Controlled Release of Proteins from Poly(L-lactic acid) Coated Polyisobutylcyanoacrylate Microcapsules

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SYNOPSIS

Poly(L-lactic acid)-coated polyisobutylcyanoacrylate microcapsules containing protein molecules were prepared by a single-step procedure based on either a double-emulsion–solvent evaporation method or a spray-drying method. First, an aqueous protein solution was emulsified in an organic phase of methylene chloride containing a wall-forming monomer (isobutylcyanoacrylate), various kinds of poly(L-lactic acid), and a surfactant. An immediate polymerization process of isobutylcyanoacrylate takes place at the W/O interface upon contact with hydroxide ion in the aqueous phase, leading to the formation of a polyisobutylcyanoacrylate wall around the aqueous droplets. This W/O emulsion was reemulsified in an aqueous solution to promote the solvent removal and, consequently, the precipitation of poly(L-lactic acid) onto polyisobutylcyanoacrylate microcapsules or was spray-dried to directly deposit the poly(L-lactic acid) on the wall. Three proteins, bovine serum albumin, horseradish peroxidase, and tetanus toxoid, were encapsulated in these poly(L-lactic acid)-coated polyisobutylcyanoacrylate microcapsules, and then their release profiles were examined *in vitro* as a function of molecular weight of poly(L-lactic acid) and its copolymers with glycolic acid. These formulations exhibited a low "burst" effect at initial incubation stages and released the proteins for extended periods of time. Subcutaneous injections of the tetanus toxoid-loaded microparticles into rats showed that the time course of immunization (antibody titer) can be controlled by the type of polymer matrices used.

INTRODUCTION

Recently, biodegradable polymers have been extensively utilized in drug-delivery systems to achieve long-term release of small and high molecular weight polypeptides.[1,2] Among them, poly(L-lactic acid) (PLA) and its copolymers with glycolic acid (PLGA) have been used for the microencapsulation of peptides[3] and proteins[4] due to their extremely low toxicity *in vivo* and due to the wide range of biodegradabilities and polymer processing techniques available. Several previous studies revealed the ability to efficiently control the release of small peptides from PLGA microspheres for up to 1 month.[2,3] Nevertheless, the possibility to control the release of proteins from PLGA matrices appears to be more difficult. In particular, a significant initial release (burst effect) followed by a slow release thereafter has been frequently reported.[5–7] This "burst" effect may be primarily caused by an imperfect entrapment of the protein molecules in the polymer matrices. For microencapsulated proteins, this may be explained by their tendency to migrate to the microsphere surface during the microencapsulation process. In addition, data reported previously suggest that the "burst effect" is affected by the microencapsulation procedure, being particularly acute for the spray-drying technique.[8–10] As the

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microspheres are placed in an aqueous environment, water penetrates into and hydrates the microspheres at the initial stage, which may not only remove protein molecules from the microsphere surface, but also generate interconnecting pores and channels within the microspheres. This problem is particularly critical for PLA and its copolymers because they degrade via bulk erosion. It has been postulated that through these aqueous porous pathways the hydrated protein molecules may be rapidly diffused out, leading to the "burst."[4]

In this article, we present a new formulation method to reduce the burst effect and to prolong the protein-release period. The new formulation consists of two biodegradable polymers, polyisobutylcyanoacrylate (PIBCA) and PLA or PLGA. Polyalkylcyanoacrylates have been used for a long time as a tissue adhesive material and recently as drug-delivery carriers for small molecular weight antibiotics[11] and polypeptides.[12,13] In Europe, polycyanoacrylate nanospheres have undergone human clinical trials in several places.[14] PIBCA was selected as a microcapsule-forming material around the protein molecules since it can be readily formed upon contact with an aqueous environment. The monomers (dissolved in the continuous phase of the organic solvent) diffuse into the dispersed phase of the aqueous solvent containing the initiator, hydroxide ions, leading to the polyalkylcyanoacrylate precipitation at the interface with microcapsule formation.[15]

PIBCA microcapsules that contain the protein molecules in their inner aqueous phase were prepared in an organic solvent where PLA or PLGA was dissolved, using a previously reported method.[15] PLA or PLGA were then coated on the wall of the PIBCA microcapsule by either spray drying or reemulsification in an aqueous phase. In this manner, the protein molecules could be securely encapsulated within the double coatings of the two biodegradable polymers. It is our hypothesis that the PLA coating of microcapsules that contain the protein molecules may reduce the burst effect, because the protein molecules could be encapsulated by the two biodegradable polymer layers. Different molecular weight (MW) PLA and PLGA were coated by the spray-drying technique. Three proteins, bovine serum albumin, horseradish peroxidase, and tetanus toxoid, were encapsulated within the various polymer-coating layers and their release profiles were examined *in vitro*. The tetanus toxoid-loaded microparticles were tested *in vivo*, using a rat model, for their ability to act as a controlled-release vaccine system.

MATERIALS AND METHODS

Materials

PLA of various MW (2,000, 50,000, 100,000) were purchased from Polysciences, Inc., Warrington, PA. PLGA 50 : 50 (50% of lactic acid) and 75 : 25 (75% of lactic acid) were obtained from Boehringer-Ingelheim, Ingelheim, Germany. Isobutylcyanoacrylate (IBCA) and poly(vinyl alcohol) (PVA) (88% hydrolyzed, MW 25,000) were from Polysciences. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC–BSA), horseradish peroxidase, and dextran (MW 80,000) were obtained from Sigma Co. Tetanus toxoid (Institut Pasteur Meriux, Serums and Vaccines, Paris, France) was provided by the World Health Organization (WHO). Pluronic L-101 surfactant was donated from BASF Co. Other chemicals were reagent grade.

Preparation of PLA-coated PIBCA Microcapsules

Fifty milligrams of protein dissolved in 1 mL of distilled water was first emulsified in 10 mL methylene chloride containing 1 g of PLA or its glycolic acid (GA) copolymers and 2.5% (w/v) Pluronic L-101 surfactant. IBCA (0.25 mL) monomer dissolved in 10 mL methylene chloride was then added in the above organic phase. The solution was sonicated for 20 s and then was stirred for 2 h at room temperature under a dry nitrogen atmosphere. During this period, IBCA monomers existing in the organic phase migrate in the W/O interface and were immediately polymerized upon contact with hydroxide ions available in the inner aqueous phase. Next, to coat the dissolved PLA existing in the outer organic phase onto the PIBCA microcapsule walls, two separate solvent-removal methods, reemulsification and spray drying, were used as follows: First, for the reemulsification method, the volume of the methylene chloride phase was reduced to about 2–3 mL using a rotary evaporator and then reemulsified in 200 mL of 0.5% dextran/0.1% PVA solution. The aqueous pH was adjusted to pH 3 or 7 with 0.1*N* HCl and/or NaOH. This W/O/W emulsion phase was maintained under stirring for 3 h to precipitate the PLA on the newly formed PIBCA microcapsule walls by extracting and evaporating methylene chloride into the aqueous phase. The hardened PLA-coated microcapsules were glass-filtered, extensively washed with deionized water, and then freeze-dried. Second, for the spray-drying method, 30 mL of methylene chloride was further added in the W/O

emulsion and spray-dried. The spray drying was carried out using a Buchi 190-Mini Spray Dryer. The operating conditions were as follows: nozzle diameter, 0.7 mm; air flow rate, 600 L/h, sample pumping speed set, 5; aspiration, −40 mbar; inlet temperature, 27°C; and outlet temperature, 22°C. Free-flowing powder was obtained in a collecting chamber, which was freeze-dried overnight under vacuum. Two other proteins, horseradish peroxidase and tetanus toxoid, were loaded using the above spray-drying procedure. The loading amounts for horseradish peroxidase and tetanus toxoid to 1 g polymer were 30 and 50 mg, respectively. Spray-drying yields were calculated based on the weight of particles carefully recovered in the wall of cyclone area and the collecting chamber. Microparticles stuck to the wall of a spray cylinder were not taken into account in calculating the spray-drying yield. All the microparticles were further dried extensively under vacuum to remove any residual solvent and water. A schematic description of preparing PLA coated PIBCA is shown in Figure 1.

Scanning Electron Microscopy (SEM)

Microparticles were coated with gold particles to a thickness of 200–500 Å and observed with an Amary 1000 A SEM at 20 kV electron-beam radiation. To observe PIBCA microcapsules with the SEM, a W/O emulsion without the PLA in the organic phase was directly cast onto the glass plate and then the solvent was evaporated.

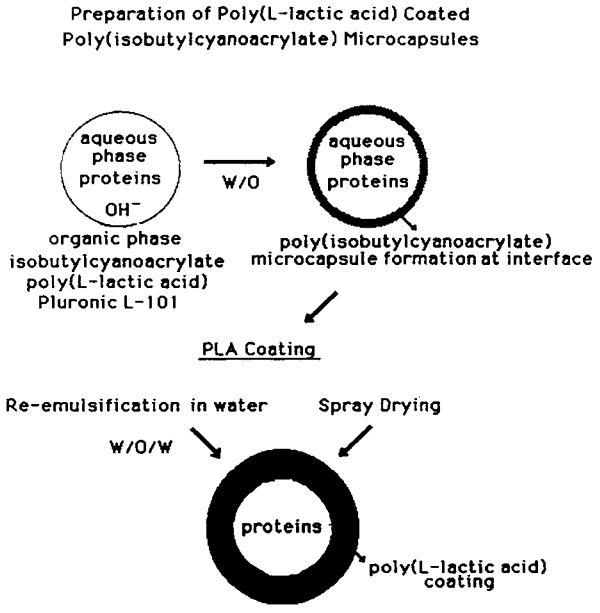

Figure 1 Schematic description of preparing PLA-coated PIBCA microcapsules that contain protein molecules.

Differential Scanning Calorimetry (DSC)

Using a Perkin-Elmer 7 Series DSC, 3–5 mg samples sealed in the aluminum pan were first heated to 200°C at 10°C/min and rapidly cooled down to −25°C and then reheated with a heating rate of 10°C/min. There were no apparent changes in the glass transition temperature and crystalline melting temperature between the first and second heating runs. Nitrogen was used as a sweeping gas.

In Vitro Release Studies

Because the size of particles obtained by the re-emulsification method was bigger (more than 100 μm) than that of particles prepared by the spray-drying method (2 μm), sample preparations for the release studies were performed in two ways: filtration and centrifugation. Particles (300 mg) obtained by the reemulsification method were added to 10 mL of PBS/0.05% (w/v) sodium azide solution, which were placed in a 10 mL disposable plastic column (Bio-Rad) having a plastic filter at the outlet end. At regular time intervals, the sample solution was withdrawn from the outlet through a syringe and then filtered with a syringe filter (Gelman 0.2 μm, low protein binding). The buffer solution was replaced at each time to maintain sink conditions. The amount of FITC–BSA released was calculated by measuring absorbance at 495 nm using a calibration curve. Particles (25 mg) prepared by the spray-drying method were placed in 1.5 mL of the buffer solution in an Eppendorf tube. At preset time intervals, the tube was centrifuged at 1000 g for 10 min, and the supernatant was filtered as described above for the determination of released BSA. The release medium was replaced at each time to maintain sink conditions. The released amounts of horseradish peroxidase and tetanus toxoid were determined by incubating 100 and 200 mg, respectively, of particles in 10 mL of PBS buffer. The supernatant after centrifugation at 3000 g for 10 min was assayed with the micro-BCA method.[16] All the samples were incubated in triplicate at 37°C with continuous orbital shaking. Fractional releases were calculated based on the theoretical protein loading. It was not possible to efficiently recover the protein molecules

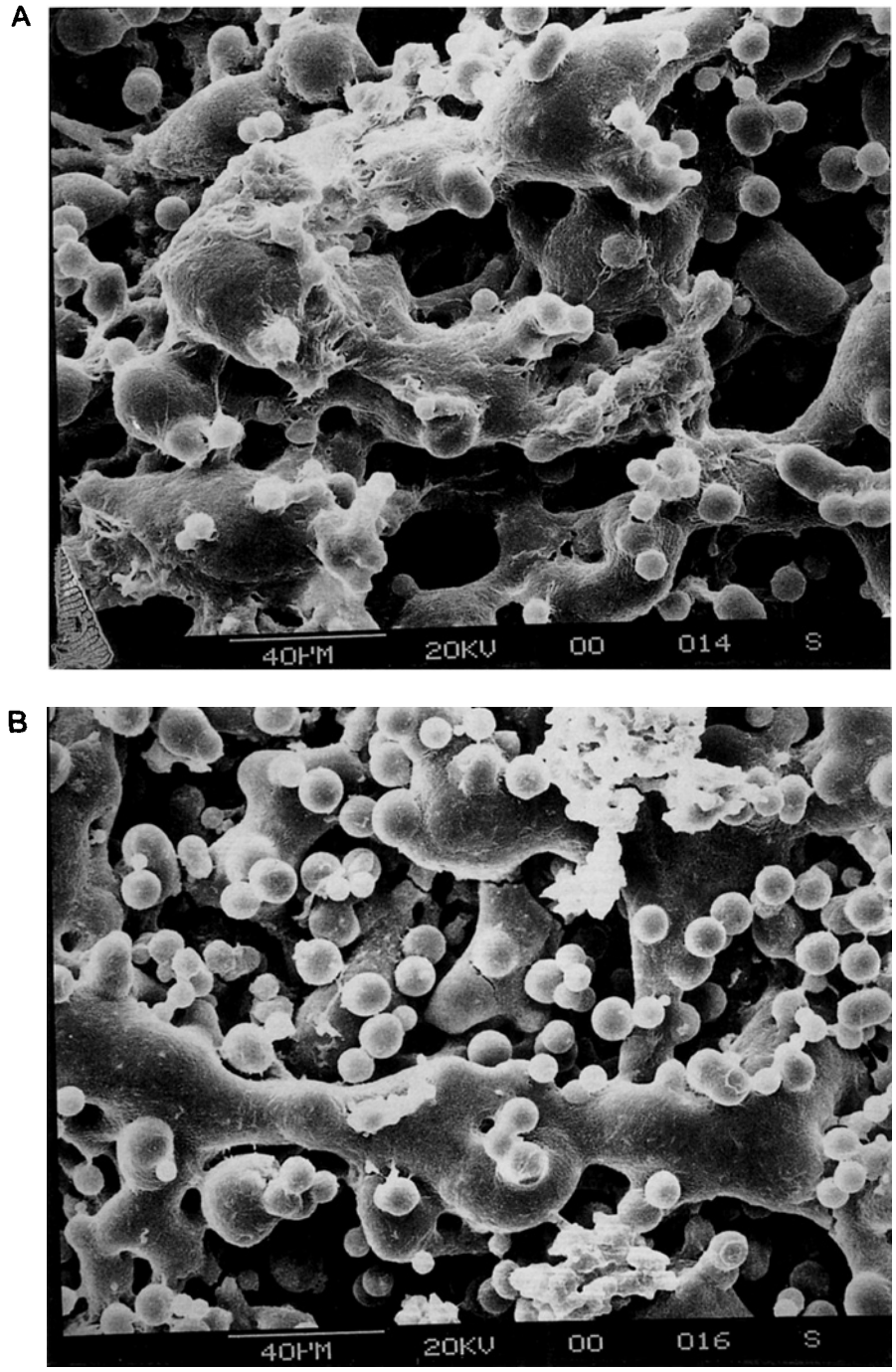

Figure 2 SEM picture of PIBCA microcapsules. The microcapsules dispersed in the methylene chloride phase were cast onto the glass plate and then the solvent was evaporated for the SEM observation: (a) day 1, (b) day 2; (c) magnified picture of (b).

entrapped in the polymer matrices into the aqueous phase by using the method of dissolving the polymer microspheres in an organic solvent and subsequent aqueous-phase extraction.

In Vivo Studies

Groups of three male Sprague Dawley rats (Charles River), 4–8 weeks and weighing 175–200 g, were used

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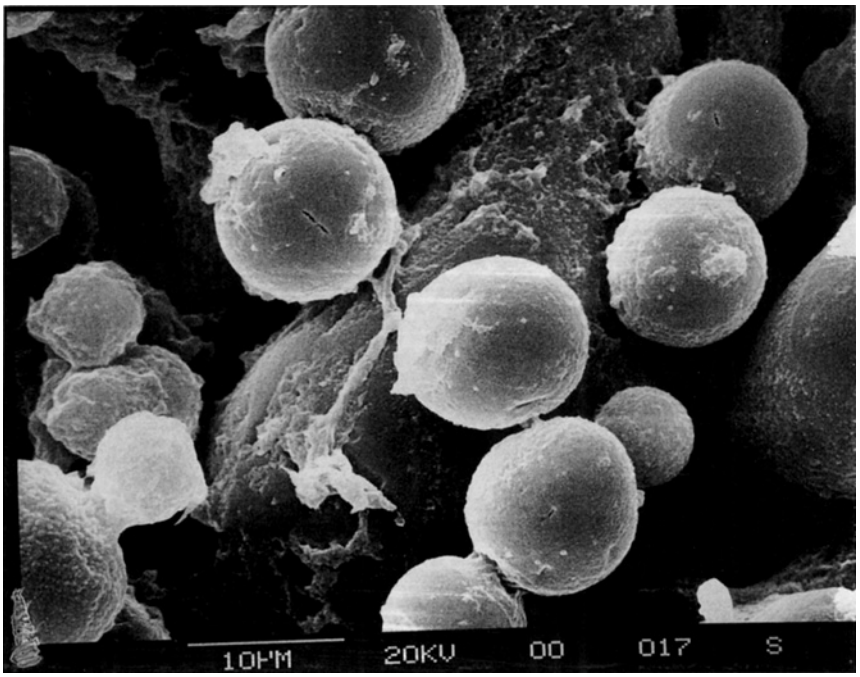

Figure 2 (*Continued from the previous page*)

in an *in vivo* immunization study. Each rat was subcutaneously injected at four different places with 12 mg of the tetanus toxoid microparticles that were suspended in 0.8 mL of a mixture of 1% (w/v) sodium carboxymethylcellulose, 0.2% Tween 80, 0.14% methyl-*p*-hydroxybenzoate, 0.014% propyl-*p*-hydroxybenzoate, and 5% sorbitol. Blood was withdrawn from the tail veins at regular time intervals, and then serum was separated and stored in a freezer until further bioassay. An enzyme-linked immunosorbent assay (ELISA) was used to determine the antitetanus toxoid titer in serum (Protocol provided by the WHO).

RESULTS AND DISCUSSION

There have been several critical problems in formulating protein-loaded PLA microspheres. On the one hand, the solvent-evaporation method often leads to the protein partitioning onto the polymer surface region due to the migrational tendency of trapped hydrophilic proteins toward the outer aqueous phase.[8] On the other hand, even though the spray-drying technique is a convenient method to prepare microspheres because of its easy scale-up possibility, microspheres prepared by this technique frequently have an imperfect and porous coating of the PLA around the protein powder due to the different interfacial energies between the protein and PLA.[9]

To minimize the initial burst protein release, it was hypothesized that once the protein molecules are first microencapsulated by a relatively hydrophobic polymeric wall it will be much easier for the PLA to be coated next onto that polymer surface because of similar solubility parameters between the two polymers. PIBCA has a solubility parameter of 11.6 $cal^{1/2}/cm^{3/2}$, whereas PLA and PLGA have solubility parameters of 11.1 and 12.4 $cal^{1/2}/cm^{3/2}$, respectively. These values were calculated based on a group contribution method.[17,18] It can be expected that this kind of formulation may exhibit a low burst with minimal protein diffusion and a prolonged protein release, which is hopefully controlled by the polymer wall biodegradation.

SEM photographs of PIBCA microcapsules that have an inner aqueous phase are shown in Figure 2. For the SEM observation, PIBCA microcapsules dispersed in the methylene chloride phase were directly cast onto the glass plate and then the solvent was evaporated. The fragile film obtained was incubated in PBS for 1 day [Fig. 2(A)] and 2 days [Fig. 2(B) and (C)]. The film disintegrates with time and the microcapsules emerged from the film. The average microcapsule diameter is around $\sim 10\ \mu m$.

Two different approaches of coating PIBCA mi-

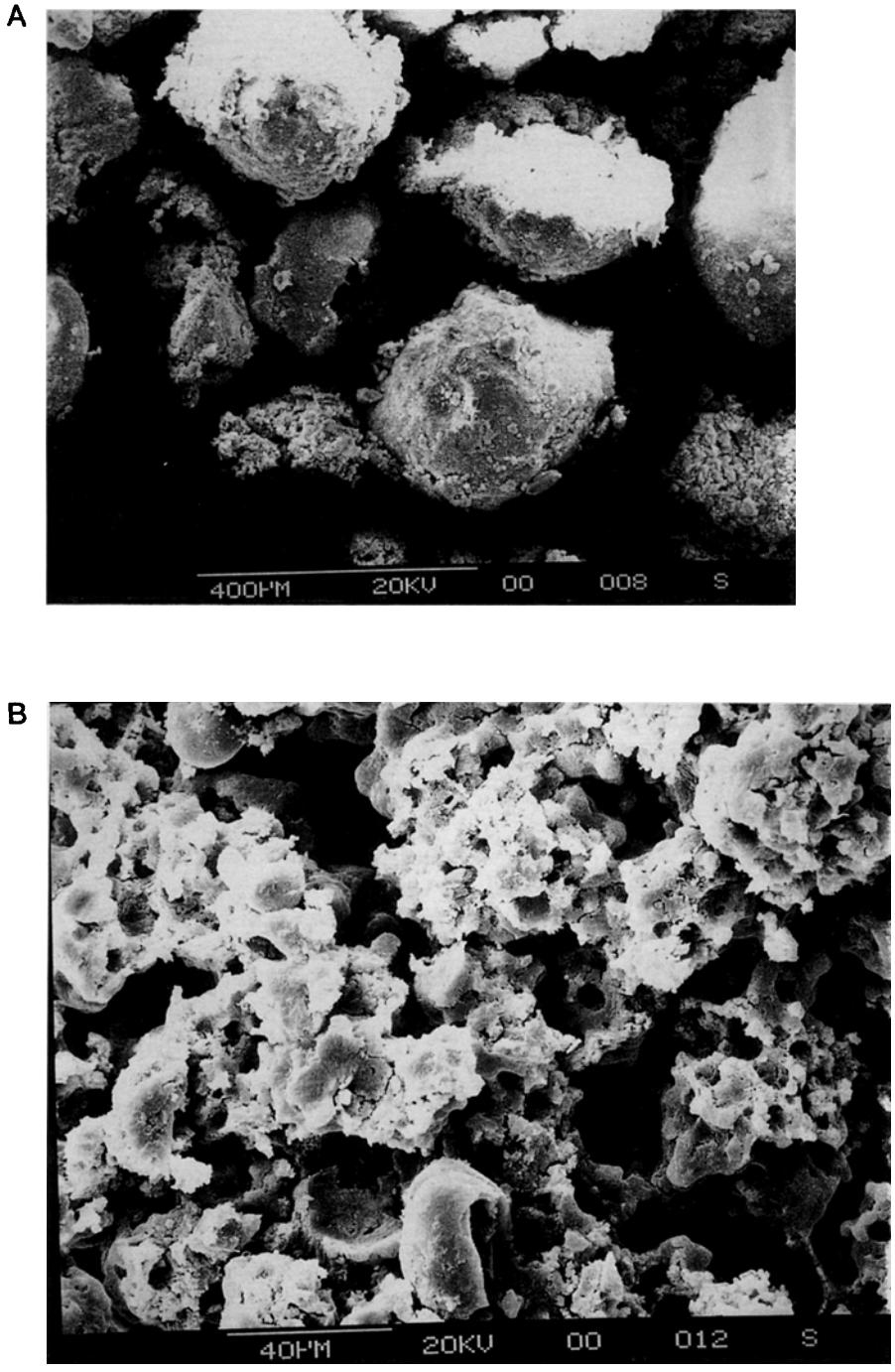

Figure 3 SEM pictures of PLA (MW 50,000)-coated PIBCA microcapsules prepared by the reemulsification method: (a) whole particles; (b) cross-sectional view of the particle.

crocapsules with PLA and PLGA were attempted: First, a double emulsion–solvent evaporation method, and, second, a spray-drying method with direct solvent removal. As illustrated in Figure 1, the reemulsification of the W/O emulsion in the aqueous phase induces PLA precipitation on the microcapsule wall by the slow removal of methylene chloride into the aqueous phase. Figure 3 shows SEM photographs of PLA-coated PIBCA microcapsules prepared by the reemulsification method. In Figure 3(A), it can be seen that relatively large and irregular-shaped particles ranging from 100 to

200 μm in diameter are obtained, suggesting that the PIBCA microcapsules were immobilized in a continuous PLA phase. A magnified view of a cross-sectional area in the particle [Fig. 3(B)] demonstrates the presence of PIBCA microcapsules embedded in the PLA matrix.

Figure 4 shows BSA release profiles from the microspheres prepared by the reemulsification method using two different aqueous external media. Since the polymerization rate of the IBCA monomer increases with increasing pH in the medium (high hydroxide ion concentration), it was thought that the thickness of the PIBCA wall and morphological structure of double-walled microspheres could be dependent on this parameter. To check this hypothesis, two reemulsification media (pH 3 and 7) were used, and the effect on the BSA release profiles from L-PLA-coated PIBCA microcapsules was investigated. *In vitro* release profiles shown in Figure 4 indicate that the fractional BSA released at both pH values exhibits near zero-order release behavior over a 100 day period regardless of the reemulsification pH values. Here, the fractional release is based on the theoretical loading amount of protein within the microspheres. The similar release profiles at both pH values suggest that the medium pH is not a major factor affecting the polymer morphology in the PLA-coating process. It is also of interest to see that there is a remarkably low initial "burst" compared to previous studies,[9,10] indicating that the PLA indeed apparently entraps the PIBCA microcapsules that contain the protein molecules. This kind of double-coating process should generate an isolated environment for the protein molecules, avoiding their location at the microsphere surface and therefore their immediate release. It should be mentioned that even though the BSA release lasted for a long period (100 days), there might be a portion of loaded BSA molecules that could not be released due to moisture-induced protein aggregation, protein adsorption to polymer, and possible covalent bond formation between protein and PIBCA.[13] Thus, it is expected that complete release of the loaded protein could not be achieved via further incubation.

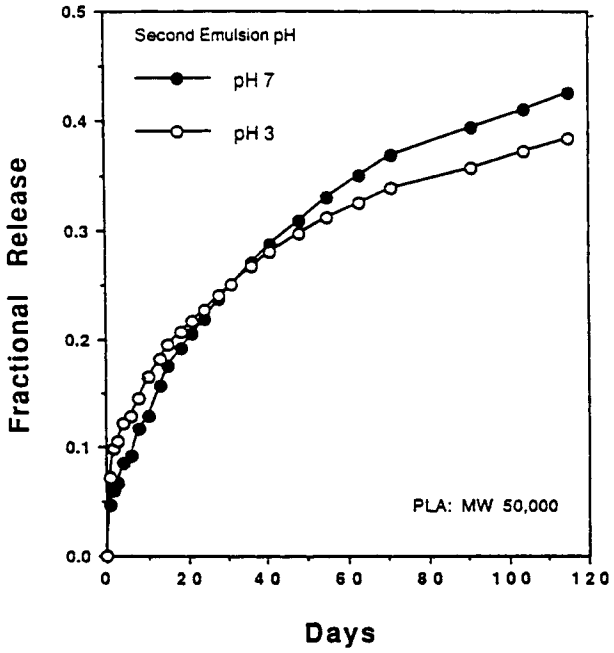

Figure 4 BSA-release profiles from the PLA MW (50,000)-coated PIBCA microcapsules prepared by the reemulsification method at two different pH values.

The microspheres prepared by spray drying generally were smaller and had more irregular shapes than those prepared by the solvent-evaporation method. The average particle size is about 2 μm, which is partly due to the fine particle formation of the polymer solution through the small orifice of an atomizer or needle by compressed air. Figure 5 shows a SEM photograph of spray-dried PLA-coated PIBCA microcapsules. It can be seen that the microspheres have a distorted and collapsed shape, but are not ruptured, and are 1–2 μm in diameter. This shrunken particle structure might be caused by the collapse of double-polymer-coated microcapsules during the spray-drying process. The polymeric wall encapsulating the aqueous protein phase may not sustain the mechanical stress in the high shear-generating spraying process. When the W/O emulsion in the absence of the PIBCA microcapsule wall was spray-dried, fused spherical particles of 1–2 μm in diameter were obtained. Thus, the collapsed microparticles seen in Figure 5 were clearly caused by the removal of the internal aqueous phase during spray drying as well as the subsequent vacuum-drying process.

To investigate the possibility of an interaction between both polymers, PLA and PIBCA, forming the microcapsules, DSC studies were carried out. Any detectable changes in the DSC thermograms are listed in Table I. PIBCA microcapsules displayed a T_g at −16.5°C. PLA and PLGA-coated PIBCA microcapsules exhibited a series of peaks, which can be assigned as glass transition (T_g), crystallization (T_c), and crystalline melting (T_m) temperatures. None of the coated microcapsules show any measurable thermal changes around the T_g of PIBCA. However, when we investigated the thermal behavior of PLA (molecular weight, 50,000) microspheres without PIBCA, we observed important changes in

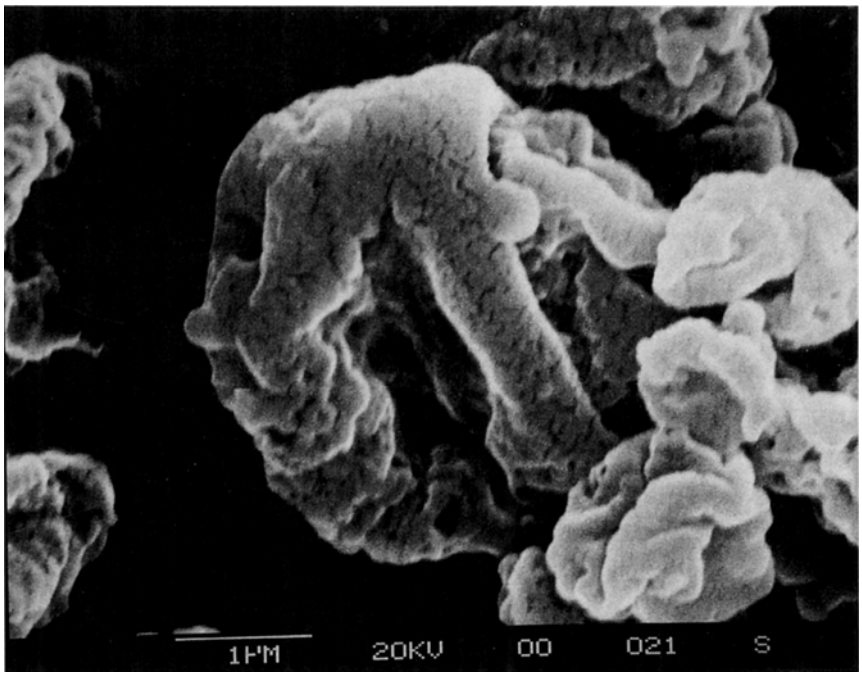

Figure 5 SEM picture of the spray-dried PLA-coated PIBCA microcapsules.

the thermal events. The T_g for these PLA microspheres was 60.3°C and the T_m was 173.8°C, whereas the corresponding values for PLA-coated PIBCA microcapsules were 36.1 and 168.4°C (Table I). The important change in the PLA's T_g caused by the presence of PIBCA may be interpreted as a result of an interaction between PLA and PIBCA, such as intermixing or interpenetration of the two polymers. Table I shows that the spray-drying yields were around 60–82%. If spray drying is performed above the glass transition temperature of the polymer, it normally yields highly aggregated particles with a low yield due to the rubbery state of the polymer.[11] In our case, the T_g's are higher than the inlet temperature of the spray drier, a fact that explains the fairly high yields of the process.

The BSA release profiles from the PLA-coated PIBCA microcapsules prepared by spray drying (2 μm average size) are shown in Figure 6. There are significantly low initial "bursts" of protein release regardless of the PLA molecular weights, which indicates that the PLA precipitates and coats the PIBCA wall without creating any porous cracks. The BSA releases from PLA-coated PIBCA microcapsules over 100 days, but the release rates appear to slow down with time. The release profile may be a

Table I Thermal Characteristics of PLA and Its Copolymers with Glycolic Acid, Which Were Coated onto PIBCA Microcapsules by a Spray-drying Technique

Polymers	MW	T_g (°C)	T_c (°C)	T_m (°C)	Spray Drying Yield (%)
PIBCA microcapsule	na	−16.5	nd	nd	nd
Poly(L-lactic acid)	2,000	nd	62.2	139.5	60.2
Poly(L-lactic acid)	50,000	36.1	66.2	168.4	78.5
Poly(L-lactic acid)	100,000	37.1	70.0	168.2	67.8
Poly(DL-lactic acid)	na	26.2	nd	nd	74.3
Poly(L-lactic-*co*-glycolic acid, 50 : 50)	22,000	34.6	nd	nd	82.7
Poly(L-lactic-*co*-glycolic acid, 75 : 25)	15,000	36.5	nd	nd	81.4

T_g: glass transition temperature; T_c: crystallization temperature; T_m: crystallnie melting temperature; na; not available, nd: not detected.

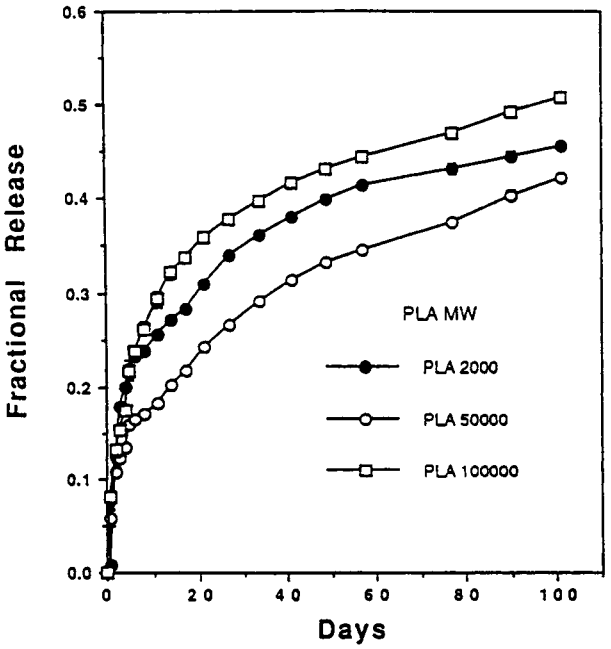

Figure 6 BSA-release profiles from the PLA-coated PIBCA microcapsules prepared by spray-drying method.

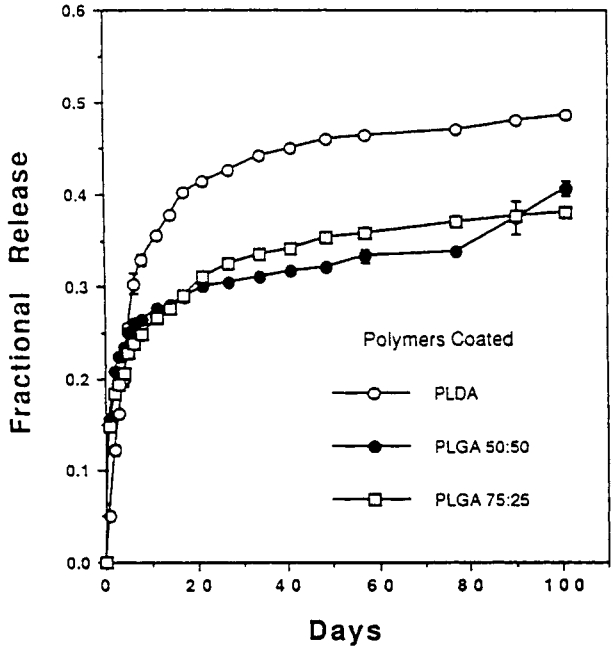

Figure 7 BSA-release profiles from poly(D,L-lactic acid) and poly(lactic-*co*-glycolic acid)-coated PIBCA microcapsules.

complicated function of BSA diffusion through the polymer walls and degradation rate of both PLA and PIBCA. Nevertheless, because degradation of PIBCA is quite fast,[19] it may be assumed that the main factor controlling the release is the erosion of the coating. The porosity of the PLA coating will be gradually increased due to the polymer degradation, allowing the slow release of BSA from the microcapsule core for extended periods of time. It could be expected that low MW PLA erodes faster because of the shorter time required to produce PLA monomers, and, therefore, encapsulated protein molecules are more rapidly released. However, our results indicate an insignificant influence of the polymer molecular weight. Our hypothesis is that PLA MW may affect polymer film properties. It is expected that low MW PLA creates more uniform and physically defect-free coatings onto the PIBCA microcapsule walls than does the high molecular weight PLA. Thus, these two opposing factors on BSA release might cancel each other, resulting in BSA release profiles independent of the PLA molecular weights.

Figure 7 shows the BSA release profiles from poly(D,L-lactic acid) (PLDA) and PLGA-coated PIBCA microcapsules. There are relatively fast release rates up to a 10 day period and a leveling-off behavior beyond that time point. These BSA release rates, clearly faster than those in Figure 7, may be due to the more rapid degradation of PLDA and PLGA than those of the PLA.[2] A minimal "burst" of BSA release (from 5 to 15%) can also be seen,

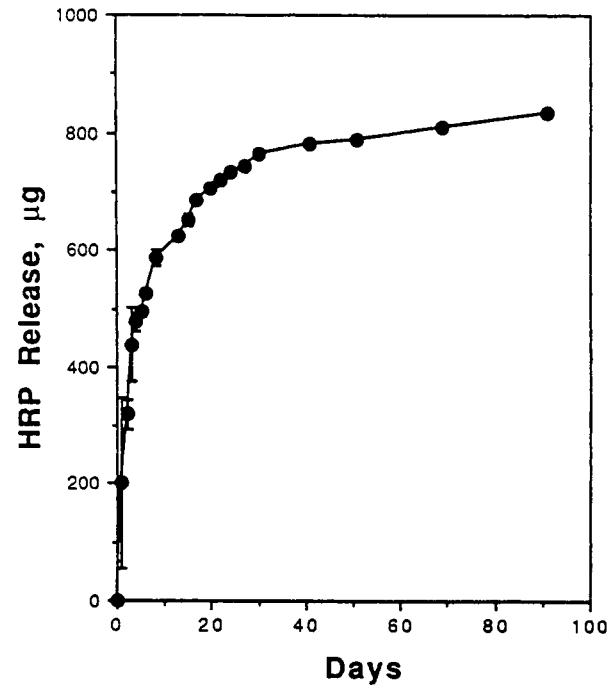

Figure 8 Horseradish peroxidase release profiles from PLA (MW 2000)-coated PIBCA microcapsules.

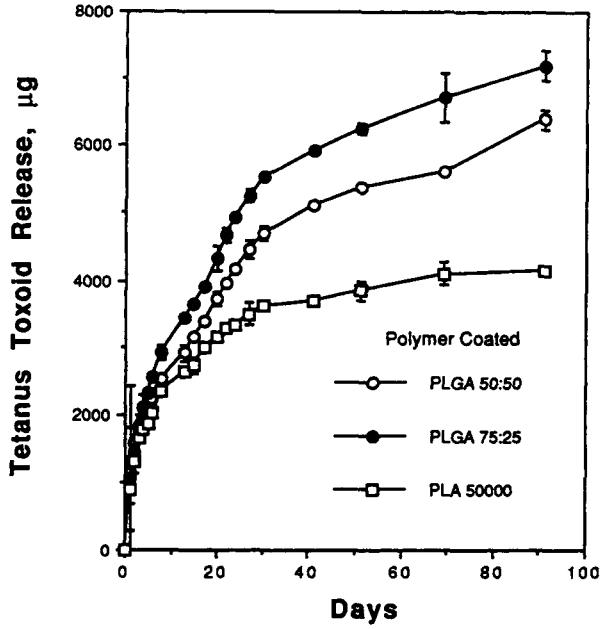

Figure 9 Tetanus toxoid release profiles from PLGA (75 : 25 and 50 : 50) and PLA (MW 50,000)-coated PIBCA microcapsules.

supporting the fact that the amorphous PLDA and PLGA form an uniform coat around PIBCA microcapsules, thus reducing protein "dumping."

Figures 8 and 9 show the release profiles of horseradish peroxidase from the PLA (MW 2000) and tetanus toxoid from two PLGA (75 : 25 and 50 : 50)- and PLA (MW 50,000)-coated PIBCA microcapsules. In both cases, long-term protein release over several months could be achieved with low initial burst effects. In particular, for tetanus toxoid, it was found that release was dependent on the composition of the polymer coat. Since it is known[2] that PLGA 50 : 50 degrades faster than PLGA 75 : 25 and PLA, a correlation was observed between the degradation rate of the polymer coating and the *in vitro* toxoid release rate. This correlation may be explained by the increasing number of porous channels generated during the polymer degradation process.

To determine the efficacy of the microencapsulation process in terms of immunogenicity of encapsulated antigens and *in vivo* antigen presentation, tetanus toxoid-loaded microparticles were subcutaneously injected into rats. Table II, which shows the ELISA titer in serum, indicates that the immune response to tetanus toxoid depends on the microcapsule-coating composition. The time course of antibody formation is related to the polymer degradation rate as well as to the *in vitro* release profiles in Figure 9. This preliminary data led us to the assumption that it may be possible to modulate antigen presentation by judiciously selecting the type of coating.

These new formulations are expected to be promising for the application of vaccine delivery in view of their long-term release patterns.[20] Since the particle size is less than 2 μm, it is possible for the particles to be engulfed by macrophages, which may enhance the immunization capabilities *in vivo*.[21] It may also be possible to achieve a pulsatile release of the vaccine, when the PLA coating layer is physically changed. For example, if the coating thickness could be controlled, several patterns of initial slow release and then a pulse of release could be achieved.

Table II ELISA Titers of Sera from Rats Subcutaneously Injected with Different Tetanus Toxoid-loaded Microparticles

		ELISA Titer[a] on Postinoculation Day					
Microparticles	Rat No.	0	8	16	30	44	58
PLGA 75 : 25	1	20	< 20	1280	1280	1280	2560
	2	< 20	< 20	160	640	640	640
	3	< 20	< 20	160	640	1280	2560
PLGA 50 : 50	4	< 20	< 20	—	—	—	—
	5	< 20	< 20	1280	1280	2560	5120
	6	< 20	< 20	< 20	20	—	40
PLA 50,000	7	< 20	< 20	< 20	80	40	40
	8	< 20	< 20	< 20	< 20	< 20	< 20
	9	< 20	< 20	40	80	320	320

—, Serum not available.
[a] Titer defined as reciprocal of the highest dilution of the serum showing an optical density of 0.15 or more in ELISA.

Consequently, when two types of vaccine-loaded particles with different coating thickness are formulated together, two main pulses of release may be attained at different times. By using this approach, the conventional method of vaccine administration requires that three shots over several months could be theoretically simulated with only one administration using this cocktail-like microsphere formulation. Nevertheless, further studies need to be done to assess the feasibility of this new approach.

In summary, it has been demonstrated that biodegradable PIBCA microcapsules containing protein molecules can be coated with biodegradable PLA and its copolymers with GA. These new formulations showed a very low protein burst and prolonged release times.

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