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Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres

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Abstract

Using lysozyme as a model protein, this study investigated protein stability, protein–polymer interaction in different release media and their influence on protein release profile and in vitro–in vivo correlation. Lysozyme was microencapsulated into PLGA 50:50 by a double emulsion–solvent extraction/evaporation method. Protein stability, protein–PLGA adsorption and protein in vitro release were studied in various test media. Differential scanning calorimetry analysis showed lysozyme to be most conformationally stable in pH 4.0 acetate buffer with highest T_m at 77.2 °C and ΔH_{cal} 83.1 kcal/mol. Lysozyme exhibited good stability in pH 2.5 glycine buffer with T_m at 63.8 °C and ΔH_{cal} 69.9 kcal/mol. In pH 7.4 phosphate-buffered saline (PBS), lysozyme showed a trend toward aggregation when the temperature was elevated. When PLGA polymer was incubated with lysozyme in the various buffers, adsorption was found to occur in PBS only. The adsorption severely limited the amount of lysozyme available for release from microspheres, resulting in slow and incomplete release in PBS. In contrast, the release of the microspheres in acetate and glycine buffers was complete within 40 and 70 days, respectively. Radiolabeled lysozyme blood levels in rats from the microspheres correlated qualitatively well with in vitro release in glycine buffer as a release medium. This study suggests that protein stability and adsorption are critical factors controlling protein release kinetics and in vitro–in vivo correlation of PLGA microspheres.

Keywords: Lysozyme; PLGA; Stability; Release; Interaction

1. Introduction

For the past decade, research has accelerated on the delivery of protein and peptide drugs in biodegradable injectable microspheres made of poly(D,L-lactide) or poly(D,L-lactide-co-glycolide) (PLGA) [1–3]. Entrapment in these polymers can protect the protein and peptide drug from proteolysis, prolong drug release and enhance therapeutic efficacy. Various peptides have been prepared in this type of delivery system including several luteinizing hormone-releasing hormone LHRH analogues [4–6], octreotide [7] and salmon calcitonin [8]. Nearly linear release after an initial burst has been achieved

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with LHRH analog loaded PLGA microspheres for 1-month therapy [4]. However, as polymeric carriers for high-molecular-mass protein drugs, there is growing concern that PLGA may not provide a suitable microenvironment for many encapsulated protein molecules. Proteins, as macromolecules, vary in their size, solubility, shape and since most proteins are subject to denaturation, it has been difficult to prepare a controlled release dosage form without loss of biological activity [9].

While most of the available reports have investigated the effect of polymer characteristics, e.g., molecular mass and copolymer ratio, on the morphology and prolonged release kinetics [10,11], the importance of the in vitro release conditions seems to have been neglected. For example, a number of studies focused on protein release kinetics without sufficient attention to protein stability. Generally, microspheres are incubated in a certain volume of release medium consisting of phosphate-buffered saline (PBS), a preservative and, optionally, a surfactant. The experiment is then performed in glass or plastic tubes or flasks at 37 °C with or without agitation [12,13]. Alternative experimental set-ups have been investigated for a non-protein drug [14]. In many release studies using microspheres, protein release kinetics are often unpredictable; the devices exhibit an initial burst release followed by a very slow release over an extended period [15–17], and then culminate with incomplete release despite significant polymer degradation [18]. Consequently, correlation between in vitro release and in vivo behavior of protein drugs from microspheres has been difficult to achieve [19,20].

For peptide and protein drugs, the influence of the composition of release medium is most important [21]. In vitro release assessment of protein or peptide from PLGA microspheres generally requires optimization of the test conditions for a given molecule especially if the goal is in vitro/in vivo correlation. For example, octreotide release slowed down with increasing ionic strength [7]. Another LHRH analog, orntide, showed nearly complete release in acetate buffer, but not in phosphate buffer [22]. Besides the release kinetics, the stability of the released material in the medium is also critical. Many proteins are not stable in buffer media at 37 °C. Chemical degradation (cleavage, oxidation, reduction, etc.) and physical changes (conformational, aggregation, adsorption on surfaces) have been reported [23,24]. Considering all these factors, it becomes obvious that in vitro release tests are complex and require careful attention and selection for each individual formulation based on the protein properties.

The goal of the present study was to further investigate the effect of protein stability and protein–polymer interaction in different buffers on protein release profiles from PLGA microspheres. Lysozyme was selected as a model protein for encapsulation into PLGA using a double emulsion solvent extraction/evaporation method. The in vivo work was performed to assess in vitro–in vivo correlation and allow a better prediction of in vivo performance. Such information is essential for the development of sustained release microsphere formulations.

2. Materials and methods

2.1. Materials

PLGA (50:50, M_w 7831, M_n 4544) with free carboxyl end groups was purchased from Boehringer Ingelheim (Ingelheim, Germany, RG 502H). The lysozyme and poly(vinyl alcohol) (M_w 3000–7000, 88% extent of hydrolysis) were obtained from Sigma. $Na^{125}I$ was purchased from ICN Biomedicals (Irvine, CA, USA) and a bicinchoninic acid (BCA) kit was from Pierce (Rockford, IL, USA).

2.2. Radiolabeling lysozyme

A 1-mg amount of lysozyme was radiolabeled with 0.1 mCi of $Na^{125}I$ by the chloramine-T method. The reaction was carried out for 10 s in PBS and quenched by the addition of sodium metabisulfite. Labeled protein was separated from the free iodine using a Sephadex G-25 column (15×1.0 cm).

2.3. Differential scanning calorimetry (DSC) studies for lysozyme stability

DSC was performed on a VP-DSC from MicroCal, LLC. (Northampton, MA, USA) over the temperature range 25–95 °C with constant scan rate 90 °C/h. Sample solutions with 1 mg/ml lysozyme were

prepared using three different buffers which include 0.1 M PBS (pH 7.4), 0.1 M acetate buffer (AB) (pH 4.0) and 0.1 M glycine–HCl buffer (GB) (pH 2.5). The samples were degassed by stirring under vacuum before scanning. Data analysis was carried out using Origin® software provided with the instrument.

2.4. Size-exclusion chromatography (SEC) for lysozyme

The intact lysozyme was analyzed by SEC (Column Phenomenex BIOSEP SEC-S2000). Samples of 20 μl were eluted with 0.1 M PBS at pH 7.4 at a flow rate of 0.5 ml/min and detected at 280 nm.

2.5. Lysozyme–PLGA interaction

Fresh lysozyme solutions at 200 μg/ml were prepared in 0.1 M PBS (pH 7.4), 0.1 M AB (pH 4.0) and 0.1 M GB (pH 2.5). A 4-ml volume of each solution was incubated with 20 mg RG502H polymer at 37 °C with orbital rotation. At predetermined time points, the samples were centrifuged and the supernatant was injected onto the SEC column. Additionally, 4 ml of the same lysozyme solution in 0.1 M PBS was incubated with 10, 20 or 40 mg PLGA at 4, 25 and 37 °C for 24 h and the supernatant was analyzed. Samples were in duplicate and controls in the absence of PLGA were included.

2.6. Preparation of microspheres

Lysozyme loaded microspheres were prepared by a double-emulsion solvent extraction/evaporation technique. The organic phase, 30% (w/w) PLGA solution in methylene chloride was added to the inner aqueous phase, cold and hot lysozyme solution dissolved in PBS, and the mixture was sonicated for 30 s with a W-370 sonicator (Ultrasonics) using a microtip probe with an output setting of 3. The primary emulsion was then added to a 100 ml aqueous solution of 6% (w/v) poly(vinyl alcohol) at 4 °C. The second emulsion was formed by homogenization for 2 min at 3000 rpm on a Silverson (East Longmeadow, MA, USA) homogenizer. The resultant water-in-oil-in-water (w/o/w) emulsion was transferred to 1 l of deionized water and stirred continuously for 1 h at 4 °C; the temperature was then elevated to 40 °C and stirring continued for another 2 h to enhance solvent removal. The hardened microspheres were centrifuged, washed three times with deionized water, freeze–dried and stored at 4 °C.

2.7. Microsphere characterization

The morphology and size of the microspheres were analyzed by scanning electron microscopy (SEM) (Hitachi Model S800) and laser light diffraction (Malvern Instrument, UK). To determine drug content, triplicate samples of 10 mg of microspheres were dissolved in 1 ml 1 M NaOH by overnight rotation; then the solution was neutralized with 1 ml 1 M HCl. A 0.5-ml volume of the above solution was counted in a γ-counter (Packard COBRA™II, Meriden, CT, USA). The same sample was also measured by BCA protein assay.

2.8. In vitro release

The in vitro lysozyme release was determined by suspending 20 mg of microspheres in 1.5 ml of the buffers and rotating the suspensions at 37 °C. At regular intervals, samples were centrifuged and the supernatant was removed for γ counting. Fresh replacement media was added to resuspend the microspheres.

2.9. In vivo study

The lysozyme loaded PLGA microspheres were tested in rats (male Sprague–Dawley, 360–420 g). The animals were housed in groups of two in a well ventilated environment under controlled temperature (22±1 °C) and humidity (60±5%), with food and water made available ad libitum. Four rats were given the microspheres by subcutaneous injection at the neck region. The dose of protein was 4.5 mg/kg. At different time intervals, 0.8 ml blood was collected from the tail vein. The serum was gathered after blood clotting and centrifugation. A 200-μl

volume of serum was counted to assess the serum lysozyme level.

3. Results and discussion

3.1. Protein stability

Protein molecules have hierarchy structure and when the secondary or tertiary structures change, the biological function of the protein is usually lost or modified. In this study, DSC measures the transition midpoint temperature (T_m) and transition enthalpy (ΔH_{cal}) for transition from folded native state to unfolded state. Thermal transitions were fitted to a single peak non two-state unfolding model, and the ΔH_{cal} determined by fitting. Higher T_m and ΔH_{cal} indicate greater protein conformational stability. In pH 4.0 AB, the lysozyme showed maximum stability with T_m of 77.2 °C and ΔH_{cal} of 83.1 kcal/mol (Fig. 1). In pH 2.5 GB, the T_m shifted to 63.8 °C with ΔH_{cal} of 69.9 kcal/mol, still exhibiting fairly high stability even though the T_m and ΔH_{cal} were slightly lower than in the acetate buffer. Lysozyme solution in pH 7.4 PBS displayed a much different DSC profile. The lack of an endothermic peak and the downward trend of the profile at 70–80 °C suggested that unstable lysozyme molecules spontaneously aggregated and precipitated from solution at increased temperature.

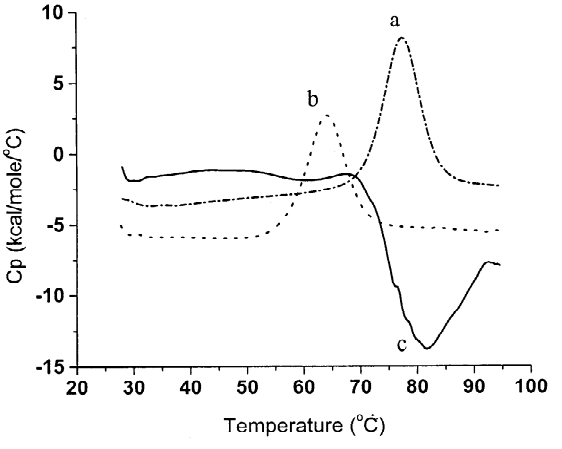

Fig. 1. Differential scanning calorimetry (DSC) of lysozyme 1 mg/ml in (a) pH 4.0 acetate buffer, (b) pH 2.5 glycine buffer and (c) pH 7.4 PBS.

3.2. Lysozme–PLGA interaction and adsorption

The emulsion processing method can generate microspheres possessing a broad degree of surface areas, governed by the extent of porosity. Accordingly, it is expected that a large amount of encapsulated protein could become non-specifically adsorbed to the polymer surface and subsequently hinder the release of protein. In a previous study, the slow release rate of calcitonin from PLGA microspheres was attributed to an adsorption phenomenon [25]. Likewise, lysozyme adsorption onto the surface of blank PLGA microspheres was observed and pegylation of the protein has been reported to result in a reduced adsorption [26]. Therefore, selection of an appropriate release medium to minimize adsorption might be important in achieving an adequate release. When lysozyme in different buffers was incubated with PLGA polymer at 37 °C, it was observed (Fig. 2a) that in PBS, 25% protein molecules adsorbed to the polymer within 1 h and after 48 h, only 63% of lysozyme remained in solution. In the SEC chromatogram, the molecular mass peak was similar, eliminating the possibility of soluble aggregates in PBS. However, lysozyme did not show any adsorption to PLGA in pH 4.0 AB or pH 2.5 GB. The lower interaction with PLGA and localization in the aqueous phase enhances retention of protein in the hydrated and folded state. A control in the absence of polymer in all three release media showed no change of lysozyme concentration. The high adsorption in PBS suggested exposure of the hydrophobic region of the protein thereby giving rise to a stronger interaction with the polymer. The irregular and incomplete release in PBS was therefore attributed to the interaction.

To have a better understanding of the lysozyme–PLGA interaction in PBS, the incubation was also carried out for 24 h at different temperatures (4, 25 and 37 °C) with varying amounts of PLGA. Fig. 2b illustrates low binding of lysozyme to PLGA at 4 °C (<5 μg/mg) but binding increased substantially at higher temperatures. For example, the lysozyme bound to 20 mg PLGA 502H at 4, 25 and 37 °C was

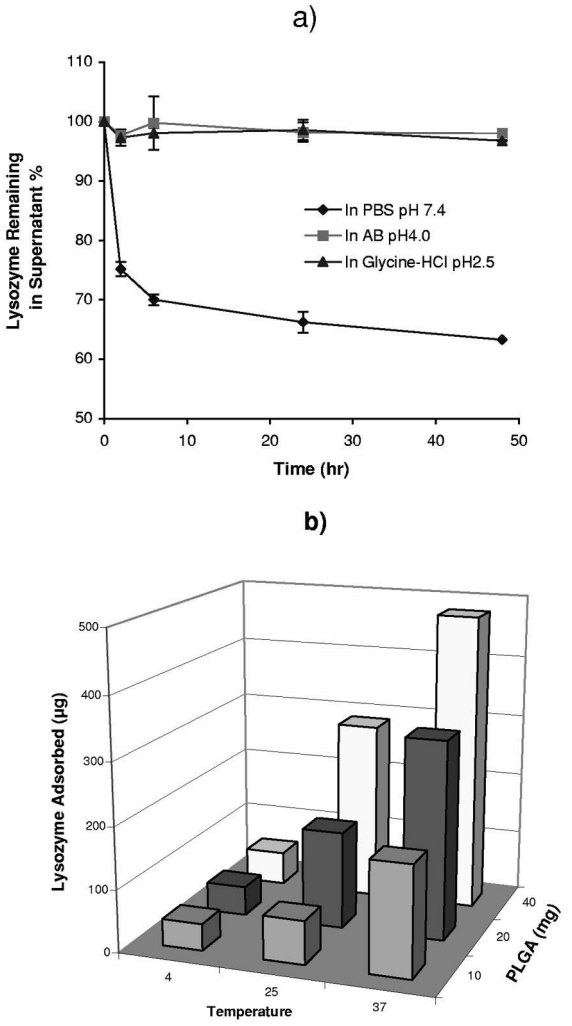

Fig. 2. (a) The influence of release media on the adsorption of lysozyme to PLGA polymer. (b) The influence of temperature and polymer amount on the adsorption of lysozyme to PLGA in 0.1 M PBS.

47, 156 and 320 μg, respectively. Protein binding increased with increase in the polymer amount. This is expected due to the increase in surface area. This result served to verify that the protein disappearance from solution was most likely due to binding to the polymer rather than self aggregation and precipitation since the latter will depend on the protein concentration but not be dramatically affected by amount of polymer.

3.3. In vitro and in vivo characterization of microspheres

3.3.1. Microsphere evaluation

Particle size distribution of the microspheres was unimodal with a mean diameter of 26.3 μm and 90% in the range of 10.3–63.6 μm. The scanning electron micrographs in Fig. 3 show spherical particles with uneven surfaces and visible pores. Apparently, the 50:50 comonomer composition and the low molecular mass of PLGA (7831D) play a critical part in determining the external morphology. The low crystallinity of the polymer and low organic phase viscosity resulted in an uneven surface. The encapsulation efficiency of lysozyme was 85% by γ counting, and BCA assay yielded a similar result. The satisfactory incorporation can be ascribed to an appropriate volume ratio of the inner aqueous phase and external organic polymer phase. After the fabrication of microspheres, the encapsulated lysozyme was extracted for both sodium dodecyl sulfate–polacrylamide gel electrophoresis (SDS–PAGE) (data not shown) and substrate assay. An intact protein band and 97.7% specific activity were observed. The residual enzyme in the microspheres during release was extracted for electrophoresis and no aggregation or degradation band was observed.

3.3.2. In vitro release kinetics

The in vitro release of lysozyme from the microspheres depended greatly on the type of release medium (Fig. 4). In PBS, pH 7.4, 14% of the protein was initially released within the first 24 h followed by very slow release for 70 days. During this interval, only 13% additional protein was released. On termination of the release study, cumulative lysozyme release reached 27%. The release profile at pH 7.4 was inconsistent with expected PLGA polymer hydrolysis and degradation. The small increments of lysozyme release provides compelling evidence that the protein either formed insoluble aggregates or adsorbed to the polymeric surface especially when the erosion of PLGA enlarged the polymeric surface and provided additional sites for protein adsorption. This is in good agreement with the protein stability and adsorption results in PBS. The stability and adsorption can be either individual phenomena or occur in a simultaneous manner. For

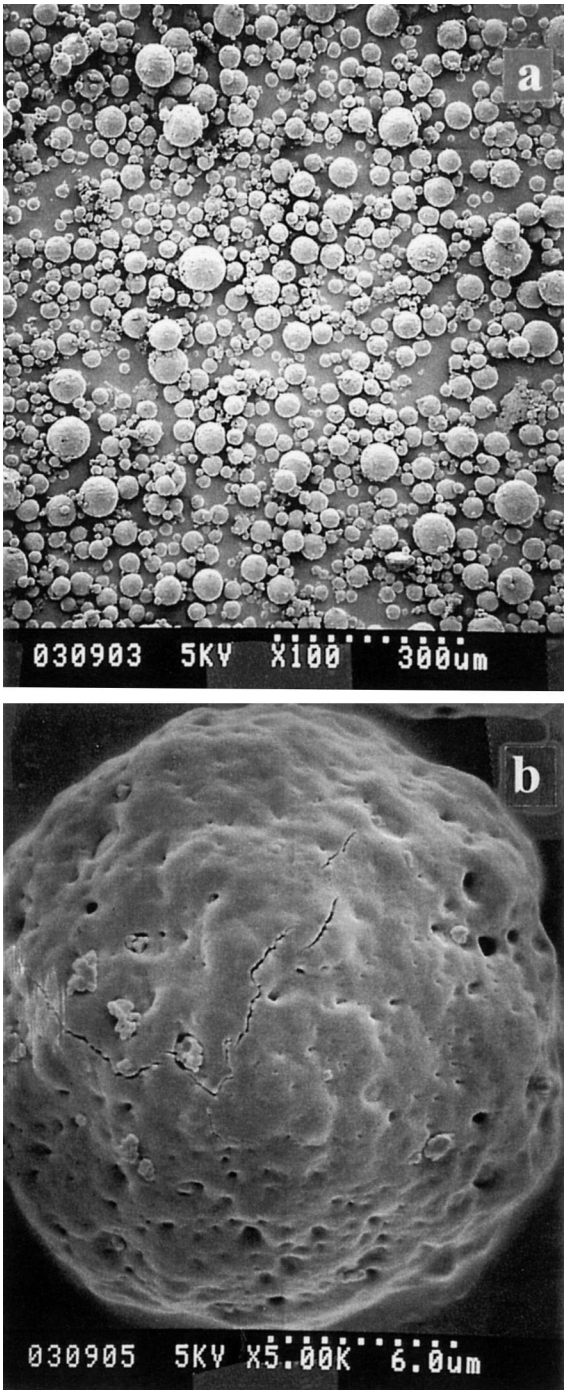

Fig. 3. Scanning electron microscopy of (a) size distribution of lysozyme loaded PLGA microspheres (100× magnification) and (b) surface morphology of lysozyme loaded PLGA microspheres (5000× magnification).

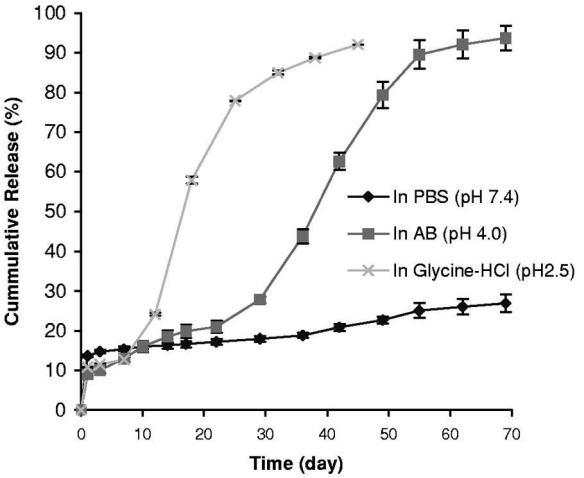

Fig. 4. In vitro release of lysozyme loaded PLGA microspheres in different release media at 37 °C for 70 days.

example, in PBS, marginal protein stability may facilitate protein molecules to partially unfold, leave the aqueous phase and adsorb to the hydrophobic polymer surface. One the other hand, the adsorbed protein molecules could form a dense local concentration, thereby further enhancing aggregation.

When the lysozyme loaded microspheres were incubated in pH 4.0 AB or pH 2.5 GB, three-phase mode release profiles were evident with an initial burst of about 10% in the first day due to the surface localized protein molecules. In the second phase, the protein molecules released via diffusion through pre-existing pores and channels in the polymer matrix. Duration of this slower diffusion-controlled pattern depended on the acidity of release media. For example, at pH 4.0, the diffusion lasted for 3 weeks. Around day 21, the polymer erosion progressed, resulting in more rapid release; this persisted to day 60. At pH 2.5, from day 1 to day 7, the release was via diffusion, which was followed by a steep slope of release due to polymer erosion in the following 3 weeks. The polymer could degrade faster to permit matrix erosion in the more acidic media due to the presence of protons [27]. The overall release at pH 4.0 and 2.5 was over 90% of the protein content in the microspheres. The complete release profiles in AB and GB correlate well with the stability and adsorption data. Good protein stability and lack of

protein adsorption in either AB or GB resulted in complete release.

3.3.3. In vivo study

After subcutaneous injection of lysozyme loaded microspheres in rats at the neck region, the lysozyme serum level showed an initial peak reaching 250 ng/ml on the first day followed by a decline through day 5 (Fig. 5). The protein level increased again to 250 ng/ml around day 10 and then decreased slowly through day 28 when the serum lysozyme fell below detectable level. The release pattern of lysozyme as a model protein may have clinical importance for therapeutic proteins because the present formulation does not have a high burst release nor the occurrence of an extended period of little or no release. The initial lysozyme serum peak is almost of the same magnitude as the broad peak induced by polymer degradation. Previous reports showed an in vivo profile exhibiting an extremely high initial peak followed by a plateau less than one sixth of the former peak height [28].

3.3.4. In vitro–in vivo correlation

Comparing the lysozyme in vivo release with the in vitro release in the three buffer systems, it was observed that GB represented the most desired in vivo behavior of the formulation. For example, the initial burst in GB was about 10% (Fig. 4) and the normalized percentage from first serum lysozyme peak area (days 0–4) was also around 10% (Figs. 5 and 6). In vitro polymer erosion lasted 3 weeks, which was qualitatively in good agreement with the serum lysozyme profile. The maximum in vitro release rate was from day 12 to day 18, approximately the same as the second serum lysozyme peak time in vivo. In Fig. 6, the in vivo area under the curve (AUC) was plotted with the in vitro release in the GB. The two curves ramp up with a similar slope and they are almost parallel. A question may be raised that the GB at pH 2.5 is much more acidic than body fluid and thus could not simulate the in vivo situation. Nevertheless, since the PLGA degrades by hydrolysis and is acid-catalyzed, a low pH environment within the microspheres would result in faster degradation. Several studies, using indirect or direct methods, have shown the presence of an acidic environment within degrading polymer devices. For example, Goepferich [29] has used pH-sensitive dyes and confocal microscopy to measure the pH immediately surrounding degrading anhydride tablets and found it to be lower than that of the surrounding bulk media. In addition, Shenderova et al. [30,31] developed several methods to examine the pH with microspheres and films, and concluded in each case

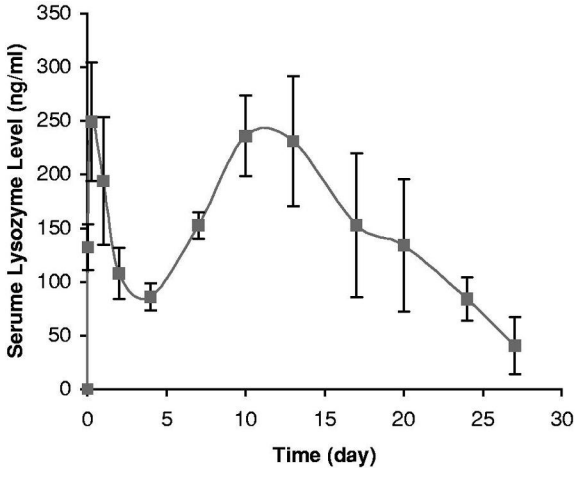

Fig. 5. In vivo serum lysozyme level after single administration of lysozyme loaded microspheres (dose 4.5 mg lysozyme/kg, $n=4$).

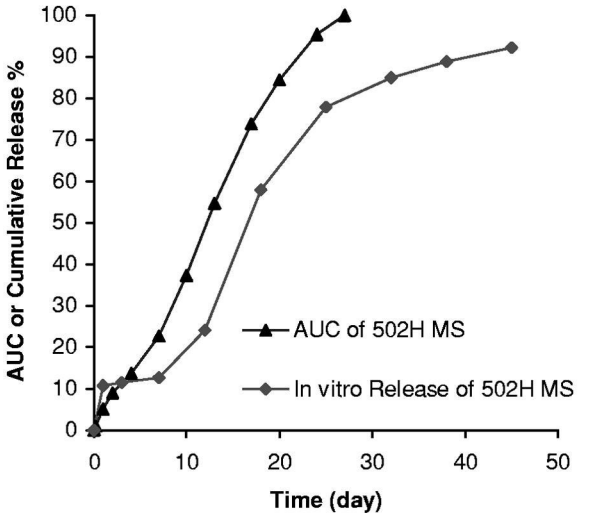

Fig. 6. In vitro lysozyme release in glycine buffer and in vivo release; the latter was plotted as cumulative area under serum level curve normalized as percent of the total area.

that the microenvironment had a pH below 5. More recently, Fu et al. [32] semiquantitatively determined the intrapolymer acidity by entrapping pH-sensitive fluoresent dyes within the microspheres and imaged them with confocal fluoresence microscopy. Their research showed the formation of a very acidic environment (1.5–3.5) within the microparticles with a pH as low as 1.5 in the center of the microsphere. Therefore, during polymer erosion, the drug located in the central of the microspheres may release first due to faster polymer degradation. In such cases, release will be greatly controlled by the intrapolymer pH rather than that of the bulk release medium. Furthermore, the subcutaneously injected microspheres are not surrounded by a large volume of buffer as in the in vitro condition. Therefore, a different hydrodynamic situation could be at play in such depot forms. Data from in vitro release studies at physiological pH may not always give good insight into the performance of a dosage form in vivo. Therefore, it should not be surprising that a better in vitro–in vivo correlation could be observed with glycine buffer.

The in vivo conditions involve a great deal of complexity which would be impossible to simulate in vitro, such as the presence of hydrolytic enzymes, the effect of plasma proteins on the polymer degradation and the pH gradient. Despite these inconsistencies, a simple release test is still required as a quality control procedure to demonstrate performance and reproducibility in the manufacturing of a microsphere product. Our findings suggest that the selection of appropriate release test media and conditions based on protein stability and adsorption could provide more meaningful in vitro release data for further development and improvement of dosage form design.

4. Conclusions

The model protein, lysozyme, was efficiently microencapsulated into end group uncapped PLGA 50:50 by a double emulsion solvent extraction/evaporation method. Lysozyme displayed high stability and lack of adsorption to PLGA in pH 4.0 acetate buffer and pH 2.5 glycine buffer, whereas in pH 7.4 PBS, the protein stability was low and significant protein adsorption was evident. The differences in release profiles observed in the above three media underline the importance of testing the in vitro release in an appropriate fluid in which protein stability and adsorption should be considered. The in vivo study demonstrated a sustained protein release pattern without high burst and the profile correlated best with in vitro release in glycine buffer, suggesting that the acidic microenvironment in the degrading PLGA is important.

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