Degradability and Antigen-Release Characteristics of Polyester Microspheres Prepared from Polymer Blends

HONGKEE SAH* and YIE W. CHIEN

Controlled Drug-Delivery Research Center, Livingston Campus, Rutgers—The State University of New Jersey, 41 Gordon Road, Suite D, Piscataway, New Jersey 08854

SYNOPSIS

Biodegradable microspheres were prepared to function as a depot system for the controlled release of a model protein antigen bovine serum albumin (BSA). As an approach to achieve its controlled release, microspheres were fabricated blending a high-molecular-weight poly-*d,l*-lactide-*co*-glycolide (M_w = 61,000) with a low-molecular-weight poly-*d,l*-lactic acid (M_w = 2000, PLA2000). The effects of PLA2000 on microsphere degradability and release characteristics of BSA from microspheres were investigated. On the basis of the pH change in microsphere suspensions and water uptake of microspheres, the kinetics of microsphere degradation was derived to describe the rate of formation of hydrogen ions due to hydrolysis of ester linkages of polymers. It substantiated that PLA2000-containing microspheres were subject to controlled degradation that was necessary to achieve continuous release profiles of the antigen. Immunization of rabbits by subcutaneous injection of BSA-containing microspheres enhanced the antigenicity of BSA, and significantly increased the duration of humoral immune responses. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Polylactide and its copolymer with glycolide have been widely used for the sustained release of antigens. The controlled release technology has been considered to increase the immunogenicity of antigens and to reduce the number of immunizations required to induce high titer of immunoglobulin responses. However, it is difficult to obtain a constant release rate of antigens from the polymeric devices. In most cases, release patterns of antigens from polymeric devices have been found to be multiphasic: an initial burst release from the surface of microspheres, followed by a period of slower release, and a rapid increase in the release rate. These release characteristics are associated with microencapsulation techniques and bulk degradation of polyesters. Taking advantage of such characteristics, several studies have reported how to evoke immunity by means of a pulsed-release kinetics of antigens from polymeric devices to mimic necessary booster injections.[1-4]

The degradation of lactic/glycolic acid polymer systems follows a bulk erosion process. As the homogeneous degradation pattern changes all properties of the polymer phase, it is hard to achieve a linear rate of drug release. In addition, when the amount of protein loaded in microspheres is less than the saturation level in the polymer phase, it is very difficult to achieve a constant release rate only by a driving force of concentration gradient. However, a careful design of microspheres, based on precise control of polymer degradation, can overcome a release rate that decreases with time. An increase in the polymer permeability due to increasing porosity could compensate for the effect of a decrease in concentration gradient. Such a precise balance should be essential in obtaining a constant release profile of antigens. One attempt to control the degradability of a polymeric device is blending polymers with different physicochemical properties.[5-9] For example, Bodmeier et al.[5] and Maudit et al.[6] added low-molecular-weight polymers into high-molecular-weight polymers to modify release profiles of small-molecular-weight compounds such as quin-

* To whom correspondence should be addressed at P.O. Box 4, WP 78-110, Merck & Co., Inc., West Point, PA 19486.

Journal of Applied Polymer Science, Vol. 58, 197-206 (1995)
 CCC 0021-8995/95/010197-10

idine and gentamycin. However, the role of added low-molecular-weight polymers was not investigated in detail. They speculated that the polymers might increase plasticity of polymeric dosage forms and leach out to the bulk during release study, thereby affecting release profiles of drugs. No such blending technique has been attempted for achieving the controlled release of proteins from poly(lactide-*co*-glycolide) microparticles.

In this study a low-molecular-weight polymer PLA2000, which can degrade much faster than a high-molecular-weight PLCG75 : 25, was mixed with PLCG75 : 25 to fabricate microspheres. We report here on our preliminary work on the characterization of PLA2000/PLCG75 : 25 microsphere degradability. These microspheres undergoing degradation in a controlled manner were found to display a continuous release of antigen *in vitro* and to enhance its immunogenicity in an animal model. Bovine serum albumin (BSA, M_w = 68,000) has been used as a model antigen throughout this study.

EXPERIMENTAL

Materials

A high-molecular-weight poly-*d*,*l*-lactide-*co*-glycolide with a lactide : glycolide 75 : 25 (M_w/M_n = 61,000/38,000; intrinsic viscosity = 0.48 dL/g in chloroform at 25°C) was purchased from Birmingham Polymers Inc. (Birmingham, AL). Henceforth, this polymer will be referred to as PLCG75 : 25. A low-molecular-weight poly-*d*,*l*-lactide (PLA2000, M_w = 2000) was purchased from Boehringer Ingelheim (Ingelheim, Germany) whereas polyvinyl alcohol (PVA, 88% hydrolyzed, M_w = 25,000) and carboxymethylcellulose sodium were obtained from Polysciences Inc. (Warrington, PA). Bovine serum albumin, gelatin, antirabbit IgG conjugated to alkaline phosphatase (antibody developed in goat), and *p*-nitrophenyl phosphate (pNPP) were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) solutions were obtained from Pierce (Rockford, IL).

Preparation of Microspheres

A modified water-in-oil-in-water technique has been used to prepare microspheres. BSA-containing water (15 mg in 0.3 mL) was mixed with polymeric solution (0.6–0.7 g of polymers dissolved in 7 mL of methylene chloride). The mixture was homogenized for 1.5 min using a Polytron homogenizer (Kinematica GmbH, Switzerland) to produce a primary water-in-oil (w/o) emulsion. The emulsion was then poured into 300 mL of 4% aqueous PVA solution to make a w/o/w emulsion, which was stirred for 30 min using a plate stirrer (400 HPS, VWR Scientific). An additional 700 mL of water was added to the emulsion over a period of 30 min using a peristaltic pump. The microspheres were collected by filtration and dried under vacuum to a constant weight.

Gel Permeation Chromatography (GPC)

The molecular weight of PLCG75 : 25 was determined by gel permeation chromatography coupled with a HPLC1050 pump and refractive index detector. PL-gel 5 μ mixed-C was used as the stationary phase, while chloroform was used as the mobile phase (flow rate = 1 mL/min). The tests were performed at 35°C. An aliquot (5 mg) of polymer samples was dissolved in 2 mL of chloroform and filtered before injection. Weight-average molecular weights were calculated using polystyrene standards.

Electron Microscopy

A scanning electron microscope (Amray 1400, Amray Inc.) was used to evaluate the surface characteristics of microspheres. The microsphere's internal structure was also revealed by embedding them into an epoxy resin and cross-sectioning. The microsphere samples were put on aluminum mount and coated with gold palladium under argon atmosphere for 60 s before examining under SEM.

In Vitro BSA Release Study

Microspheres (65 mg) were suspended in 4 mL of isotonic phosphate-buffered saline (PBS, pH 7.4; 120 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer) containing polyvinyl alcohol (0.02%) and sodium azide (0.02%). The vials containing microsphere suspensions were kept at 37°C in a shaking water bath (Model 129, Fisher Scientific). At predetermined intervals, microsphere suspensions were centrifuged at 2000 rpm using a CU-5000 Centrifuge. Aliquots (0.5 mL) of the supernatant were withdrawn and the suspensions were replaced with fresh PBS. The BSA released from microspheres was quantitated using a modified BCA protein assay.[10] In addition, the pH change in microsphere suspensions was continuously monitored using a microelectrode (MI4154, Microelectrodes Inc.).

BCA Protein Assay Using a Microtiter Plate

Briefly, bicinchoninic acid (BCA) working reagent was prepared by combining 50 parts of BCA stock solution and 1 part of 4% $CuSO_4/5H_2O$. A set of BSA standards was prepared using the microsphere suspension vehicle used for *in vitro* release study, and the concentration ranged from 36 to 288 μg/mL. Forty microliters of each standard and unknown samples were pipetted into microtiter plate wells to which was added 200 μL of BCA working reagent using an 8-channel pipette. The microtiter plate was covered with a lid and incubated in a shaking water bath (Model 127, Fisher Scientific) at room temperature for 2 h. Absorbance of the water-soluble purple product was measured at 550 nm using a microplate reader (Model 450, Bio-Rad Lab).

Determination of Water Uptake

Microspheres (50 mg) were suspended in 4 mL of distilled water containing sodium azide (0.02%) at 37°C for varying lengths of time to measure the ability of microspheres to absorb water. They were then collected by filtration, weighed immediately and dried to a constant weight. The water uptake of microspheres was expressed as:

$$\text{Water uptake (\%)} = [100 \times (W_1 - W_2)]/W_2 \quad (1)$$

where W_1 and W_2 are weights of the fully hydrated and the dried microspheres, respectively.

Immunization of Rabbits

New Zealand White female rabbits (3.3–3.5 kg, Davidson Mill Farm, NJ) were injected subcutaneously with 5 mg of BSA either dissolved in 0.9% NaCl solution or encapsulated in microspheres. Before injection, microspheres were suspended in an aqueous solution containing sodium carboxymethylcellulose (0.8%) and Tween 20 (0.2%). Blood samples were collected from ear marginal vein and sera were prepared for serological assay.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was carried out to assess humoral immunoglobulin responses of rabbits. Polystyrene microtiter plates were coated with BSA in carbonate–bicarbonate buffer (0.05M, pH 9.6) (30 μg/mL), and kept overnight at 4°C. Plates were washed with Tris-buffered saline (TBS) containing Tween 20 (0.05%). To avoid nonspecific antibody binding, 200 μL of 1% gelatin in TBS was added to each well. Plates were incubated at 37°C for 1 h and washed using TBS. Serial twofold dilutions of sera were prepared in TBS. One hundred microliters of sera samples were loaded into each well of plates. After incubation at room temperature for 2 h, they were washed and 100 μL of antirabbit IgG alkaline phosphatase conjugate (diluted to 1 : 1000 in TBS) was dispensed into each well. The microplates were incubated at room temperature for 2 h. After extensive washings, 200 μL of pNPP substrate solution (Sigma Fast®) was added to each well and incubated again at room temperature for 15 min. The absorbance of the colored enzymatic product was measured at 405 nm using a microplate reader (Model 450, Bio-Rad Lab).

RESULTS AND DISCUSSION

Microspheres prepared in this study had a smooth and texture-free external surface with spherical geometry, whereas their internal structure was designed to possess multiple cavities by adjusting the shear force applied to produce a primary water-in-oil emulsion and the volume ratio of protein-containing water to polymeric solution. Microspheres with different formulations had diameters in the range of 10–100 μm.

Microspheres prepared from 0.6–0.7 g of PLCG75 : 25 were subject to very little pH change, when they were dispersed in PBS (10 mM phosphate ions) for 23 days. This indicates that these microspheres, due to their insignificant degradation, do not release enough hydrogen ion to exceed buffer capacity of the release medium. As random hydrolytic chain scission of ester linkages of the polymer generates free carboxylic acid end groups, its extensive degradation will lead to a decrease in the pH of microsphere suspensions. BSA was also unreleasable from these stable microspheres, suggesting the protein cannot diffuse efficiently through the intact membrane of PLCG75 : 25.

In this study a low-molecular-weight polymer PLA2000 was mixed with PLCG75 : 25 to fabricate microspheres. The faster degradation of PLA2000 is expected to increase the porosity of the polymeric matrix, enhancing the permeability of microspheres. The effect of PLA2000 on the degradation of microspheres is demonstrated by pH changes in microsphere suspensions during release study [Fig. 1(a)]. If the ratio of PLA2000 to PLCG75 : 25 increases, the corresponding microsphere suspension shows a much faster pH change due to degradable

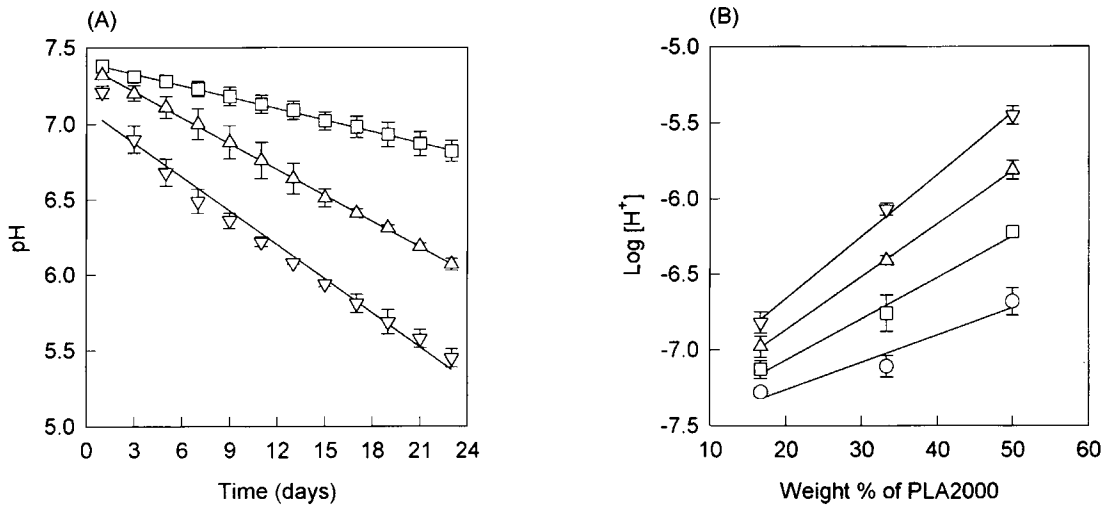

Figure 1 (a) Effect of PLA2000 on pH profiles of microsphere suspensions. Microspheres were prepared from PLCG75 : 25/PLA2000 (g/g): (□) 0.5/0.1; (△) 0.4/0.2; (▽) 0.3/0.3. (b) Relationship between log[H^+] in the bulk and weight percentage of PLA2000 in microspheres. The pH of microsphere suspensions was measured after (○) 5, (□) 11, (△) 17, and (▽) 23 days.

ester linkages in abundance. A linear relationship exists between log [H^+] in the bulk medium and the weight percentage of PLA2000 in the microspheres [Fig. 1(b)]. These results clearly demonstrate that the inclusion of PLA2000 into microspheres makes a contribution to their degradation.

Preliminary experiments indicated that buffer capacity of PBS (10 mM phosphate buffer containing 127 mM NaCl and 2.7 mM KCl) used in our experiments was weak and therefore microsphere degradation could be monitored using pH as a marker. For example, in a control experiment 28 mg of PLA2000 raw polymer was suspended in 4 mL of the same buffer system; this is equivalent to the amount contained in 65 mg of PLCG75 : 25/PLA2000 (0.4 g/0.3 g ratio). In 3-day incubation the buffer pH decreased by 1.23 (±0.005) unit, and its pH declined further as increasing incubation time. The mass of PLA2000 recovered in 3 days was 93.4%. This suggests that H^+ is generated from the ionization of terminal carboxylic acid end groups of intact PLA2000 as well as those of degraded fragments. It has been found that 5.18 mM of [H^+], or 0.0207 mmol of H^+, can decrease buffer pH by 1 unit.

Precision of the measurement of water uptake by microspheres was determined using six replicates of PLCG75 : 25/PLA2000 (0.5/0.1, g/g) microspheres incubated for 1 day in distilled water. According to eq. (1), the mean water uptake (±SD) was determined to be 60.7% (±3.3%), and the relative standard deviation (RSD) was 5.4%. The method precision seems to be acceptable. Considering that the microspheres were prepared from total 0.6 g of polymers and 0.3 g of water, the theoretical water uptake of microspheres should be at least 50% if they become fully hydrated. The experimental value obtained suggests that water is freely permeable to intact membranes of microspheres. This degree of water uptake may account for the amount of water needed to fill cavities or micropores inside microspheres and also to form a hydrodynamic diffusion layer around microspheres.

The water uptake of microspheres with different polymer formulations was then compared, after suspending them in distilled water containing sodium azide (0.02%). Prominent differences were noticed as seen in Figure 2. In the initial period (3 days), the microspheres prepared from PLCG75 : 25 alone showed the water uptake of 54.3% (±9.5%). The PLCG75 : 25 only microspheres did not exhibit a noticeable increase in the degree of water uptake after 3 days; the water content of the microspheres incubated for 24 days was determined to be 61.6% (±2.3%). This suggests that the microspheres maintained the same morphology and did not significantly degrade over the period. In contrast, as seen in Figure 2, the inclusion of PLA2000 into PLCG75 : 25 microspheres significantly enhanced the degree of water uptake. The increase in hydration might have been caused by additional holes or pores formed by the extensive degradation of mi-

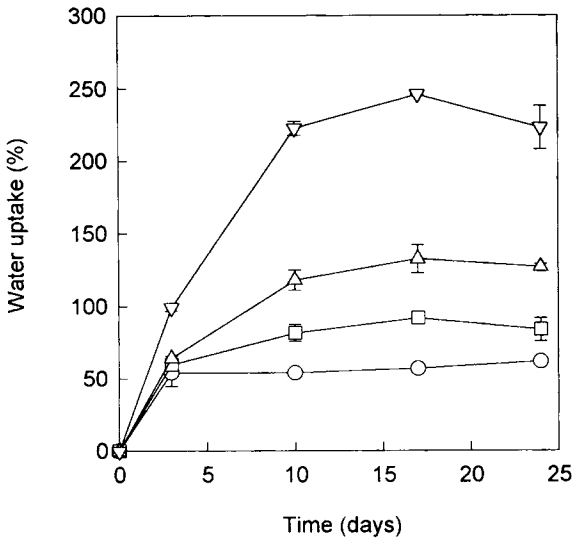

Figure 2 Water uptake of microspheres prepared from different polymer compositions. Ratio of PLCG75 : 25/PLA2000 (g/g): (○) 0.7/0.0; (□) 0.6/0.1; (△) 0.5/0.2; (▽) 0.4/0.3.

crospheres as well as the increasing amount of polar carboxylic acid end groups emerging from degraded fragments of PLA2000 and PLCG75 : 25. The data in Figure 3 indicate that except for those obtained on day 3, percentage water uptake by microspheres increases exponentially with increasing the weight percentage of PLA2000 in microspheres. The contribution of polymer degradation to water uptake has been previously reported.[11] Poly-*d,l*-lactide-*co*-glycolide 67 : 33 cast films displayed a high water uptake with levels up to 74% over 9 days; at that time its M_w decreased from 66,500 to 49,100, with exhibition of drastic changes in their physical properties. Their same studies showed only 15% or less water uptake in McIlvaine buffer (pH 3.2 ~ 6.6). However, the effect of buffer medium on the water uptake is very different from that reported by Shah et al.[12]; the water content of polylactide-*co*-glycolide 50 : 50 film (M_w = 18,000) in deionized water increased to 23% over 15 days, whereas those in phosphate-buffered saline (pH 7.4) increased to more than 40%. On the basis of these results, it can be concluded that characteristics of polymeric dosage forms and types of incubation media as well as the rate of polymer degradation are related to the water uptake.

GPC experiments also demonstrated that the inclusion of PLA2000 accelerated the degradation of PLCG75 : 25. After being kept at 37°C in distilled water for 12 days, the weight-average molecular weight of PLCG75 : 25 microspheres was slightly changed from 61,000 to 56,000. This substantiates the stability of PLCG75 : 25 only microspheres over that period. On the contrary, PLCG75 : 25 molecular weight drastically decreased when it was blended with PLA2000 to fabricate microspheres. For example, in 12-day incubation its molecular weight decreased to 42,500, 26,200, and 19,500 in the presence of 14.3, 28.6, and 42.9% of PLA2000, respectively. To further study the role of PLA2000 on microsphere degradation, a scanning electron microscope was used. SEM photographs of internal structures of PLCG75 : 25 only or PLCG75 : 25/PLA2000 microspheres were obtained after incubating them in PBS at 37°C for 21 days (Fig. 4). It illustrates in detail the presence of micropores and channels produced by degradation of PLA2000 in the microspheres. Those were not observed in PLCG75 : 25 only microspheres.

Degradation of lactic/glycolic acid polymers occurs by hydrolytic scission of polyester linkages, producing one terminal carboxylic acid group per polymer fragment. The normal kinetic equation governing the hydrolysis of polyester was derived to predict the appearance of terminal carboxylic acid end groups.[13-15] When hydrolytic cleavage of an aliphatic polyester is autocatalyzed by the generated carboxylic acid groups, the rate of appearance of carboxylic acid end groups is described by eq. (2):

$$d[\text{COOH}]/dt = K[\text{H}_2\text{O}][\text{Ester}][\text{COOH}] \quad (2)$$

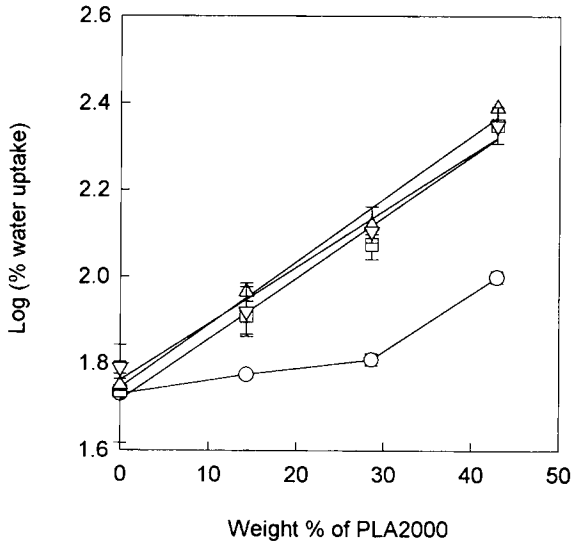

Figure 3 Linear relationship between weight percentage of PLA2000 and log (percentage water uptake of microspheres). Prior to the water uptake experiment, microspheres containing 14.3, 28.6, and 42.9% of PLA2000 were incubated in distilled water for (○) 3, (□) 10, (△) 17, and (▽) 24 days.

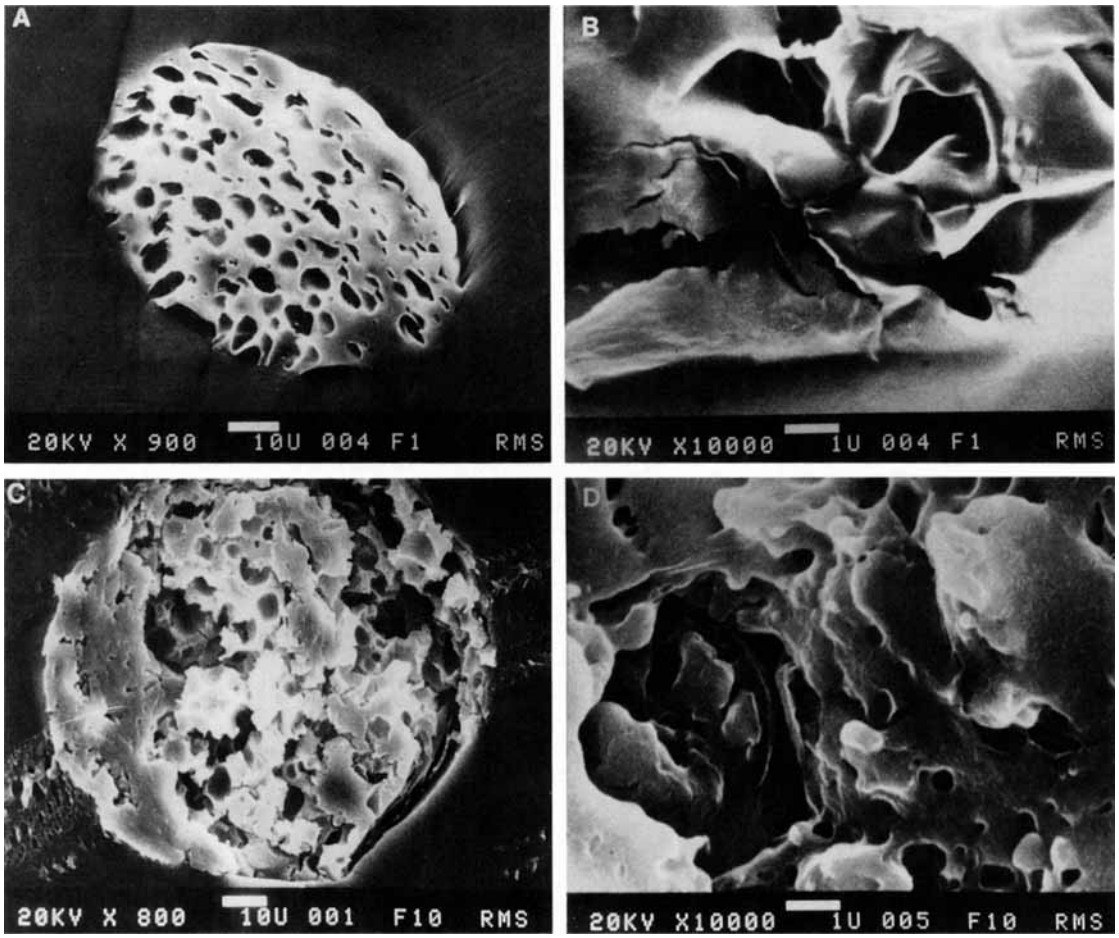

Figure 4 SEM photograph of the internal structure of microspheres that were incubated at 37°C for 21 days. (a) is observed with PLCG75 : 25 (0.7 g) only microspheres and (c) with microspheres prepared from 0.5 g of PLCG75 : 25 and 0.2 g of PLA2000. The size of the bar is 10 μm. (b) and (d) are a higher magnification of (a) and (c), respectively. The size of the bar is 1 μm.

where [Ester] and [RCOOH] are concentrations of ester and carboxylic acid end groups. Equation (2) assumes that terminal carboxylic acid end groups in hydrophobic polymer are not ionized. However, the water uptake of microspheres and the pH change in their suspensions indicate that terminal carboxyl end groups present in microspheres are ionized by water permeating through microsphere membranes. In addition, the pKa of carboxylic groups in poly-*d,l*-lactic acid is very likely to be close to that of lactoyl lactic acid (pKa = 3.0). Therefore, a different kinetic treatment can be applied if two assumptions are followed: (a) the appearing terminal carboxyl end groups are quickly ionized under neutral to weakly acidic conditions; and (b) catalytic effects of buffer ions are negligible. Then, the rate of formation of terminal carboxylic acid end groups, due to hydrolysis of ester linkages, can be written as:

$$d[\mathrm{RCOO^-}]/dt = k_1[\mathrm{Ester}][\mathrm{RCOO^-}] + k_2[\mathrm{Ester}][\mathrm{H^+}] + k_3[\mathrm{Ester}][\mathrm{OH^-}] \quad (3)$$

where [Ester], [$RCOO^-$], [H^+], and [OH^-] stand for the concentrations of ester in microspheres, ionized carboxylic acid end groups in microspheres, hydrogen and hydroxide ions, respectively. If microsphere suspensions are maintained at pH below 7.4, the catalytic effect of hydroxide ion is negligible. As the concentration of ester in microspheres can be considered constant prior to significant weight loss of polymer, eq. (3) can be approximated by:

$$d[\mathrm{RCOO^-}]/dt = k_4[\mathrm{RCOO^-}] + k_5[\mathrm{H^+}] \quad (4)$$

where k_4 and k_5 are k_1[Ester] and k_2[Ester], respectively. Unless microsphere suspensions become very

acidic, $[H^+]$ is equal to $[RCOO^-]$ since most terminal carboxyl end groups ionize above pH 4.0. Then, eq. (4) can be represented as:

$$d[RCOO^-]/dt = k_4[RCOO^-] + k_5[RCOO^-] \quad (5)$$

$$\log[RCOO^-]_t - \log[RCOO^-]_0 = (k_4 + k_5)t/2.303 \quad (6)$$

If $[RCOO^-]_0$ and $[RCOO^-]_t$ are replaced by $K_{d,0}[RCOOH]_0/[H^+]_0$ and $K_{d,t}[RCOOH]_t/[H^+]_t$, eq. (6) appears as:

$$pH_t - pH_0 = \frac{k_4 + k_5}{2.303} t + \log \frac{K_{d,0}[COOH]_0}{K_{d,t}[COOH]_t} = k_{obs}t + C \quad (7)$$

where $[RCOO^-]_{t,0}$ stands for concentrations of $RCOO^-$ at times t and 0; $K_{d,0}$ and $K_{d,t}$ are average dissociation constants at times 0 and t; $pH_{t,0}$ are pH at times t and 0; and C is a constant. The last term in eq. (7) can be eliminated, provided that $K_{d,0}$ and $K_{d,t}$ are equal and that carboxyl end groups are ionized as soon as they appear. This consideration is supported by the fact that the final equation is the same as the one derived by direct substitution of $[RCOO^-]$ for $[H^+]$ in eq. (4).

The pH of microsphere suspensions with different polymer compositions was monitored and plotted in Figure 5(a) in order to demonstrate a linear function of pH change with incubation time, as described by eq. (7). The observed rate constant (k_{obs}) of each microsphere suspension was obtained from the slope of regression line of the plot of (pH_t–pH_0) against incubation time. The k_{obs} was 0.0251 (±0.0023), 0.0571 (±0.0036), and 0.0751 (±0.0076) day^{-1}, when the respective PLCG75 : 25/PLA2000 ratio (g/g) of microspheres was 0.5/0.1, 0.4/0.2, and 0.3/0.3. These results emphasize the contribution of PLA2000 toward the rate constant and suggest that the hydrolytic rate of ester linkages of PLCG75 : 25/PLA2000 microspheres increases with increasing weight percentage of PLA2000 in microspheres [Fig. 5(b)]. It is also noteworthy that the rate of the formation of hydrogen ion, due to the appearance of a terminal carboxylic acid end group per polymer fragment, is constant without a sudden increase. This indicates that the degradation of PLA2000-containing microsphere takes place in a controlled manner. These constant rates of degradation may be of great advantage to achieving controlled release of drugs. In contrast, our long-term study demonstrated that PLCG75:25 only microspheres are subject to not a linear but a biphasic pH change as a function of time. This can be ascribed to hydrophobic nature of the microspheres that requires a long induction period before the onset of significant bulk erosion.

In vitro release study was carried out to investigate whether or not the modulation of microsphere degradability by blending PLCG75 : 25 with PLA2000 can provide a continuous release of BSA from microspheres. Microspheres loaded with BSA (2.4%, w/w) were prepared from total 0.6 g of polymers with different PLCG75 : 25/PLA2000 ratios. Figure 6(a) shows release profiles of BSA from microspheres with different polymer compositions. These data indicate the controlled degradation of the microspheres, being supported by a linear pH change of their suspension in Figure 6(b), is responsible for the continuous release of the protein. In addition to the ratio of PLCG75 : 25 to PLA2000, the total amount of polymers used is also associated with release patterns of BSA. When the amount of PLCG75 : 25 was decreased from 0.5 to 0.4 with a fixed amount of PLA2000 (0.1 g), the cumulative release of BSA was further accelerated and the release profile was very similar to that observed with PLCG75 : 25/PLA2000 (0.4/0.2, g/g) microspheres. However, microspheres prepared using less than 0.4 g of polymers displayed a significant burst effect. Our previous experiments focusing on the optimization of microsphere formulations showed that duration and release characteristics can be controlled by manipulating PLCG75 : 25/PLA2000 ratio, total amount of polymers used, the loading degree of protein, and how the primary water-in-oil emulsion is made.[16] These release patterns are encouraging because the release of antigen from (PLA2000/PLCG75 : 25)-based microspheres can be controllable and predictable. Discontinuous biphasic or triphasic release patterns with lag time have often been reported in delivering macromolecules from lactic/glycolic acid polymers.

A preliminary *in vivo* experiment also was carried out to investigate immune responses of rabbits that were vaccinated with a single subcutaneous injection of either BSA in saline solution or BSA entrapped in microspheres. When rabbit #1 was immunized by BSA in saline solution, humoral antibody response culminated at 2 weeks postinjection but rapidly declined thereafter. In contrast, the immune responses of rabbits #2 and #3 were significantly stimulated for prolonged periods when they were immunized by BSA-loaded microspheres prepared from 0.45 g of PLCG75 : 25 and 0.15 g of PLA2000 (Table I). Pulsatile release patterns of antigens from small

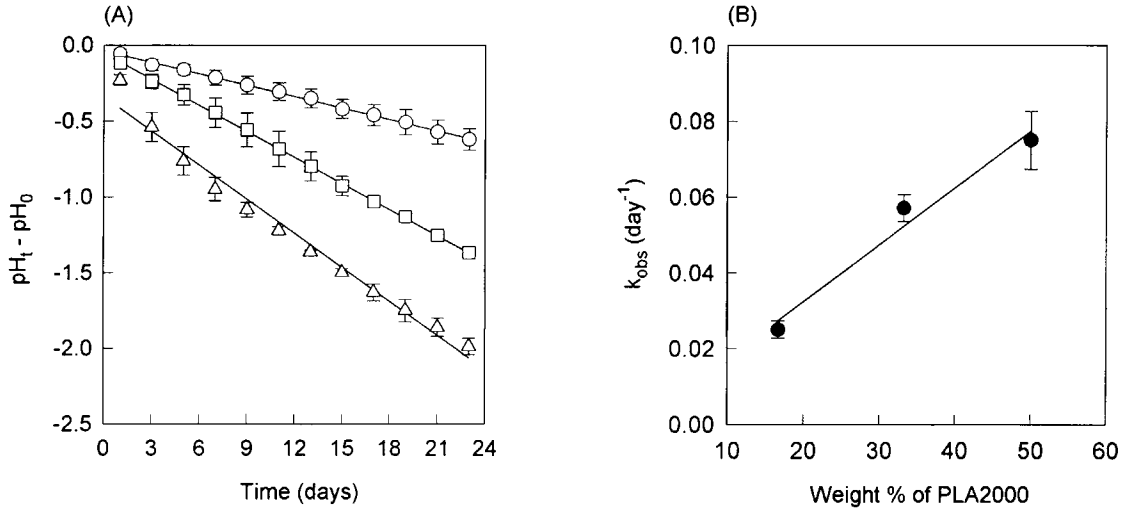

Figure 5 (a) Linear relationship between (pH_t–pH_0) and incubation time. Microspheres were prepared from the following PLCG75 : 25/PLA2000 (g/g) ratio: (○) 0.5/0.1; (□) 0.4/0.2; (△) 0.3/0.3. (b) The contribution of weight percentage of PLA2000 in microspheres to the observed rate constant k_{obs}. Hydrolytic rates of ester linkages available from microspheres increase with increasing weight percentage of PLA2000.

microparticles (1–10 μm) have been currently considered as the most effective regimen for their immunopotentiation since the release profiles mimic conventional vaccination programs including booster injections and small particles can be phagocytosed by immune accessory cells.[1,4,17] The preliminary results described in this study, however, demonstrate that microspheres providing *in vitro* continuous quasi-linear release of antigen can stimulate its immunogenicity to induce long-lived and strong immune responses, even though they (10–100 μm in diameter) remain at the site of injection without being phagocytosed by immune accessory cells such as macrophages. Further *in vivo* studies using mice revealed that the adjuvanticity of the microspheres was superior to that of aluminum hydroxide and blank PLCG75 : 25/PLA2000 microspheres did not possess adjuvanticity (manuscript in preparation). Therefore, these data support a hypothesis that repeated exposure of antigen and continuous stimu-

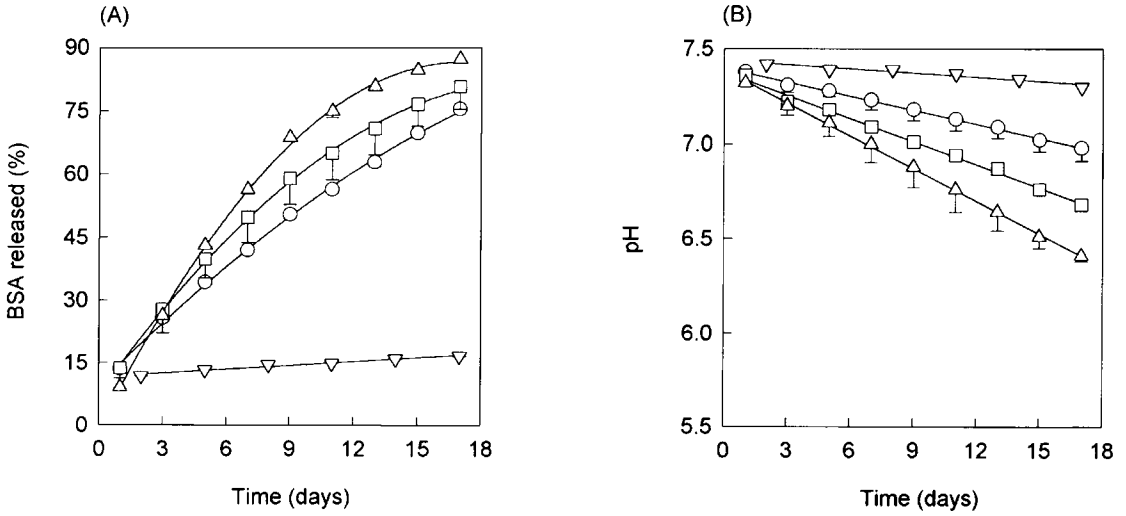

Figure 6 (a) Effect of PLA2000 on the release of BSA from microspheres. Ratio of PLCG75 : 25/PLA2000 (g/g): (▽) 0.6/0.0; (○) 0.5/0.1; (□) 0.45/0.15; (△) 0.4/0.2. (b) The pH change in the same microsphere suspensions.

Table I IgG Immune Responses of Rabbits Immunized by 5 mg of BSA Either in Saline Solution (#1) or Loaded in Microspheres (#2 and #3)

Postinjection (Week)	Rabbit (#)	Absorbance at Reciprocal Dilution of Sera (×100)[a]					
		4	8	16	32	64	128
2	1	1.20	0.53	0.22	0.08	0.03	0.00
	2	> 2.0	1.20	0.62	0.29	0.12	0.00
	3	> 2.0	1.93	0.99	0.49	0.18	0.09
4	1	0.56	0.26	0.11	0.03	0.02	0.00
	2	> 2.0	1.37	0.65	0.31	0.13	0.00
	3	> 2.0	> 2.0	1.17	0.59	0.30	0.13
6	1	0.32	0.10	0.01	0.00	0.00	0.00
	2	> 2.0	1.24	0.65	0.34	0.14	0.00
	3	> 2.0	> 2.0	> 2.0	1.28	0.86	0.40
8	1	0.01	0.00	0.00	0.00	0.00	0.00
	2	> 2.0	1.80	1.06	0.54	0.26	0.13
	3	> 2.0	> 2.0	> 2.0	1.82	1.09	0.59
10	1	0.03	0.00	0.00	0.00	0.00	0.00
	2	> 2.0	> 2.0	1.68	0.90	0.47	0.19
	3	> 2.0	> 2.0	> 2.0	> 2.0	1.86	0.92
12	1	0.00	0.00	0.00	0.00	0.00	0.00
	2	> 2.0	> 2.0	1.27	0.64	0.30	0.14
	3	> 2.0	> 2.0	> 2.0	> 2.0	1.26	0.60

[a] The levels of anti-BSA antibody in the blood are expressed as the absorbance at 405 nm.

lation of immune systems are very important with regard to immunostimulatory mechanisms as previously suggested by Kohn et al.[18] However, we cannot currently conclude whether immunogenicity of antigen is enhanced by its continuous release, since *in vivo* situations may differ from *in vitro* experimental conditions with respect to buffering capacity and cellular responses. We are further interested in pursuing effects of various antigen delivery profiles, differing in release rates of antigen and duration of antigen delivery, on immune responses.

CONCLUSIONS

The inclusion of PLA2000 into PLCG75 : 25 microspheres was a very effective way to adjust the microcapsule degradability and permeability to a model antigen BSA. The hydrolysis kinetics indicates that the degradation process of PLA2000/PLCG75 : 25 can be designed to take place in a controlled manner, and need not be a bulky and massive process. Such a system was found to provide continuous and controlled release of antigen, which can be best characterized by first-order kinetics. The microspheres enhanced the immunogenicity of BSA and induced high-titered and long-term immune responses in rabbits, suggesting its potential in devising a better vaccine formulation.

The authors wish to express their appreciation to the Johnson and Johnson Corporation for providing the fellowship to Mr. Hongkee Sah and to Parke-Davis Chair Endowment at Rutgers College of Pharmacy for research support.

REFERENCES

1. J. H. Eldridge, J. K. Staas, J. A. Meulbroek, T. R. Tice, and R. M. Gilley, *Infect. Immun.*, **59,** 2978 (1991).
2. B. Gander, C. Thomasin, H. P. Merkle, Y. Men, and G. Corradin, *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, **20,** 65 (1993).
3. A. M. Hazrati, D. H. Lewis, and G. P. Atkins, *Pharm. Res.*, **8,** 958 (1991).
4. D. T. O'Hagan, D. Rahman, J. P. McGee, H. Jeffery, M. C. Davis, P. Williams, S. S. Davis, and S. J. Challacombe, *Immunology,* **73,** 239 (1991).

5. R. Bodmeier, K. H. Oh, and H. Chen, *Int. J. Pharm.*, **51,** 1 (1989).
6. J. Maudit, N. Bukh, and M. Vert, *J. Control. Rel.*, **23,** 209 (1993).
7. K. Juni, J. Ogata, N. Matsui, M. Kubota, and M. Nakano, *Chem. Pharm. Bull.*, **33,** 1734 (1985).
8. C. G. Pitt, Y. Cha, S. S. Shah, and K. J. Zhu, *J. Control. Rel.*, **19,** 189 (1992).
9. F. G. Hutchinson, U. S. Pat. 5,004,602 (1991).
10. M. G. Redingbaugh, and R. B. Turley, *Anal. Biochem.*, **153,** 267 (1986).
11. A. Demirdere, T. Kissel, U. Siemann, and H. Sucker, *Eur. J. Pharm. Biopharm.*, **37,** 42 (1991).
12. S. S. Shah, Y. Cha, and C. G. Pitt, *J. Control. Rel.*, **18,** 261 (1992).
13. F. G. Hutchinson, and B. J. A. Furr, *J. Control. Rel.*, **13,** 279 (1990).
14. C. G. Pitt, F. I. Chaslow, Y. M. Hibionada, D. M. Klimas, and A. Schindler, *J. Appl. Poly. Sci.*, **26,** 3779 (1981).
15. C. G. Pitt, and Z. W. Gu, *J. Control. Rel.*, **4,** 283 (1987).
16. H. K. Sah, R. Toddywala, and Y. W. Chien, *J. Control. Rel.*, **30,** 201 (1994).
17. Y. Tabata, and Y. Ikada, *Biomaterials*, **9,** 356 (1988).
18. J. Kohn, S. M. Niemi, E. C. Albert, J. C. Murphy, R. Langer, and J. G. Fox, *J. Immunol. Methods*, **95,** 31 (1986).

Received January 12, 1995
Accepted April 8, 1995