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Report

Release of Human Serum Albumin from Poly(lactide-co-glycolide) Microspheres[1]

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Human serum albumin (HSA) was encapsulated in a 50:50 copolymer of DL-lactide/glycolide in the form of microspheres. These microspheres were used as a model formulation to study the feasibility of controlling the release of large proteins over a 20- to 30-day period. We show that HSA can be successfully incorporated into microspheres and released intact from these microspheres into various buffer systems at 37°C. A continuous release of the protein could be achieved in physiological buffers at 37°C over a 20- to 30-day period from microspheres with high protein loadings (11.6%). These results demonstrate the potential of poly(DL-lactide-co-glycolide) microspheres for continuous delivery of large proteins.

KEY WORDS: protein release; human serum albumin; protein stability; biodegradable microspheres; poly(lactide-co-glycolide).

INTRODUCTION

Recent progress in the development of proteins has shown their importance as therapeutic agents. A new challenge is to develop controlled-release, parenteral delivery systems for these agents. Several approaches, such as chemical modification (1), liposomal encapsulation (2), and use of polymeric systems (3), for protein delivery have been investigated. Polymer-based protein delivery systems may be further subdivided into those based on nonbiodegradable and biodegradable polymers. The use of nonbiodegradable polymers for the controlled delivery of macromolecules has shown considerable potential as evidenced by numerous reports on the subject (4–7). For biodegradable polymers, the use of polyesters for controlled delivery of macromolecules was recently reviewed (8). One particular polyester system that holds considerable promise for the controlled delivery of proteins is based on biocompatible, biodegradable poly(lactide-co-glycolide)s (PLG). PLG microspheres have been investigated extensively for delivery of low molecular weight drugs (9,10). The use of polylactide and poly(lactide-co-glycolide) implants for the delivery of proteins has also been reported and promising results have been obtained (11,12). Recent research has established the usefulness of PLG microspheres for the delivery of peptides (13,14). These results, however, cannot be directly extrapolated to large proteins because of the many physical chemical differences that exist between peptides and proteins. In contrast to peptides, proteins are larger in size (15) and are further characterized by their complex and well-defined tertiary and/or quarterinary structure. These differences have important implications regarding the release behavior of proteins and their stability during encapsulation, as well as during residence within microspheres. In the present work, we evaluated the stability and *in vitro* release properties of human serum albumin (HSA) from PLG microspheres. HSA was chosen as a model protein for these studies because it is a well-characterized protein (16), is large in size (molecular weight, ~66,000 daltons), and is readily soluble in water (solubility at 25°C is greater than 250 mg/ml).

MATERIALS AND METHODS

Commercially available HSA (derived from normal human serum, heat treated and stabilized, 25% solution, U.S.P., Hyland) was lyophilized and used for microencapsulation. Lyophilization of HSA was carried out using a Lyolab G freeze-dryer (LSL Secfroid, SA). Vials containing HSA solution were frozen at −50°C for 4 hr, followed by sublimation for 24 hr at −30°C and 35-μm pressure and then drying at 15°C for 18 hr at the same pressure. Microspheres were prepared using an emulsion-based process whereby a mixture of PLG, lyophilized HSA, and methylene chloride was dispersed in water with subsequent removal of the solvent to afford discrete particles. The microspheres were then collected by filtration and dried in vacuum. The 52:48 poly(DL-lactide-co-glycolide) (DL-PLG) excipient used had an inherent viscosity of 0.73 dl/g (hexafluoroisopropanol, 30°C, 0.5 g/dl).

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The *in vitro* release of HSA from the microspheres was performed by incubating 20 mg of a microsphere sample in 2 ml of a desired buffer receiving fluid maintained at 37°C. The receiving fluid was periodically withdrawn and the release study was continued after replacement with fresh buffer. The withdrawn receiving fluid was quantified for the amount of HSA released by using the Lowry–Peterson assay (17). All receiving fluids was based on salts of sodium phosphate with 0.02% sodium azide as a bacteriostatic agent.

The amounts of HSA encapsulated per unit weight of microspheres was determined by digesting microspheres overnight in 0.1 *N* sodium hydroxide containing 5% sodium dodecyl sulfate (SDS). Sodium hydroxide catalyzes the hydrolysis of the PLG and SDS ensures the complete solubilization of the protein during the PLG hydrolysis. The resultant solution was then neutralized to pH 7 by stepwise addition of 1 *N* HCl. The Lowry–Peterson procedure was used to quantify HSA and thus the protein loading of the microspheres. As compared to an HSA standard, our results by size-exclusion chromatography [Superose 12 column (Pharmacia); mobile phase, 0.1 *M* ammonium sulfate, 0.01 *M* sodium phosphate, pH 7.0; UV detection; flow rate, 0.5 ml/min] indicated that there was no degradation of the protein during the sodium hydroxide treatment.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of HSA before encapsulation and after release into buffers was done as follows. Protein samples were mixed with 80 m*M* Tris-H_3PO_4 loading buffer (pH 6.6) containing 1% SDS and 5% β-mercaptoethanol, 25% glycerol, and 0.01% bromophenol blue, heated at 95°C for 3 min, and subjected to electrophoresis at 40 mA in vertical slab gels (18). Proteins were visualized by Coomassie blue (0.1%) in water–methanol–acetic acid (45:45:10, by volume). For isoelectric focusing (IEF) protein samples were applied to vertical slab gels (Ampholine PAG plates, pH 4.0–6.5, LKB) and run at 15 W constant power for 2.5 hr as described by Righetti *et al.* (19). Proteins were visualized by Coomassie blue as described above.

Scanning electron micrographs (SEMs) of microsphere samples were taken using a Cambridge Stereoscan instrument. The microsphere suspensions were placed on aluminum stubs, dried at room temperature, and coated with gold–palladium. These preparations were viewed at an accelerating voltage of 15 kV.

RESULTS AND DISCUSSION

Characterization of Microspheres

Poly(DL-lactide-co-glycolide) microspheres containing HSA were spherical in shape and fairly uniform in size. The diameter of these microspheres varied between 50 and 150 μm and the average diameter was 107 ± 40 μm as determined by SEM (n = 54). Microspheres loaded with 1.6 to 12.0% (w/w) HSA were used for these studies. Figure 1 shows a typical population of these microspheres.

Stability of the HSA During Encapsulation and Residence Within Microspheres

During microsphere formation, the HSA is subjected to conditions that may induce irreversible denaturation and/or self-aggregation, such as contact with methylene chloride, exposure to large air–water interfaces, and mechanical agitation. These conditions might convert the HSA into a form which is either partially or completely insoluble in aqueous media and, thus, render the protein unavailable for release from the microspheres. The presence of a surface active agent in the release medium would help to resolubilize the HSA. HSA-release experiments performed in the presence or absence of such an agent would thus serve as a good indicator to assess the effect of microencapsulation conditions on the protein. Toward this goal, we monitored the release of HSA from DL-PLG microspheres in buffers with and without 0.1% SDS; the amount of HSA released was determined by the Lowry–Peterson assay. Typical results are shown in Fig. 2. The two release profiles are similar and the bulk of the encapsulated protein (>90%) is released in 26 days of incubation. These results indicate that there is little

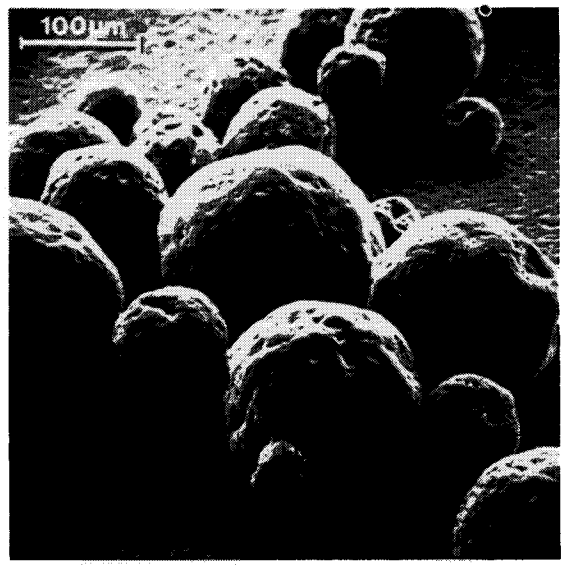

Fig. 1. Scanning electron micrographs of 11.6% HSA-loaded DL-PLG microspheres. 200×; reduced 20% for reproduction.

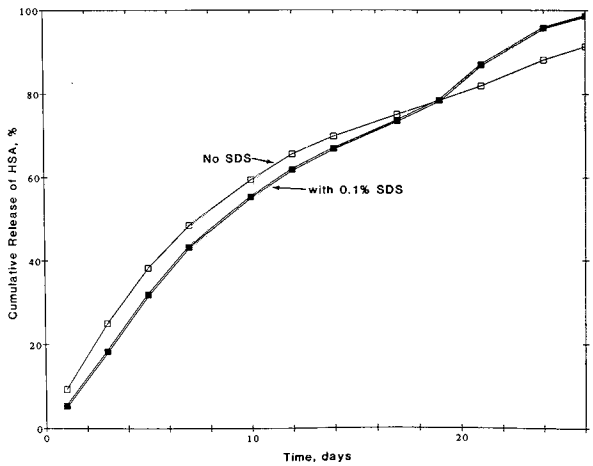

Fig. 2. Cumulative release from 12% HSA-loaded DL-PLG microspheres (as percentage of the originally encapsulated amount) in 20 m*M* sodium phosphate buffer (pH 7.5), with or without 0.1% SDS, plotted as a function of time (in days).

or no significant effect of the microencapsulation conditions on human serum albumin.

Next we wished to investigate if the HSA was irreversibly damaged during its residence in the hydrophobic polymer core after encapsulation and/or during incubation. We characterized the released HSA for nonreducible dimer and covalent oligomer formation by SDS-PAGE and for formation of any new charged species by IEF, using standard biochemical procedures. Figure 3 presents the SDS-PAGE profile of randomly selected release samples. As seen in this figure, no higher molecular weight species were seen in the released-HSA samples. Similarly, no change in the IEF pattern of HSA was seen after release in buffers at 37°C (Fig. 4). The SDS-PAGE and IEF data, taken together, indicate that the purity of HSA is not affected during encapsulation and residence within the microspheres and the protein is released in its pure form. The above characterization methods alone, however, do not completely rule out the possibility of generation of oxidized and reduced forms of HSA, formation of covalent adducts of HSA with lactic or glycolic acids, etc. These and other reactions and their degradation products should be considered when formulating a delivery system for an active protein.

Factors Affecting Release

In vitro release of HSA in various release media was monitored to evaluate parameters that could affect the release of HSA from microspheres. Specifically, we studied the effect of (1) ionic strength of the medium on the release of HSA to evaluate the performance of this delivery system under physiological ionic strength conditions and (2) protein loading of the microspheres on the HSA release to assess how this factor influenced the overall release kinetics. Similar studies done to characterize other polymeric delivery systems (4) suggest that these factors can have significant effects on the release kinetics of the encapsulated molecule.

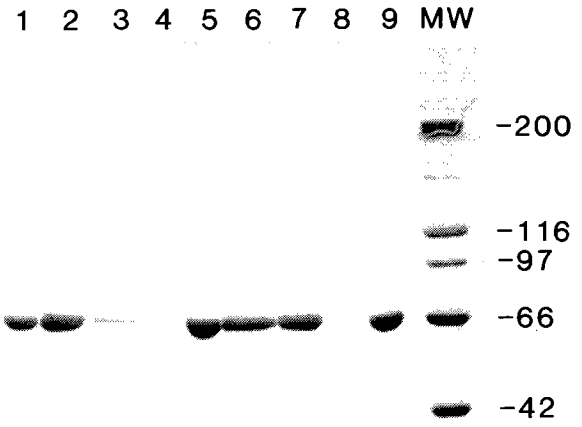

Fig. 3. SDS-PAGE behavior of HSA before encapsulation and after release from DL-PLG microspheres containing 11.6% HSA into buffers at 37°C. Lanes represent HSA released in PBS on Day 1 (1), Day 28 (2), and Day 42 (3); sample buffer (4); HSA released in 20 m*M* phosphate (pH 7.5) on Day 1 (5), Day 7 (6), and Day 21 (7); sample buffer (8); and HSA before encapsulation (9). Molecular weight standards are shown in the extreme right lane.

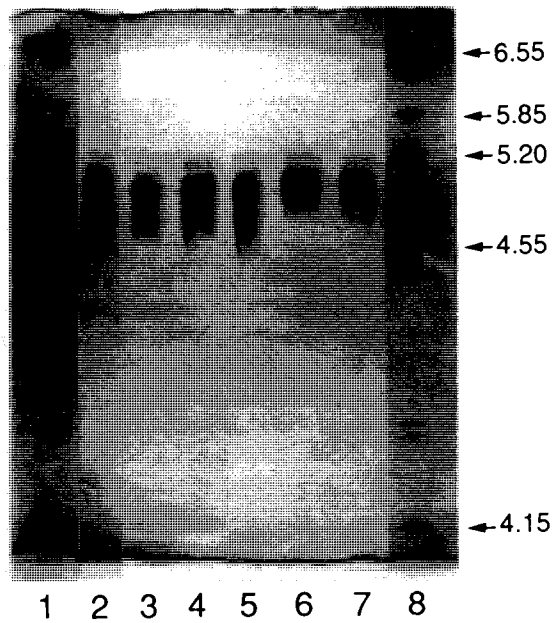

Fig. 4. Isoelectric focusing of HSA before encapsulation and after release from DL-PLG microspheres containing 11.6% HSA into buffers. Lanes represent pI standards (1), HSA before encapsulation (2), HSA released in PBS on Day 1 (3) and on Day 28 (4), HSA released in 20 m*M* phosphate (pH 7.5) on Day 1 (5), Day 7 (6), and Day 21 (7), and pI standards (8).

Ionic Strength of the Buffer. We studied the release of HSA from DL-PLG microspheres in sodium phosphate buffers (pH 7.5) of various ionic strengths and also in phosphate-buffered saline (PBS). Representative results are shown in Fig. 5. At a constant protein loading of 11.6 wt%, release of HSA from microspheres having similar size distributions decreased as the ionic strength of the buffer was increased. The release rate in PBS, which has an ionic strength of 154 m*M*, also fits into this pattern.

These release data are consistent with a triphasic profile

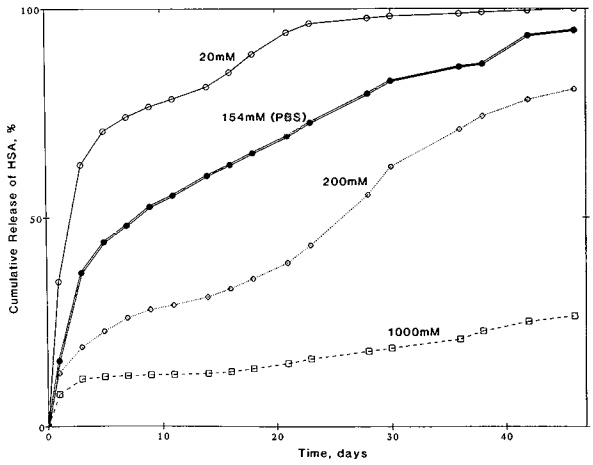

Fig. 5. Cumulative percentage release from 11.6% HSA-loaded DL-PLG microspheres versus time as a function of increasing ionic strength of the incubation in sodium phosphate (pH 7.5) buffer.

seen for the release of low molecular weight drugs (20), peptides (11,21), and proteins (13,14) from DL-PLG matrices. The three phases are (1) an initial burst comprising release of surface bound and poorly encapsulated drug, (2) a second phase which is derived from diffusional release (or a lack thereof, in which case it is known as an "induction period") during the period before bioerosion of the encapsulating polymer begins, and (3) release due to the bioerosion of the polymer matrix. As indicated above, if there is not enough diffusional release during the second phase, a period with very little or no release of the drug is observed. This is known as an induction period. For proteins, the diffusional release during the second phase probably occurs through water-filled networks of pores and channels. A similar mechanism has been proposed by Bawa *et al.* (7) for release of macromolecules from nondegradable polymers. Also, as suggested by Brown *et al.* for the release of insulin through pores and channels of nondegradable polymers (4), the cumulative release of the protein from these microspheres would be linear with respect to the square root of time during the diffusional phase. Such data treatment would, therefore, be useful for identification of the diffusional release phase from these microspheres.

The above results can also be visualized by plotting the cumulative release values as a function of the square root of time ($\sqrt{t}$) to evaluate the contribution of diffusional release in the period between the initial burst and the onset of bioerosion of the encapsulating polymer. As seen in Fig. 6, the release profiles in low-ionic strength buffers (20 m*M* phosphate and PBS) are marked by a burst phase during the first 3 days, followed by an approximately linear relationship up to Day 20, consistent with diffusion-controlled kinetics during this period. In 1 *M* phosphate buffer, practically no release of the protein is seen between Day 3 and Day 20, whereas release in 200 m*M* phosphate falls somewhere in between with a shorter induction period (Days 9 through 16). One possibility is that an increase in the ionic strength of the incubation buffer may reduce the swelling of the polymeric backbone due to an ion-shielding effect. This effect has been attributed to a reduction in swelling of hydrogel copolymer membranes of poly(hydroxyethyl methacrylate-*N,N*-dimethylaminoethyl methacrylate) (22). Lower swelling of the polymer backbone would also cause a reduction in the diffusional release of the protein through the microsphere excipient. Because the ionic strength of PBS (154 m*M*) is similar to that in the physiological environment (23), further studies were conducted in PBS.

Protein Loading. An increase in the protein loading of DL-PLG microspheres having similar size distributions resulted in higher rates of HSA release in PBS. Figure 7 presents typical HSA release data from microspheres by plotting cumulative release as a function of $\sqrt{t}$ at three different HSA loading levels. As seen in the figure, no protein is released during the first 18 days of incubation at the lowest protein loading (1.7 wt%) studied. At the highest protein loading (11.6%), a high initial burst of HSA release is observed in the first 3 days. During Days 3 through 18, only the highest-loaded microspheres (11.6 wt% HSA) showed a near-linear release of HSA as a function of $\sqrt{t}$ with no induction period.

From the protein loading/*in vitro* release experiments, a triphasic pattern of HSA release *in vitro* from DL-PLG microspheres is observed. The burst period occurs during the first 3 days of incubation. At the lowest protein loading, an absence of this phase indicates that the entire protein is internalized during the process of microsphere formation. The second phase is characterized by an induction period (Days 3 through 18) at low protein loadings and by a diffusional release at the high protein loading. At high loading levels, the protein is well distributed in the microsphere such that enough is present near the surface region to be available for release initially and that elution of this protein leaves an extensive pore structure to facilitate further elution of the protein from the inner portion of the microsphere. The third phase of release occurs when the polymer weight loss begins

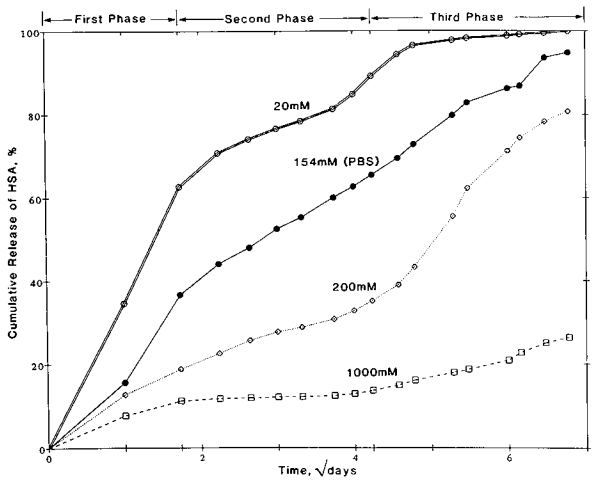

Fig. 6. Cumulative percentage release from 11.6% HSA-loaded DL-PLG microspheres versus square root of time as a function of increasing ionic strength of the incubation buffer. The three phases of release are indicated at the top of the graph (see text for the description).

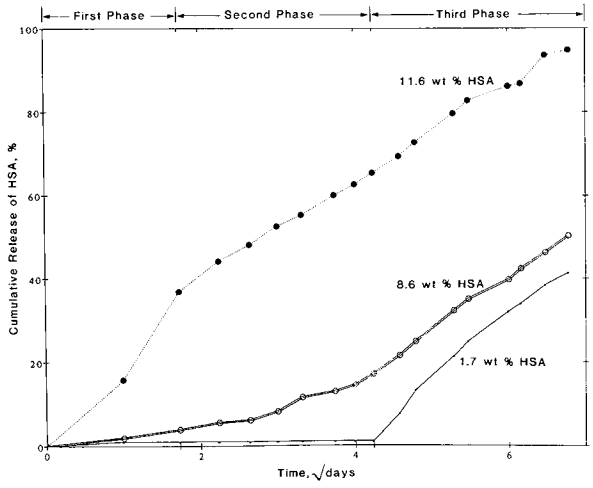

Fig. 7. Cumulative percentage release of HSA from DL-PLG microspheres in phosphate-buffered saline (154 m*M*) versus square root of time as a function of increasing protein loading. The three phases of protein release are indicated at the top of the graph (see text for further details).

to take place and the protein diffuses through the existing pores and channels, and those which are created following bioerosion. The third phase is separated from the second only at the two lower loadings (1.7 and 8.6 wt%). At the highest loading studied (11.6 wt%), merger of the second and third phases is observed.

CONCLUSIONS

Human serum albumin can be successfully incorporated in poly(DL-lactide-co-glycolide) microspheres and can be released intact into various buffer systems at 37°C. A continuous release of the protein could be achieved into physiological buffers at 37°C over a 20- to 30-day period from microspheres with high protein loadings (11.6 wt%). In contrast, release of the protein from lower protein-loaded microspheres in buffers containing physiological concentration of salt was marked by the presence of an induction period. These results suggest that at high HSA loadings enough protein is present near the surface of the microspheres for release initially and that depletion of this protein would leave an extensive pore structure to facilitate more rapid diffusional release of HSA from the internal core of the microspheres before the onset of bioerosion. These studies demonstrate the potential of poly(DL-lactide-co-glycolide) microspheres for continuous delivery of large proteins over a 20- to 30-day period.

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