In Vitro Protein Release and Degradation of Poly-*dl*-lactide-poly(ethylene glycol) Microspheres with Entrapped Human Serum Albumin: Quantitative Evaluation of the Factors Involved in Protein Release Phases

Xiaohong Li,[1,2,3] Xianmo Deng, [1] and Zhitang Huang[2]

Received July 18, 2000; accepted October 14, 2000

Purpose. To quantitatively evaluate the correlations between the amount of initial burst release and the surface-associated protein, and between the onset time for the second burst release and the matrix polymer degradation.

Methods. Human serum albumin (HSA) was microencapsulated in polylactide (PLA) and poly-*dl*-lactide-poly(ethylene glycol) (PELA) with PEG contents of 5, 10, 20, and 30%, respectively, using the solvent extraction procedure based on formation of double emulsion w/o/w. Microspheres with similar particle size (1.7–2.0μm), similar protein entrapment (2.1–2.8%) but different surface-associated proteins (9.3–53.6%) were used to evaluate the *in vitro* matrix degradation and protein release profiles. Degradation was characterized by studying the intrinsic viscosity decrease, medium pH change, and weight loss of the microspheres.

Results. The matrix degradation and protein release profiles were highly dependent on the polymer composition of the microspheres. Faster decreases in the intrinsic viscosity of recovered matrix polymer, the microspheres weight, and the pH of degradation medium, and earlier onsets for the break in intrinsic viscosity reduction and the mass loss were detected for PELA microspheres with higher PEG content. The hydration and swelling of microspheres matrix contributed greatly to the degradation of matrix polymer. The HSA release showed triphasic profile and involved two mechanisms for all the microsphere samples. Smaller amount of initial burst release, larger gradual release rate, and earlier onset for the second burst release were observed for HSA from matrix polymer with higher PEG content. The extent of the initial burst release was quantitatively related with the surface-associated protein. The second burst release of HSA was observed to occur within 1 week after the onset for mass loss, which was also the break in the intrinsic viscosity reduction rate.

Conclusion. Protein release profiles could be rationalized by optimizing the matrix polymer degradation and microsphere characteristics.

KEY WORDS: poly-*dl*-lactide-poly(ethylene glycol) microspheres; polymer degradation; protein release; burst release; surface-associated protein.

INTRODUCTION

Increasing applications for polymers in the pharmaceutical industry as matrices for drug delivery systems have been investigated. With the success of biotechnology and recombinant technology, proteins are being looked upon as future therapeutic agents. The short biological half-life and oral instability are inherent problems with most of the biologically active proteins and hence their development as controlled delivery systems is a greater challenge.

The potential applicability of biodegradable microspheres as drug, peptide, protein, and antigen delivery systems is based not only on the protection from acidic and proteolytic degradation, but also on the fact that the controlled release profile could be adjusted by polymer degradation. In particular, sustained-release microspheres using biodegradable products such as polylactide (PLA) (1), poly-*dl*-lactide-glycolide (PLGA) (2), and poly-*dl*-lactide-poly(ethylene glycol) (PELA) (3) have been investigated and satisfactory results have been obtained.

Because of the existence of a certain amount of hydrophilic poly(ethylene glycol) (PEG) segments in polymer chains, compared with the commonly used PLA and PLGA, PELA shows much potential for use in protein delivery systems (4). In our previous work, the preparation of PELA microspheres with entrapped antigen proteins of *Vibrio cholera* and *Leptospira interrogans* were studied by investigating factors influencing particle size, antigen encapsulation efficiency (4), and immune responses (5). To mimic the multiple injection procedure of vaccinization by a single-dose microsphere formulation, the amount of initial release and the time of onset for the second burst release are essential to achieve preventive immune responses.

Because of the large molecular size and insolubility in PLA, PLGA, and PELA, release of proteins from these polyesters by classical partition-dependent diffusion is minimal. Consequently, the degradation of matrix polymer might be critical in determining the release of protein from the matrices. It is known that systems based on PLA and PLGA undergo hydration in aqueous environment, followed by bulk hydrolysis of ester bonds, a decrease in molecular weight, and then loss of microsphere weight (6). Microspheres prepared with these polymers, which hydrated and degraded slowly, displayed a larger initial burst release followed a lag phase characterized by no protein release (7). Inserting hydrophilic PEG into relatively hydrophobic PLA blocks can promote the water uptake and swelling of the microsphere matrix, and modulate diffusion of proteins from the carrier system. In a previous investigation, the protein-loaded PELA microspheres revealed triphasic release profiles; that is, initial burst release during the first day, gradual release over 1 month, and finally, the second burst release (8). The initial burst release measured during *in vitro* incubation test for PLA, PLGA, and PELA delivery systems was thought to be caused by the desorption of protein molecules absorbed at or located near the surface of the microspheres. However, the relationships between the burst release and surface-associated protein have not been quantitatively determined. The second burst release occurred after 35 days of incubation for PELA with 10% of PEG content in our previous investigation (8). However, no systematic work had been carried out to investigate the quan-

[1] Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Chengdu 610041, P. R. China.

[2] Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, P. R. China.

[3] To whom correspondence should be addressed at: The Ohio State University, College of Pharmacy, 500 West 12th Avenue, Parks Hall, Columbus, OH 43210. (e-mail: li.441@osu.edu)

0724-8741/01/0100-0117$19.50/0 © 2001 Plenum Publishing Corporation

titative relationship between the onset for the second burst release and the onset for matrix polymer degradation.

The possibility of controlled protein release through the degradation of matrix polymer and microsphere characteristics represents an attractive feature for protein delivery. To rationalize the design of protein delivery microspheres, the present work aimed at interpreting the *in vitro* degradation and release profiles of PELA microspheres with an entrapped model protein, human serum albumin (HSA). The work was also undertaken to achieve an insight into mechanisms involved in polymer degradation and protein release, and a quantitative understanding of the correlations between the extent of initial burst release and the surface-associated protein, and between the onset time for the second burst release and matrix polymer degradation. PELA copolymers with PEG contents of 5, 10, 20, and 30% were synthesized by bulk ring-opening polymerization. HSA/PELA microspheres with similar size distribution, similar protein entrapment, but different surface-associated protein were prepared by solvent extraction process based on formation of double emulsion water-in-oil-in-water (w/o/w). The degradation profiles of these microspheres were characterized by checking the molecular weight decrease, weight loss of microspheres, and medium pH change. The extent of initial burst release and the onset time for the second burst release were evaluated from the protein release procedures.

MATERIALS AND METHODS

Materials

PEG (Mw = 6000 Da) and polyvinyl alcohol (PVA, 88% hydrolyzed, Mw = 130,000 Da) were from Guangzhou Chemical Reagent Department, China. Copolymer PELA with 5, 10, 20, and 30% of PEG contents and PLA homopolymer were prepared by bulk ring-opening polymerization of lactide/PEG or lactide using stannous chloride as initiator (9). The actual PEG content of PELA was calculated from the integral height of hydrogen shown by ^{1}H-NMR (Varian FT-80A, Harbor City, CA). The Mw and Mw/Mn of PELA and PLA were obtained with gel permeation chromatography (GPC, Waters ALC/GPC 244, Milford, MA), using polystyrene as standards. HSA was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of reagent grade or better.

Preparation of HSA/PELA Microspheres

HSA-encapsulated PELA microspheres were prepared by the solvent extraction method based on the formation of a modified double emulsion $w_1/o/w_2$ reported earlier (10). Briefly, the w_1 phase, containing aqueous solution of HSA was dispersed into the organic phase (o), consisting of the polymer dissolved in dichloromethane, using a high-speed stirrer for 60 s at room temperature. The primary water-in-oil emulsion (w_1/o) was then immediately added to the external aqueous phase (PVA solution) and further emulsified again by a high-speed homogenizer. The organic solvent was extracted by adding 6% isopropanol and the mixture was stirred at a moderate speed at ambient temperature for 3 h. The microspheres were spun in a centrifuge at 5000 ×*g* for 8 min, washed three times with double-distilled water, lyophilized overnight, and stored at 4°C in a desiccator.

Characterization of HSA/PELA Microspheres

Microspheres (ca. 5 mg) were suspended in 4 ml aqueous solution for 5 min using an ultrasonic bath. The particle size and distribution were determined by laser light diffractometry (Shimazu SALD-2009, Japan), and expressed as volume mean diameter. At least three different batches were detected to give an average value and standard deviation for diameter of the microspheres.

Core loading of HSA in microspheres was determined by extracting the protein from microspheres and assaying the protein content of the extracted solution. In brief, a known amount of microspheres (ca. 100 mg) was dissolved in 500 μl of methylene chloride and extracted three times with 600 μl of double-distilled water. The HSA content of the extracted solution was determined using Bradford's method (11), and compared with a standard curve of data obtained by assaying known concentrations of HSA solutions. Different calibration values for inefficient extraction were obtained for each matrix polymer by adding the same quantity of HSA solution in polymer solution, and extracting under the same conditions as above. The HSA content was determined in triplicate for at least three different batches. The amount of encapsulated HSA (AE) in microspheres, given as a percentage, indicates the amount (in milligrams) of HSA encapsulated per 100 mg of microspheres. The encapsulation efficiency of the process indicates the percentage of HSA encapsulated with respect to the total amount used for the preparation of microspheres.

The analysis of HSA located at the surface of the microspheres was based on removing the adsorbed proteins by trypsin (EC 3.4.21.4, Sigma Chemical Co.) and determining the protein content of the supernatant after centrifugation (12). Briefly, 50 mg of microspheres was weighed and dispersed in 2.0 ml 0.05% trypsin aqueous solution. The mixture was shaken at 25°C for 24 h. After centrifugation, the supernatant was collected and the content of HSA was determined and calibrated following a similar procedure as that described above. The amount of the surface-associated protein indicates the percentage of the HSA located at the surface with respect to the total amount of HSA entrapped in microspheres.

In Vitro Degradation of Microspheres

The degree of degradation was estimated from the decrease in intrinsic viscosity, from the mass loss, and from the change of medium pH, respectively. Preweighed microspheres (ca. 400 mg) were suspended in 10.0 ml of 154 mM phosphate buffered saline (PBS), pH 7.4 ± 0.1, containing 0.02% sodium azide as a bacteriostatic agent. The microsphere suspension was kept in a thermostated shaking water bath (Jiangsu Taichang Medical Apparatus Co., China) that was maintained at 37°C and 60 cycles/min. The degradation medium was removed weekly from the vessel containing microspheres by centrifugation and replaced with the same volume of fresh PBS. At predetermined intervals, triplicate samples for each kind of microsphere were recovered, rinsed with distilled water to remove residual buffer salts, and dried to constant weight in a vacuum desiccator.

Mass loss of microsphere samples was determined gravi-

metrically by comparing the dry weight remaining at a specific time with the initial weight. The recovered and dried microspheres were dissolved in tetrahydrofuran (THF) and filtered to eliminate insoluble proteins. The intrinsic viscosity was measured with an Ubbelohde viscometer on 0.4 g/dl solution of polymer in THF at 30.0 ± 0.1°C. The determination of pH variation of the phosphate buffer (initial pH 7.35), in which the *in vitro* degradation tests were performed, was carried out with a model pHS-3B pH meter (Shanghai Scientific Instrument Co., China) equipped with a combined glass electrode.

In Vitro Release Studies

Microspheres (ca. 200 mg) were suspended, in triplicate, in 5.0 ml of pH 7.4 ± 0.1 PBS, containing 0.02% sodium azide as a bacteriostatic agent. The suspensions were incubated at 37°C under continuous orbital shaking at 60 cycles/min. At predetermined intervals, the aqueous media were removed with a syringe after centrifugation, while the same amount of fresh PBS was added back to the release medium. The HSA content was determined as described above, using a calibration curve obtained with placebo microspheres treated under the same procedure.

RESULTS AND DISCUSSION

Protein encapsulation and delivery from biodegradable microspheres have been the objective of extensive investigation because a large number of recombinant proteins are now being developed for pharmaceutical applications. However, the therapeutic and prophylactic effects are highly associated with the protein release profiles from polymeric carriers, such as the total amount of protein released, the release rate, and the amount of protein released at different intervals. As indicated before (8), encapsulated protein release is mainly polymer degradation dependent in the present microparticulate controlled-release system. *In vitro* investigation provides a convenient method by which to inspect the *in vivo* behavior of polymer degradation and protein release. Thus, the purpose of this study was to interpret the *in vitro* degradation and HSA release profiles from PELA microspheres. The effects of the extent of initial burst release and the onset time for the second burst release have been quantitatively examined in the triphasic release profile of HSA/PELA microspheres.

Characterization of HSA/PELA Microspheres

Copolymers PELA with 5, 10, 20, and 30% of PEG contents were synthesized by bulk copolymerization initiated by stannous chloride. The block structure of the copolymers was confirmed by ^{13}C-NMR, and the actual PEG content was estimated from the integral height of hydrogen shown in ^{1}H-NMR according to a previous study (13). The estimated PEG contents and the amount added before polymerization are compared in Table 1, indicating that the hydrophilic PEG segments have been quantitatively inserted into PLA backbone, thus the hydrophilicity/lipophilicity of copolymer PELA can be delicately manipulated according to the present polymerization system.

In our previous study (10), the evaluation of particle size and protein encapsulation efficiency as a function of different preparation parameters was studied in detail. The particle size was associated with the mixing efficiency of emulsification procedure and the stability of the primary w_1/o emulsion. An increase in the viscosity of polymer organic solution and/or the volume of external aqueous phase led to reduction in the mixing efficiency, resulting in large particle size. The amount of protein expelled from the internal aqueous phase to the external aqueous phase during the second emulsification determined the protein loading efficiency. The small transfer area (due to large size), high mass transfer resistance (due to stable primary w/o emulsion, viscous organic phase, and thick oily polymer layer), and high precipitation rate of polymer solution into microspheres (due to less solvent) were beneficial to elaborate microspheres with high protein loading efficiency. In the present investigation, HSA was encapsulated into copolymers with PEG contents of 5, 10, 20, and 30%, respectively. It was indicated that the particle size and the amount of protein entrapment have great influence on the protein release (4). Thus, HSA/PELA microspheres with similar size (1.7–2.0 μm) and similar HSA entrapment (2.1–2.8%), summarized in Table 2, were prepared based on the optimized parameters. For comparison, HSA-loaded PLA microspheres with similar size and HSA entrapment were also included to determine the protein release profiles as a function of microsphere characteristics and matrix polymer degradation.

The surface-associated protein indicates the percentage of the amount of protein located at or near the surface of the microspheres with respect to the total amount of protein entrapped in the microspheres. In the preparation of protein-loaded microspheres by solvent extraction process based on formation of w/o/w emulsion, protein molecules would unavoidably expel from the internal aqueous phase into the external aqueous phase during the second emulsification procedure. After solvent extraction and solidification of the microspheres, protein molecules were absorbed at or located near the surface of the microspheres. However, the accumulated PEG segments may create a hydrophilic microenvironment within microspheres, and protein molecules may preferentially localize within deeper sections of the microspheres matrix. Thus, a smaller amount of surface-associated protein was achieved for matrix polymer with higher PEG content. As shown in Table 2, an increase in the PEG contents of matrix polymer PELA from 0 to 30% resulted in decrease in the surface-associated protein from 53.6 to 9.3%.

Table 1. Characteristics of Matrix Polymer Under Investigation

Samples	PLA	PELA-5	PELA-10	PELA-20	PELA-30
PEG (%, add.)	0	5.0	10.0	20.0	30.0
PEG (%, cal.)[a]	0	6.1	11.5	23.3	32.9
Mw (K Da)[b]	58.1	98.4	52.2	25.8	18.2
Mw/Mn[b]	3.05	2.75	2.63	2.89	3.06
[η] (dL/g)[c]	0.389	0.543	0.359	0.228	0.181

[a] Estimated from the integral height of hydrogen shown in ^{1}H-NMR spectrum.
[b] Weight average molecular weight and its distribution (Mw/Mn) measured by gel permeation chromatography (GPC) and calibrated with polystyrene standards.
[c] Intrinsic viscosity determined by Ubbelhode viscometer at 30.0 ± 0.1°C.

Table 2. Characteristics of HSA-Loaded Microspheres Under Investigation

Microspheres	HSA/ PLA	HSA/ PELA-5	HSA/ PELA-10	HSA/ PELA-20	HSA/ PELA-30
Diameter (μm)[a]	1.88	1.79	1.92	1.83	1.74
SD[a]	0.284	0.277	0.263	0.255	0.252
HSA entrapment (%)[b]	2.36	2.14	2.75	2.44	2.18
SP (%)[c]	53.6	44.7	23.3	17.4	9.3

[a] The average diameter and its standard deviation measured by laser diffraction particle size analyzer.

[b] Protein extracted from microspheres and detected by Bradford's method.

[c] The surface-associated protein (SP) removed from microspheres surface and determined by Bradford's method.

In Vitro Degradation Profile of HSA/PELA Microspheres

The intrinsic viscosity [η] of all microsphere samples decreased continuously after being exposed to PBS. Fig. 1(A) shows the decrease in $[\eta]/[\eta]_0$ compared to incubation time. The intrinsic viscosity of PLA sample showed a slight reduction during the experimental period. The increase in the PEG content resulted in a faster decrease in the intrinsic viscosity of recovered matrix polymer PELA. As detected from Fig. 1(A), which shows HSA-loaded PLA and PELA microspheres independent of PEG content, the plots of $[\eta]/[\eta]_0$ versus degradation time showed biphasic patterns. Faster decrease in $[\eta]/[\eta]_0$ was observed prior to the slowdown decrease region. The breaks in reduction rates occurred approximately at week 8, 6, 5, 4, and 2 after incubation for HSA-loaded PLA, PELA-5, PELA-10, PELA-20, and PELA-30 microspheres, respectively. A possible explanation for the breaks was that the intrinsic viscosity was measured on the solid fraction of the matrix polymer. As a result of degradation, a fraction of polymer with low molecular weight would have been dissolved in the test buffer solution and would be removed from the solid fraction so that the dissolved part were no longer taken into account in the calculation of intrinsic viscosity.

Gravimetric evaluation of loss of microsphere mass during incubation is summarized in Fig. 1(B), and also indicates apparent biphasic profiles. Note that no mass loss occurred in all microsphere samples over the initial several weeks, although significant changes in the intrinsic viscosity of matrix polymer were detected [Fig. 1(A)]. It suggested that the mass loss began when a fraction of oligomers that could be soluble in degradation medium were generated from the polymers. The onset for mass loss lagged behind molecular weight reduction, thus, the hydrolysis of copolymer PELA with different PEG contents from 5 to 30% should proceed through the bulk of polymer structure, which was identical to the bulk degradation mechanism of PLA (14). An alternative mechanism, surface layer hydrolysis and erosion, requires that the erosion preceeded molecular weight reduction of retrieved matrix polymer. As seen from Fig. 1(A) and (B), the onset for mass loss of microspheres was observed approximately at the breaks in the reduction rates of the intrinsic viscosity of matrix polymers. Coincident with erosion onset, low molecular weight polymer fractions dissolved into buffer solution and became unavailable for analysis of intrinsic viscosity on retrieved samples, resulting in a lower reduction rate of the intrinsic viscosity.

During the experiment, the degradation medium in which microspheres were suspended and incubated at 37°C was not changed, which allowed to pH change to be determined. Fig. 1(C) shows that the pH of PBS decreased in all samples over the incubation time. The pH decrease for HSA/PELA-30 molecules displayed an almost linear behavior ($\gamma^2 = 0.980$) throughout the investigation period. But for HSA/PLA microspheres, a slight decrease in the pH of degradation medium was observed during the initial weeks and a sharp pH decrease was detected 9 weeks after incubation. As seen from Fig. 1(C), during the initial 9 weeks, the pH value of test buffer decreased faster for matrix polymer with higher PEG content. However, in the latter incubation intervals, the HSA/PELA microspheres with higher PEG content observed a slower pH decrease. This was because of the much higher number of free carboxylic acid end-groups formed by degradation of matrix polymer PLA and PELA with lower PEG content. After 15 weeks of incubation, the pH reached final values of 3.36, 3.11, 3.61, 4.10, and 4.56 for PLA, PELA with PEG contents of 5, 10, 20, and 30%, respectively.

As seen from Fig. 1, faster decreases in the intrinsic viscosity of recovered matrix polymer [Fig. 1(A)], the weight of microspheres [Fig. 1(B)], and the pH values of degradation medium [Fig. 1(C)] were detected for PELA microspheres with higher PEG content. HSA/PELA microspheres with higher PEG content showed earlier onsets for the break in the intrinsic viscosity reduction [Fig. 1(A)] and the mass loss [Fig. 1 (B)]. This may be due to the increasing hydrophilicity of PELA microspheres matrix with larger amount of PEG segments in the copolymer backbones. The introduction of hydrophilic PEG domains into PLA promoted the water uptake and the swelling of microspheres matrix, which both enhanced the hydrolysis rate of polymers.

To inspect the contribution of hydration and swelling of the microspheres matrix to the degradation of matrix polymer, HSA/PELA-10 microspheres were incubated into pH 7.4 PBS of different concentration, and the degradation profiles were characterized. It was anticipated that an increase in the ionic strength of the incubation buffer might reduce the swelling of microspheres matrix due to an ion-shielding effect (15). The mass loss of HSA/PELA-10 microspheres incubated in PBS of different concentration is presented in Fig. 2(A).

All of the samples showed a slight loss during the initial 5 weeks and exhibited a significant weight loss in the following period. During the initial weeks of incubation, the mass loss showed a similar profile, but microspheres lost a slightly smaller amount of weight when incubated in PBS of higher concentration. This can be explained by the fact that during

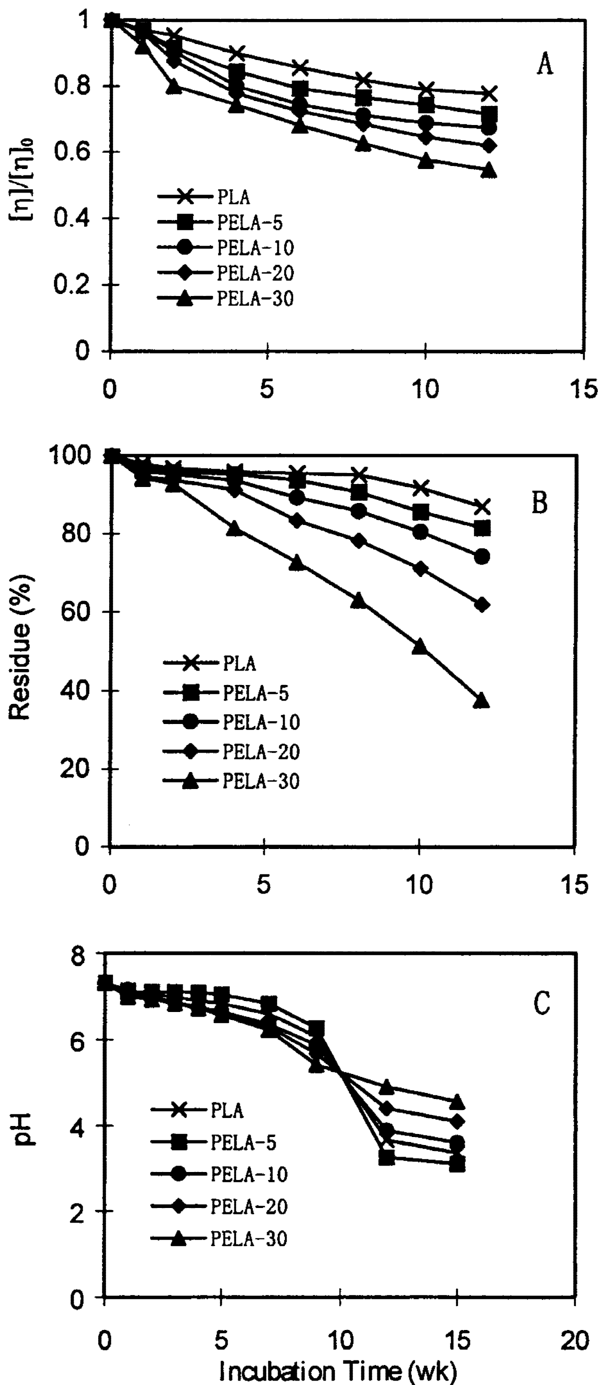

Fig. 1. The changes of the intrinsic viscosity of retrieved matrix polymer (A), the residual weight of recovered microspheres (B), and the medium pH (C), against degradation time of HSA-loaded PLA (×), PELA-5 (■), PELA-10 (•), PELA-20 (♦), and PELA-30 (▲) microspheres incubated in pH 7.4 ± 0.01 PBS at 37°C.

incubation an important amount of buffer penetrated deeply into the polymer matrix. These salts could not be eliminated, especially for high concentration of buffer solution, during the collecting step so that they might produce a weight increase, which was balanced by the protein release (16). During the latter incubation intervals, the mass loss was larger when microspheres were tested in lower concentrations of PBS. At week 12 after incubation in 20, 154, 400, and 1000 mM of PBS, microspheres remained 75.2, 77.2, 79.6, and 88.3% of the initial weight, respectively. Because of the ion-shielding effect, it was beneficial for lower concentration of PBS to penetrate throughout the microspheres matrix, causing higher hydration and swelling of polymer backbone. The hydrated and swollen inner structure of microspheres facilitated the hydrolysis of matrix polymer.

The degradation of polyesters has been shown to take place by random hydrolysis of ester bonds, and the degradation of PLA and its copolymer can be described as follows (17):

$$[\eta] = [\eta]_0 \exp(-\alpha k t)$$

where α is exponent in the Mark-Howink formation $[\eta] = KM^{\alpha}$.

$$\ln([\eta]/[\eta]_0) = -\alpha k t$$

thus, the intrinsic viscosity of matrix polymer retrieved from microspheres incubated in different concentration of PBS was determined and the plot of $\ln([\eta]/[\eta]_0)$ versus time is shown in Fig. 2(B). The plots were nearly linear for all samples during the initial 4 weeks of degradation, and the apparent rate constant (αk) can be obtained from the slope of the plot of $\ln([\eta]/[\eta]_0)$ against time. The linear coefficients (γ^2) were 0.987,

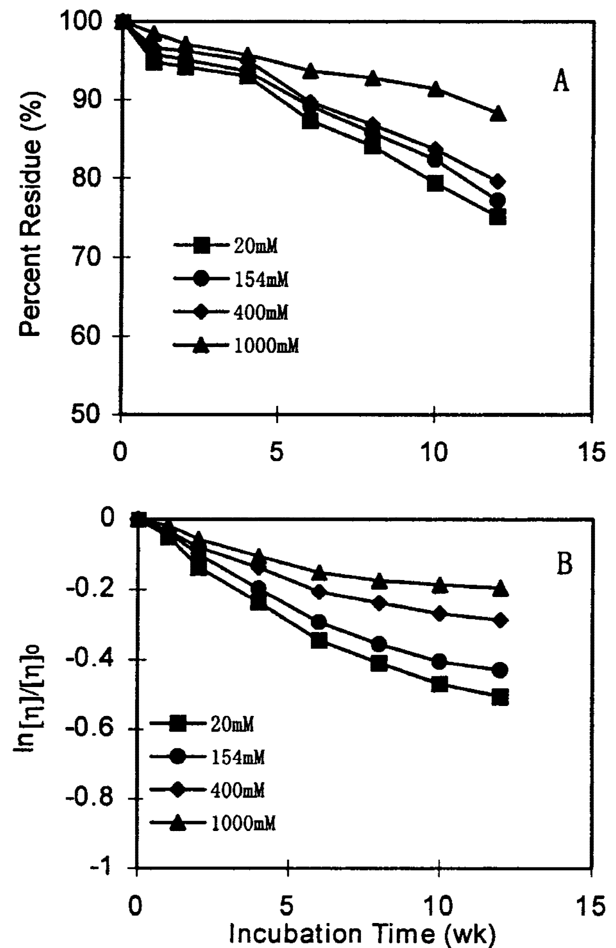

Fig. 2. The mass loss (A) and semilogarithmic relationship between the intrinsic viscosity $[\eta]/[\eta]_0$ and degradation time (B) of HSA-loaded PELA-10 microspheres incubated in PBS of 20 mM (■), 154 mM (•), 400 mM (♦), and 1000 mM (▲), respectively.

0.996, 0.991, and 0.988, and the evaluated values of αk were 5.91, 4.95, 3.47, and 2.66 (10^{-2} week^{-1}) for HSA/PELA microspheres incubated into 20, 154, 400, and 1000 mM of PBS, respectively. Results indicated that the apparent degradation rate constant of PELA when incubated in 20 mM of PBS was over 2 times higher than that when incubated in 1000 mM. Thus, when comparing the mass loss profiles [Fig. 2(A)] and the rate constants as a function of buffer concentration, it appeared that the different degradation profiles of the investigated microsphere samples could also be explained from the apparent degradation rate constants.

From above discussion, the degradation of PELA could be described by penetration of water into microspheres matrix, followed by the bulk hydrolysis of ester bonds, and resulted in decrease in molecular weight and loss of weight of microspheres. The degradation involved three stages. The initial stage of degradation displayed a random chain scission process. Here, the intrinsic viscosity of the matrix polymer deceased significantly, but there was no appreciable weight loss and trace soluble monomeric products were formed. In the middle stage, a decrease in the intrinsic viscosity was accompanied by rapid mass loss and soluble oligomeric and monomeric products were formed. The final stage, which was not investigated in present work, should be characterized as rapid formation of soluble monomeric products and complete polymer solubilization.

In Vitro HSA Release from HSA/PELA Microspheres

Results indicated that systems based on PLA and PELA, in an aqueous environment, undergo hydration followed by bulk erosion. During erosion, the porosity of the matrix increased and the release of protein by diffusion was facilitated. Assuming this behavior, not only the degradation rate of the polymer, but also the initial inner structure and the dispersion pattern of protein within the microspheres matrix should be considered as critical factors controlling the release process.

Figure 3 shows the release profiles of HSA from PLA and PELA microspheres.

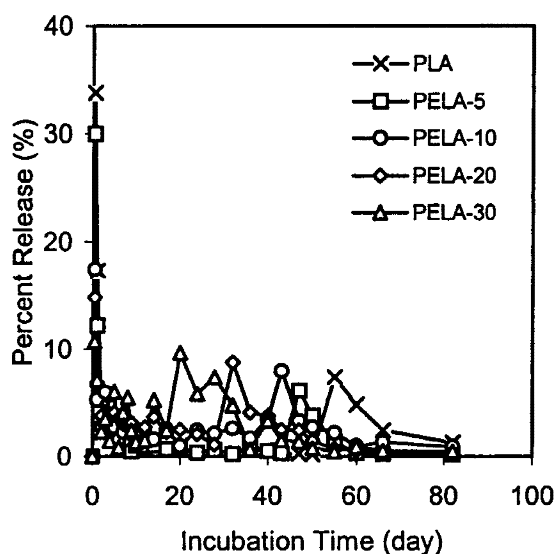

Fig. 3. Percent release of HSA from PLA (×), PELA-5 (□), PELA-10 (○), PELA-20 (◇), and PELA-30 (△) microspheres incubated in pH 7.4 ± 0.01 PBS at 37°C.

The release profiles of all systems were characterized by a typical triphasic pattern. In the initial phase, an important amount of burst release during the first day was observed, and then a gradual release followed and proceeded for several weeks, whereas in the later incubation time, the second burst release occurred to release the remaining HSA within microspheres.

As shown in Fig. 3, lower amount of initial burst release, larger gradual release rate, and earlier onset for the second burst release were detected for HSA release from matrix polymer with higher PEG content.

The burst release of protein was anticipated to be associated with protein molecules distributed close to the surface of the microspheres. Table 3 summarizes the amount of surface-associated protein of microspheres and the extent of the initial burst release of *in vitro* test. Although the quantitative formula was difficult to achieve for all matrix polymers in present investigation, it was concluded that the higher amount of surface-associated protein would result in a larger burst effect. The protein molecules dispersing close to the surface of the microspheres were those adsorbed at or loosely bound near the surface, which diffused out in the initial incubation time. The adsorption might result from the fraction of HSA that leaked from the inner aqueous phase toward the external aqueous phase during the final emulsion process or during the solvent extraction process. The HSA might be readsorbed on the dichloromethane/water interface, because of its highly tension-active properties. As seen from Table 3, the burst release of HSA in 24 h from the microspheres matrix with lower PEG content, such as PLA, PELA-5, and PELA-10, showed little difference with the evaluated amount of surface-associated protein. When the PEG content increased above 10%, such as for PELA-20 and PELA-30, the detected burst release was larger than the evaluated surface-associated protein. This may be because water-soluble protein molecules diffused out more easily through the more hydrophilic microspheres matrix. For therapeutic protein delivery system, the burst release should be limited within some extent to achieve a sustained release behavior (18). For a vaccine delivery system, a certain amount of initial release of antigen protein is essential to stimulate the immune response (19). Thus, to rationalize the extent of burst release, the surface-associated protein should be optimized from process parameters of microspheres formation. In present HSA/PELA microspheres prepared by w/o/w method, the amount and distribution of protein had been shown to depend critically on formation conditions (20), such as the concentration of polymer solution, the surfactants in protein and external aqueous phases, shear conditions, and the stability of the primary w_1/o emulsion.

After the desorption of protein molecules located at the surface of microspheres during the initial burst release, the release of fully encapsulated protein must be preceeded by diffusion of protein molecules from the inner sections of microspheres matrix through the swollen and porous inner structure. The swollen and porous inner structure, which was involved in the gradual release phase, might be formed by hydration and hydrolysis of matrix polymer after contacting with the aqueous release medium and the diffusion of protein into the medium. As indicated in Table 3, the gradual release rate was significantly higher for HSA from microspheres matrix with higher PEG content. This is because of the more

Table 3. Relationship Between the Surface-Associated Protein and the Protein Release Profile

Microspheres	HSA/ PLA	HSA/ PELA-5	HSA/ PELA-10	HSA/ PELA-20	HSA/ PELA-30
SP (%)[a]	53.6	44.7	23.3	17.4	9.3
Release in 12 h (%)[b]	33.8	30.0	17.4	14.9	10.7
Release in 24 h (%)[b]	51.1	42.2	22.7	21.7	17.8
Gradual release (%)[b]	26.6	31.0	43.0	42.7	33.9
Gradual release rate (%/day)[b]	0.493	0.674	1.02	1.38	1.78

[a] The surface-associated protein (SP) removed from microspheres surface and determined by Bradford's method.
[b] Determined from Fig. 3.

hydrophilic microspheres matrix and the higher hydrolysis rate of matrix polymer. Both of them contributed to the formation of swollen and porous network, through which easier and faster diffusion of protein molecules was achieved. As seen from Fig. 3, microspheres prepared with PLA displayed a larger initial burst release followed a lower amount of gradual release. It was suggested that a large amount of entrapped protein was located close to the surface of the microspheres and no significant release by diffusion was characterized before significant degradation of polymer occurred. Low swelling of the polymer backbone due to the hydrophobic nature of PLA would also cause a reduction in the diffusion release of protein through the microspheres excipient. To overcome the discontinuous and biphasic release profiles of protein from PLA matrix, the introduction of hydrophilic domains, promoting the water uptake and swelling of microspheres was an attractive strategy. In this investigation, microspheres prepared with PELA with 30% of PEG content displayed an almost linear sustained-release profile, characterized by low amount of initial burst release and high gradual release rate (Table 3).

Following the gradual release of HSA for several weeks, the second burst release phase was detected for all samples of microspheres. As shown in Fig. 3, the onset for the second burst release was greatly matrix polymer dependent. Matrix polymer with higher PEG content led to earlier onset for the second burst release. As compared in Table 4, a close correspondence between the onset for the second burst release and breakdown of microspheres matrix was detected. The burst release of HSA was observed to occur within 1 week after the onset for the mass loss [Fig. 1(B)], which was also the break in the intrinsic viscosity reduction rate [Fig. 1(A)]. When the reduction of molecular weight became significant and the mass loss had begun, a critical increase in the porosity and even the breakdown of the microspheres matrix were achieved and higher protein release rate proceeded.

From above discussion, the *in vitro* HSA release from PELA microspheres was characterized by three phases. The release involved two different mechanisms, that is, diffusion of protein molecules from microspheres matrix and degradation of matrix polymer.

Diffusional release mainly contributed to the initial release, which involved the surface-associated protein that had been partially or incompletely entrapped. They were released rapidly, giving rise to the initial burst release. The gradual release phase involved both of the mechanisms. It could be described as diffusion of protein molecules through aqueous pores or channels, which could be formed during preparation of microspheres or after dissolving and releasing of certain protein domains. However, the swollen and porous network mainly resulted from the hydrolysis of the polymer backbone. The erosion of matrix polymer contributed to the second burst release. The protein molecules completely entrapped in microspheres matrix could not be released at higher release rate until the matrix started losing its integrity.

CONCLUSION

The matrix degradation and protein release profiles were highly dependent on the polymer composition of the microspheres. Close correlations were quantitatively evaluated between the extent of initial burst release and the surface-associated protein, and between the onset for the second burst release and the onset for mass loss of microspheres. It was concluded that HSA release profiles could be rationalized by optimizing the matrix polymer and characteristics of the microspheres.

ACNOWLEDGMENTS

This work was supported by National Science Foundation of China (grant number 20004009 and 29774034).

Table 4. Comparison of the Onset for the Mass Loss with the Onset for the Second Burst Release

Microspheres	HSA/ PLA	HSA/ PELA-5	HSA/ PELA-10	HSA/ PELA-20	HSA/ PELA-30
Onset for mass loss (week)[a]	8	6	5	4	2
Onset for the second burst release (d)[b]	55	47	43	32	20

[a] Determined from Fig. 1.
[b] Determined from Fig. 3.

REFERENCES

1. I. Yamakawa, Y. Tsushima, R. Machida, and S. Watanabe. *In vitro* and in vivo release of poly(*dl*-lactic acid) microspheres containing neurotensin analog prepared by novel oil-in-water solvent evaporation method. *J. Pharm. Sci.* **81**:808–811 (1992).
2. A. J. Hudson, K. J. Lewis, M. V. Rao, and S. Alchar. Biodegradable polymer matrix for the sustained exogenous delivery of a biologically active c-myc hammerhead riboenzyme**.** *Int. J. Pharm.* **136**:23–29 (1996).
3. T. Kissel, Y. X. Li, C. Volland, S. Gorich, and R. Koneberg. Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks. *J. Control. Release* **39**:315–326 (1996).
4. X. M. Deng, X. H. Li, M. L. Yuan, and Z. T. Huang. Optimization of preparative parameters for poly-*dl*-lactide-poly(ethylene glycol) microspheres with entrapped Vibrio Cholera antigens. *J. Control. Release* **58**:123–131 (1999).
5. W. X. Jia, W. B. Zhang, X. W. Li, and X. M. Deng. Experimental study on administration of microencapsulated Vibrio Cholera vaccine. *Chin. J. Microbiol. Immunol.* **17**:342–347 (1997).
6. M. Ramchandani, M. Pankaskie, and D. Robinson. The influence of manufacturing procedure on the degradation of poly(lactide-co-glycolide) 85:15 and 50:50 implants. *J. Control. Release* **43**:161–173 (1997).
7. T. Uchida, S. Goto, and T. P. Foster. Particle size studies for subcutaneous delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin as vaccine formulation. *J. Pharm. Pharmacol.* **47**:556–560 (1994).
8. X. H. Li, X. M. Deng, and Z. T. Huang. *In vitro* degradation and release profiles of poly-*dl*-lactide-poly(ethylene glycol) microspheres with entrapped proteins. *J. Appl. Polym. Sci.* **78**:140–148 (2000).
9. X. M. Deng, C. D. Xiong, and L. M. Cheng. Synthesis and characterization of block copolymer from lactide and poly(ethylene glycol). *J. Polym. Sci. Polym. Lett.* **28**:411–416 (1990).
10. X. H. Li, X. M. Deng, M. L. Yuan, and Z. T. Huang. Investigation on process parameters involved in preparation of poly-*dl*-lactide-poly(ethylene glycol) microspheres containing Leptospira Interrogans antigens. *Int. J. Pharm.* **178**:245–255 (1999).
11. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
12. H. Rafati, A. G. A. Coomes, J. Adler, J. Holland, and S. S. Davis. Protein-loaded poly(*dl*-lactide-co-glycolide) microparticles for oral administration: formulation, structural and release characteristics. *J. Controlled Release* **43**:89–102 (1997).
13. X. M. Deng, X. H. Li, and Z. T. Huang. Ring-opening copolymerization of *dl*-lactide and poly(ethylene glycol) initiated by lanthanum acetate and application as matrix of microspheres containing Vibrio Cholera antigens. *Chin. J. Polym. Sci.* **17**:265–270 (1999).
14. S. J. Holland, B. J. Tighe, and P. L. Gould. Polymers for biodegradable medical devices. 1. The potential of polymers as controlled macromolecular release systems. *J. Control. Release* **4**: 155–180 (1986).
15. M. S. Hora, R. K. Rana, J. H. Nunberg, T. R. Tice, R. M. Gilley, and M. E. Hudson. Release of human serum albumin from poly-(lactide-glycolide) microspheres. *Pharm. Res.* **7**:1190–1194 (1990).
16. S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang, and R. Langer. Controlled delivery systems for proteins based on poly-(lactic/glycolic acid) microspheres. *Pharm. Res.* **8**:713–720 (1991).
17. L. M. Sanders, T. J. Kent, and G. I. McRae. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *J. Pharm. Sci.* **73**:1294–1297 (1984).
18. J. K. Li, N. Wang, and X. S. Wu. A novel biodegradable system based on gelatin nanoparticles and poly(lactide-co-glycolide) microspheres for protein and peptide drug delivery. *J. Pharm. Sci.* **86**:891–895 (1997).
19. S. Cohen, L. Chen, and R. N. Apte. Controlled release of peptides and proteins from biodegradable polyester microspheres: an approach for treating infectious diseases and malignancies. *React. Polym.* **25**:177–187 (1995).
20. X. H. Li, Y. H. Zhang, W. X. Jia, X. M. Deng, and Z. T. Huang. Influence of process parameters on the protein stability encapsulated in poly-*dl*-lactide-poly(ethylene glycol) microspheres. *J. Control. Release* **68**:41–52 (2000).