass (%); (b) ratio of intrinsic viscosity; (c) molecular weight ratio (degraded vs initial) are shown as a function of time.

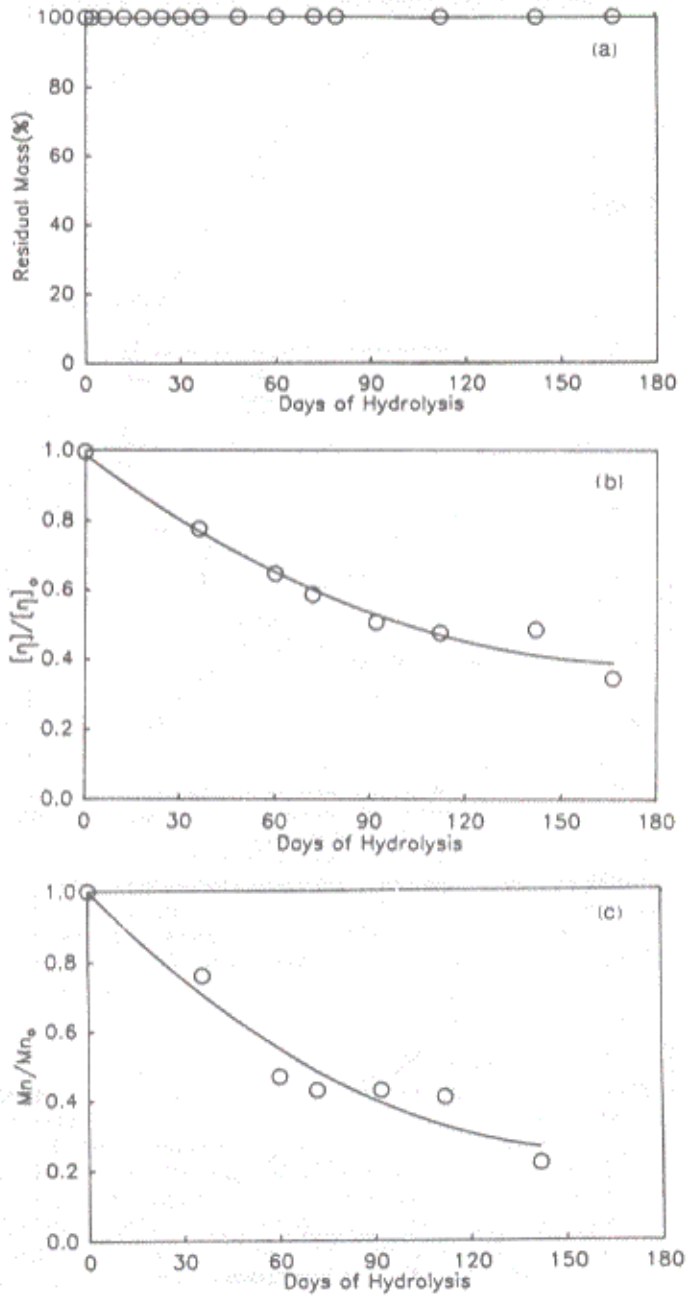

Figure 3. Degradation studies on poly(L-lactide), (Polysciences) microspheres by various methods: (a) Residual mass (%); (b) ratio of intrinsic viscosity; (c) molecular weight ratio (degraded vs initial) are shown as a function of time.

detected (Figure 3b & 3c). These results suggest that polymer chain scission begins as soon as the polymer is exposed to PBS, but that even over a period of 180 days, the polymer molecular weight is not reduced sufficiently to produce water-soluble oligomers. Other experiments in our laboratory have shown that poly(L-lactide) microspheres maintained >60% of their initial mass after exposure to PBS at 37°C for 10 months.

These results indicate that the degradation profiles can be separated into three phases. Initially there is a constant residual mass with no formation of soluble monomeric or oligomeric products but a rapid decrease in polymer intrinsic viscosity and molecular weight is observed. This implies a random-chain scission process throughout the bulk of the polymer microspheres. Secondly there is simultaneous loss in residual mass, decrease in polymer intrinsic viscosity and molecular weight, and production of soluble monomeric and oligomeric products.[30] The final phase involve a reduced rate of residual mass loss, probably due to rate-determining dissolution of monomeric and oligomeric products.

3. *In vitro* Release Studies

In vitro release studies of BSA-containing microspheres of poly(DL-lactide:glycolide, 50:50) of molecular weights varying from 31,000 to 93,000 daltons showed that the release of the BSA is not very sensitive to variation in polymer molecular weight (Figure 4). The time at which half of the loaded BSA loading had been released was less than 1 day, irrespective of the polymer molecular weight.

The release of BSA from poly(DL-lactide:glycolide, 50:50 or 85:15) microspheres with different BSA loadings (Figures 5a & 6b) indicated that an initial burst release of BSA was observed followed by a linear release period. A significant burst-effect was found for microspheres loaded with 5 and 10% BSA in both polymers. In poly(DL-lactide:glycolide, 50:50) microspheres loaded with 5.5 and 10.37% BSA, half of the BSA dose was released within 1 day. The copolymer microspheres loaded with 5% BSA showed a slightly lower burst-effect than those loaded with 10% BSA. In

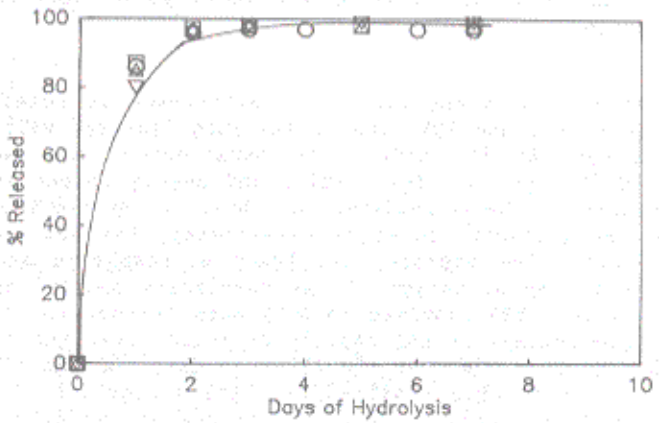

Figure 4. BSA release profiles from poly(DL-lactide:glycolide, 50:50), (Henley Chemicals) microspheres of various molecular weights. Molecular weights: 31,000 (o), 42,000 (△), 74,000 (□) and 93,000 (▽) are shown as a function of time.

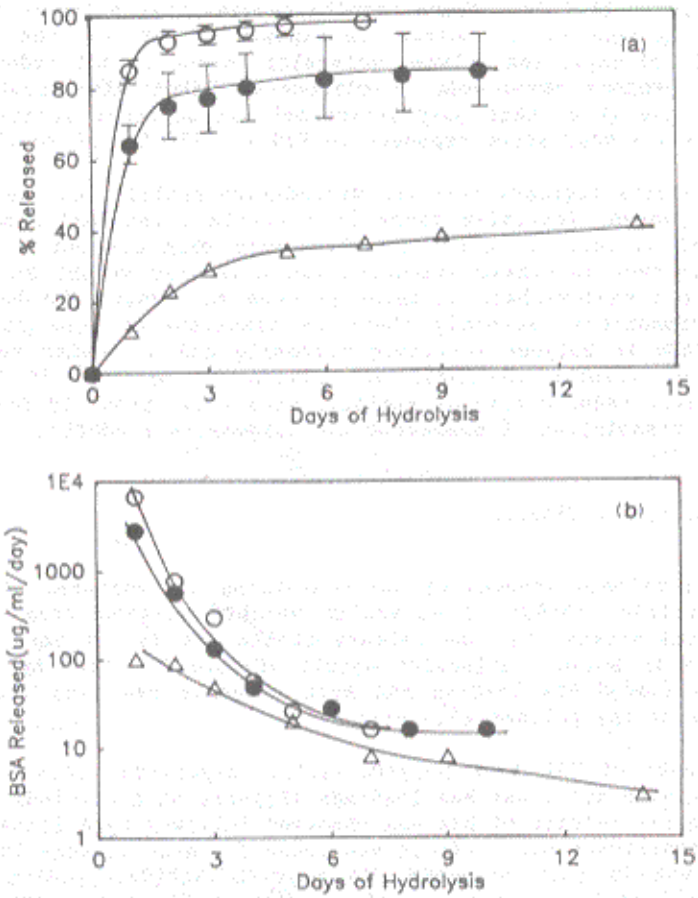

Figure 5. Effect of BSA loading on protein release from poly-(DL-lactide:glycolide), 50:50, (Dupont): (a) cumulative percents released; (b) daily release from microspheres with 10.37% (o), 5.50% (●), and 1.02% (△) (w/w) BSA loading are shown as a function of time.

microspheres of the 85:15 copolymer, the difference in release profiles of microspheres with 5 and 10% loading was much less and well within the experimental error. At 1% loading the burst-effect was substantially lower in the 50:50 copolymer formulation and nearly absent in the 85:15 copolymer formulation. These loadings may, however, be too low for application in vaccine delivery. In these experiments, the BSA remaining was confirmed by drying the degraded copolymer microspheres and measuring their BSA content. The summation of the cumulative percent released and the remaining percent BSA resulted in 100% mass balance for the delivery system. The daily release of BSA rapidly decreased over the first 7 days for the 50:50 copolymer and over the first 15 days for the 85:15 copolymer (Figures 5b & 6c), then remained relatively constant at a low rate for the duration of the release studies. Because of the detection limit, of the Bradford assay (5 µg/mL),[31] the daily release of BSA could only be measured for 14 days for the 50:50 copolymer and 30 days for the 85:15 copolymer.

The *in vitro* profiles of BSA release from poly(L-lactide) micro-

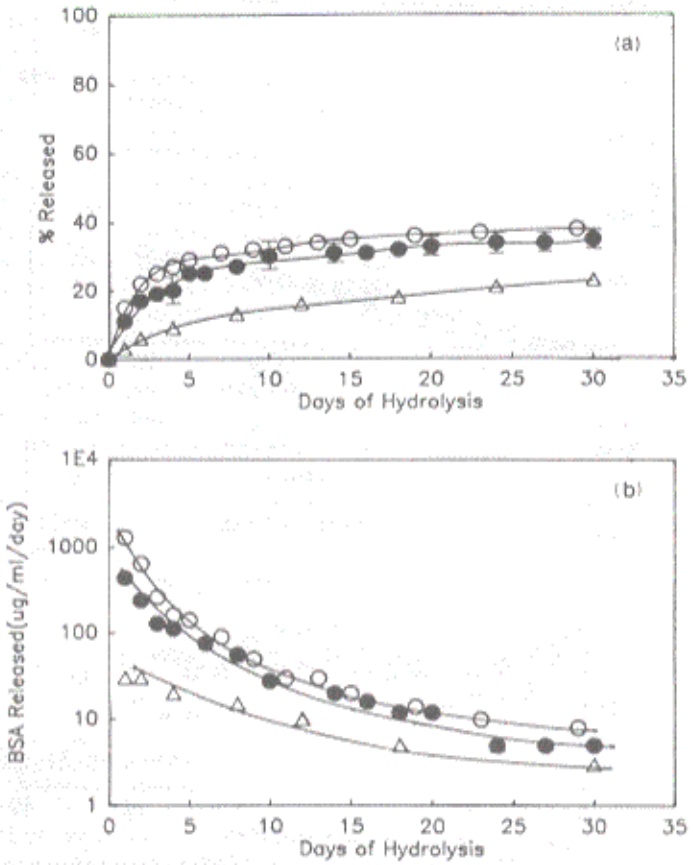

Figure 6. Effect of BSA loading on protein release from poly-(DL-lactide:glycolide, 85:15), (Dupont): (a) cumulative percent released; (b) daily release from microspheres with 10.75% (o), 5.18% (●), and 1.60% (△) (w/w) BSA loading are shown as a function of time.

spheres (Figure 7a) were nearly linear over the time period studied for both microsphere preparations. A burst effect was not observed. The daily BSA release (Figure 7b) ranged between 10-100 µg level throughout a 36 day period. The different release behavior (i.e., the absence of a burst effect and a nearly linear release profile) between poly(L-lactide) and copolymer of lactide and glycolide may be due to the partially crystalline character of poly(L-lactide). The crystalline domain inside the BSA loaded poly(L-lactide) microspheres is probably slowly degraded compared to the amorphous domain. This may serve to block the diffusional release of BSA from the poly(L-lactide) microspheres.

To confirm the identity of the protein measured by Bradford assay,[31] *in vitro* release of BSA (5% loading) from poly(DL-lactide:glycolide, 50:50) microspheres was followed using SDS-PAGE (Figure 8a). The last two lanes corresponding to the first and third day's release, reflected the large burst-effect observed using the Bradford assay (Figure 5). The first lane was loaded with a 20 µg BSA standard. The BSA release could be confirmed throughout the entire 27 day period. Quantification of BSA using this method is probably not reliable because of the precipitation

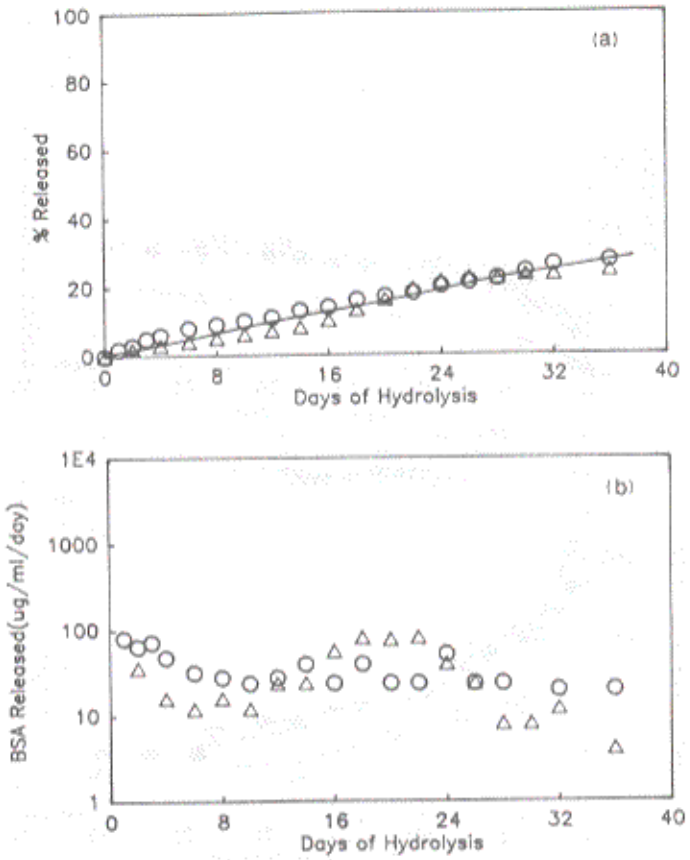

Figure 7. BSA released from poly(L-lactide) microspheres: (a) cumulative percent released; (b) daily release from microspheres prepared with polymer from Polysciences (o); Birmingham Polymers (Δ) as a function of time.

of protein that occurs during dialysis and reconstitution.

SDS-PAGE was also applied to the release studies of copolymer microspheres containing FPL-K and EcoBac^R-Plus (Figures 8b & c). These two vaccines contained BSA, hence, the BSA band in the SDS-PAGE gel could be regarded as a marker to establish overall release of the vaccine. A similar burst effect was observed in the SDS-PAGE gel during the first three days of the release study on FPL-K (Figure 8b), while no burst effect was detected for the microspheres containing EcoBac^R-Plus (Figure 8c). The reason for this difference might be due to the low concentration of BSA contained in the EcoBac^R-Plus vaccine. The SDS-PAGE results (Figures 8b & c) suggested that release of vaccine from poly(DL-lactide: glycolide, 85:15) microspheres could be sustained for up to 33 days.

CONCLUSIONS

Degradation profiles of poly(ester) microspheres prepared by spray drying showed a lag period during which the residual mass remained con-

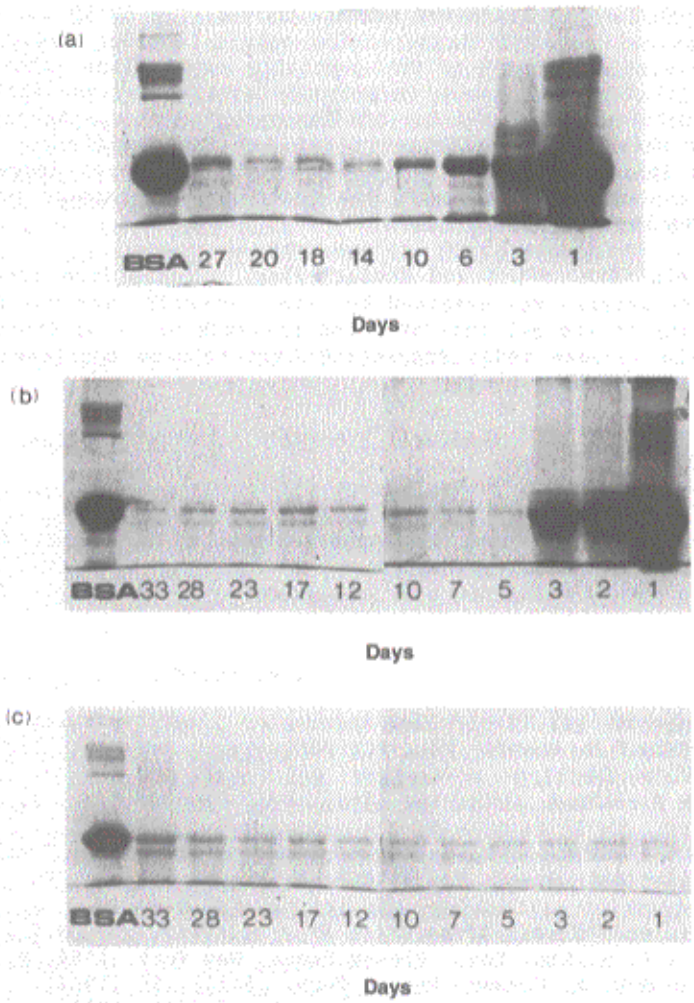

Figure 8. SDS-PAGE analysis of protein released from formulated microspheres: (a) BSA-poly(DL-lactide:glycolide, 50:50), (Dupont) (b) FPL-K-poly(DL-lactide: glycolide, 85:15), (Dupont) (c) EcoBac®-Plus-poly-(DL-lactide:glycolide, 85:15), (Dupont) microspheres are shown as a function of time.

stant. The length of this period was a function of monomer structure and copolymer ratio, ranging from 10 to 30 days for 50:50 and 85:15 copolymers to 6-10 months for poly(L-lactide). The decrease in polymer intrinsic viscosity and molecular weight during this period suggests that the hydrolysis of microspheres proceeds by random-chain scission.

The release profiles of BSA from microspheres prepared from poly(DL-lactide:glycolide, 50:50) having molecular weights, 31,000, 42,000, 74,000 and 93,000, were identical. This indicated that variation in molecular weight of polymers between 31,000 and 93,000 would not change the release characteristics of BSA loaded polymeric microspheres.

The monomer structure, copolymer ratio and BSA loading of the poly-

meric microspheres gave different release curves. The 50:50 and the 85:15 copolymers showed burst effects, while poly(L-lactide) did not. This difference could be related to the partially crystalline nature of the poly(L-lactide). The difference in poly(DL-lactide-co-glycolide) copolymer ratio (50:50 versus 85:15) for BSA incorporated microspheres resulted in different rates of degradation in PBS and hence different rates of release. The increase in BSA loading of the microspheres increased the volume fraction of BSA, decreasing the protective entrapment of BSA and increasing the rate of BSA release.

The protein (BSA, FPL-K and EcoBac^R-Plus) release from incorporated polymeric microspheres was examined by using SDS-PAGE. The BSA band (released protein) in the gel indicated the presence of >1 µg quantities of protein in the release media suggesting that release continued to take place over a one month period.

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