Sustained Release of Human Growth Hormone from PLGA Solution Depots

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Received May 17, 1999; accepted August 30, 1999

Purpose. The effects of altering the dynamics of phase inversion of a polylactic glycolic acid (PLGA) solution depot on the sustained-release delivery profile of human growth hormone (hGH) were evaluated. The impact of adjusting the protein particle composition was also studied in a slow phase-inverting formulation.

Methods. Protein release profiles of depots prepared from four model solvents were generated by injecting formulations into the subcutaneous space of normal rats and monitoring hGH serum levels over the course of 1 month. Scanning electron microscopy, Coulometric Karl Fischer titration, size-exclusion liquid chromatography, and reversed-phase liquid chromatography were used to observe depot morphologies, bulk water absorption, PLGA degradation, and protein particle dissolution rates, respectively.

Results. An extended-release profile and significantly reduced burst effect resulted when the aqueous affinity of the depot solvent was reduced. As seen earlier in *in vitro* experiments, lowering the solvent's aqueous affinity slows the phase inversion rate, which in turn produces depot morphologies favorable to prolonged release. Protein burst on injection was entirely eliminated in a slow phase-inverting formulation by densifying the lyophilized protein particles. Unlike the use of metal cations to prolong release of some proteins in PLGA microsphere depots, this technique is more universal, and thus is potentially usable with any protein or highly soluble drug agent. The onset of biodegradation was observed to occur at 14 days for all depot formulations, however the bulk biodegradation rate slowed as the aqueous affinity of the depot solvent decreased. This result supports the hypothesis that, in a slow phase-inverting system, drug release over the first few weeks is governed by the diffusion rate of drug through the polymer solution.

Conclusions. By taking advantage of the effects of low aqueous affinity and protein particle densification, a PLGA solution depot was produced with the capability of sustaining hGH levels in normal rats at a serum level of 10 to 200 ng/ml for 28 days.

KEY WORDS: phase inversion; sustained-release depot; human growth hormone.

INTRODUCTION

A number of drug delivery companies are developing injectable biodegradable implants for the sustained delivery of peptide and protein therapies (1–5). These technologies include hydrogels, microparticles, and the emerging field of polymer solutions. A well-developed protein delivery system must stabilize the protein, limit the burst effect on injection, and release drug within the therapeutic requirements for the duration of treatment. The ease of administration and the cost-effectiveness of the manufacturing process must also be considered.

Injectable microparticle and solid matrix depots formulated from polylactic glycolic acid (PLGA) have long been used for the delivery of pharmaceutical agents (6–7). Although these systems are well proven for small molecules, there are only a few successful applications for peptide and protein therapies (8–10). In these systems the need to encapsulate the protein into a solid matrix and the total mass requiring encapsulation limit the type of protein that can be successfully stabilized and processed.

The emerging polymer solution platforms consisting of PLGA and a biocompatable solvent have tremendous potential for meeting the needs of biopharmaceutical macromolecule therapies. In polymer solution formulations the protein is not processed into a solid PLGA matrix. Instead, a protein particle is prepared using standard lyophilization or spray-drying techniques, then mixed into the polymer solution to form a suspension. PLGA solutions undergo liquid-liquid phase separation on injection into the subcutaneous space; this phenomenon is referred to as phase inversion (11–13). In previous in vitro work, (14,15) drug burst and the subsequent protein release-rate profile was shown to be integrally tied to the dynamics of phase inversion, which in turn is governed by the thermodynamics and mass transfer properties of the PLGA solution.

PLGA solution depots can be divided into two groups, distinguished by the rate at which the system undergoes phase inversion. In previous work (15), *in vitro* techniques demonstrated that protein release from a polymer solution depot is controlled by the phase inversion dynamics. Altering the solvent strength and solvent/non-solvent affinity of the PLGA solution was used to control the rate of phase inversion. In the classical system studied, the strong affinity between the solvent 1-methyl-2-pyrrolidinone (NMP) and non-solvent (water) enhanced the formation of a rigid, interconnecting porous structure in the precipitated gel. A burst effect was observed when the depot was injected into an aqueous environment because of the rapid release of a major fraction of the protein that resided in or near pores. Once the porous volume was depleted, the system shut down as the remaining drug was trapped in the hardened polymer matrix. When the strength and non-solvent affinity of the depot solvent were decreased, a new class of PLGA solution depot emerged, characterized by slow phase inversion dynamics that produce a nonporous, viscous-to-semi-solid mass on injection into an aqueous environment. The latter depots were found to produce a greatly reduced initial release and a prolonged sustained release, governed by the rate of diffusion of protein through the gel-like depot.

The addition of divalent cations to a lyophilized human growth hormone (hGH) formulation is known to decrease solubility and dissolution (16–22). This technique was successfully used to reduce burst in a microsphere depot in which zinc is bound to the histidine sites in the protein, forming a less soluble complex (9). Alternatively, densification of a solid substrate can significantly decrease the solubility rate of solid drugs taken

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ABBREVIATIONS: hGH, human growth hormone; ICMA, immunochemiluminescent assay; NMP, 1-methyl-2-pyrrolidinone; PLGA, polylactic glycolic acid; SEM, scanning electron microscope; THF, tetrahydrofuran; UV, ultraviolet.

0724-8741/99/1200-1825$16.00/0

orally. Here, reducing the surface-to-volume ratio slows drug dissolution in the gastrointestinal tract. Similar techniques may prove useful in further controlling injection burst in PLGA solution depots.

In this work, protein release from PLGA solution depots was examined in a small animal model. Four solvents, with strong to mild solvent/non-solvent (water) affinity and solvent strength, were used to elucidate the dependencies of the *in vivo* protein release on the system thermodynamics and mass transfer characteristics. Additionally, the concept of further controlling injection burst and prolonging release by engineering the protein particle formulation to dissolve more slowly and attract less water was examined.

MATERIALS AND METHODS

Polymer solutions consisted of a 1:1 weight ratio of poly-lactic-co-glycolic acid (PLGA Resomer 502, MW 12,000, Boehringer-Ingelheim) and solvent. The solvents, reagent grade 1-methyl-2-pyrrolidinone (NMP), triacetin, ethyl benzoate and benzyl benzoate from Sigma, all of which are currently utilized in injectable delivery systems (23), were used as received. Polymer solutions were prepared by mixing PLGA with solvent in a 10 ml glass vial. Solutions readily formed in NMP at room temperature. In order to effect good solubilization with the other three solvents, it was necessary to seal the mixture and heat it to 37°C for 24 to 48 hours, with periodic stirring to break up lumps and remove trapped air bubbles.

Human growth hormone particles were suspended in the PLGA solution at a level of 10% by weight. The hGH, with methionine attached, derived from *E. coli* (Bresagen), was supplied with the stabilizing excipients mannitol and glycine. The protein was dialfiltered to remove the excipients, then relyophilized. Protein particles consisted of 3 to 5% hGH, 0.1 to 5.1% Tris buffer, 0 to 5% zinc acetate (ZnAc), and up to 5% stearic acid used to densify the particle. Densification was achieved by compressing the lyophilized protein and excipients in a Carver press at 3,000 psi and room temperature. The densified material was reground to a powder and then passed through a 212 μm screen. When used, ZnAc was added to the solubilized protein and incubated at room temperature to form a zinc-hGH complex prior to lyophilization. In the water absorption experiments, lysozyme was substituted whenever possible for hGH to conserve the expensive drug material. In the lyophilized form, both proteins dissolve immediately in water and demonstrate the same ability to be compacted. The lysozyme used was chicken egg white lysozyme (Muramidase, mucopeptide N-acetyl-hydrolase: EC 3.2.1.17, Sigma L 6876), triple-crystallized, dialyzed, and lyophilized to a 95% protein level, with the balance primarily the buffer salts, sodium acetate, and sodium chloride.

In vivo release rate profiles were generated by injection of normal rats with 300 mg of depot gels loaded with 15 mg (approximately 5% by weight hGH, 10% total solid particles) of active protein. Research adhered to the "Principles of Laboratory Care" (NIH publication #85-23). hGH levels were determined in serum using an immunochemiluminescent (ICMA) assay. Sampling was conducted at 1, 4, and 24 hours in order to detect injection burst effects. The antibody response to the foreign hGH in rats was measured using an ELISA. hGH recoveries were evaluated by comparing areas under the serum concentration-time curves following depot injection to those for a bolus injection of a known amount. For experiments that ran 28 days, a 10 mg/kg dose of cyclosporin was given five times to the rats for the first 2 weeks, then three times per week thereafter.

Protein particle dissolution experiments were conducted as follows: Approximately 0.3 grams of protein/excipient particles was placed in 2-cm diameter, stainless-steel mesh baskets, which were in turn suspended in the USP dissolution baths by a drive shaft. The baskets were rotated at 100 rpm in solution containing 400 ml of phosphate buffer, controlled to 37°C. Samples were withdrawn from the receptor bath at regular intervals and analyzed for protein content using ultraviolet (UV) absorbance spectrophotometry.

Biodegradation and water absorption rate experiments entailed placing approximately 0.5 cc of formulated hGH depot solutions into a 37°C agitated release-rate apparatus containing phosphate-buffered solution. The samples were removed from the heated bath at regular intervals, and a fragment was taken from the approximate center of the collected mass for analysis. The PLGA molecular weight (number and weight average) was determined using size exclusion chromatography. Samples were dissolved in tetrahydrofuran (THF). In the water absorption experiments the water content was determined using Coulometric Karl Fischer titration (USP 921).

Morphologies of the hardened depots, explanted from subcutaneous space of the rats, were examined by scanning electron microscopy on a Hitachi Model S2460N instrument equipped with a Peltier cooled stage, operating at −20°C. Use of the cooling chamber allowed examination of the triacetin- and alkyl benzoate-based gels, which would otherwise melt rapidly under the focused electron beam. Samples were chilled to −20°C immediately after explantation to prevent further morphological changes (triacetin and alkyl benzoate depots can revert back to a single-phase solution if water is allowed to evaporate on removal from the bath). The depots were fractured in liquid nitrogen immediately prior to placing on the scanning electron microscope (SEM) cold stage for analysis and were viewed without prior sputter coating.

RESULTS AND DISCUSSION

Sustained Release of hGH

Serum hGH levels were monitored for the model depots, which had been made with densified protein particles, over 4 weeks after a single injection into the subcutaneous tissue of normal rats. As shown in Fig. 1, the profiles were dramatically different depending on the depot solvent. With the exception of the TA-based depots, the sustained-release hGH profile is seen to flatten as the solvent strength and affinity of the PLGA solution was decreased. This behavior is consistent with *in vitro* experiments (15), in which lower aqueous-affinity systems were shown to produce more consistent protein release profiles. The benzyl benzoate depot clearly showed optimal behavior, maintaining a continuous delivered dose within the desired therapeutic window of 10 to 200 ng/ml.

The morphologies of the injected depots exhibited characteristics that further distinguish fast and slow phase-inverting systems. Examination of NMP depots explanted 2 weeks after injection into subcutaneous space revealed a morphology typical of a rapid phase inversion system (13), consisting of a highly evolved network of interconnecting pores, framed by a

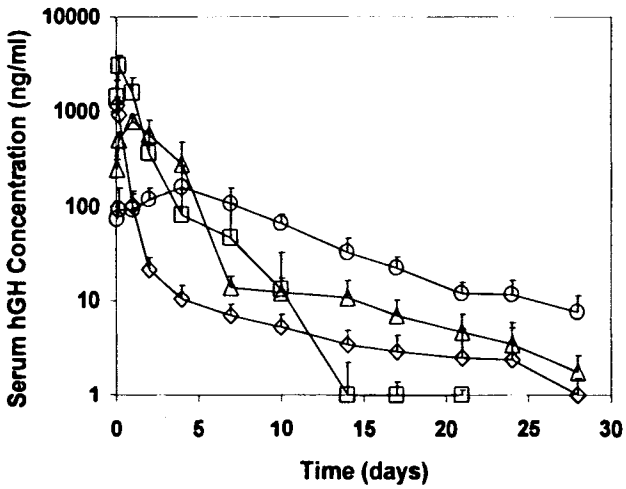

Fig. 1. Time-dependence of serum human growth hormone levels in normal rats receiving a single depot injection of one of the following: NMP depot (◇), triacetin depot (□), ethyl benzoate depot (△), or benzyl benzoate depot (○), n = 4.

brittle polymer skeleton (Fig. 2.a). This morphology supports the hypothesis suggested in our earlier studies (14,15) that the initial burst is due to rapid diffusion of the protein residing in or near the interconnected pores, while the shutdown is caused by the remainder of the drug being trapped within the hardened matrix.

The triacetin and benzoate depots explanted from the rats were found to be rubbery and somewhat flexible. While the triacetin depot exhibited some pores (Fig. 2.b), an interconnected network was not seen. Moreover, the center of the triacetin system was found to be softer than the exterior, indicating a greater presence of solvent in the interior. The ethyl and benzyl benzoate depots, on the other hand, were somewhat homogeneous in appearance, with very few apparent pores (Fig. 2.c & d). The extracted materials also exhibited elastic character when flexed. Given the absence of pores in these systems, drug release is believed to occur via diffusion through the polymer solution until hydrolysis begins to significantly degrade the depot.

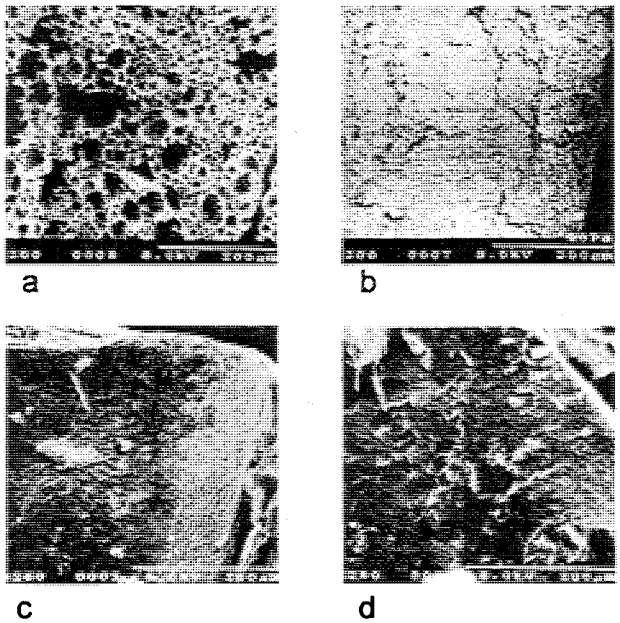

Fig. 2. Morphologies of depots explanted 14 days after injection into rats: a) NMP depot, b) triacetin depot, c) ethyl benzoate depot, and d) benzyl benzoate depot.

Figure 3 shows that significant bioerosion did not occur for approximately 2 weeks in all of the model systems. Moreover, degradation occurred more slowly in the alkyl benzoate/PLGA solutions than in the triacetin and NMP systems. Thus a reduction in the PLGA solution solvent affinity not only impacted the dynamics of the phase inversion, morphology, and drug release, but also prolonged the onset of biodegradation.

The low affinity systems were also found to provide an additional degree of protein stability not observed in the NMP depot. The total amount of hGH released as bioactive protein from the depot formulations containing triacetin, ethyl benzoate or benzyl benzoate was found to be 70% or greater, while the total drug released from the NMP depot was only 10%. This behavior was not observed in the *in vitro* release rate experiments where lysozyme was used as the protein model. However, whether the hGH is degraded within the NMP depot, trapped within the rapidly solidified matrix after injection, or both, the general shape of the *in vivo* NMP release rate curve matches that observed *in vitro*.

Burst Reduction

To further control the release of drug and to reduce the initial burst, protein particle properties were altered. Two methods were employed to slow the dissolution rate of hGH in the presence of water: 1) hGH complexation with zinc, and 2) densification of the solid protein particle. Figure 4 compares the effects of these methods on the dissolution rate. When 15 mM of zinc acetate was complexed with hGH in the lyophilized protein particle, the dissolution time-scale slowed from seconds to hours. On the other hand, densification of the hGH had only a slight impact on the rate of protein dissolution. In this case,

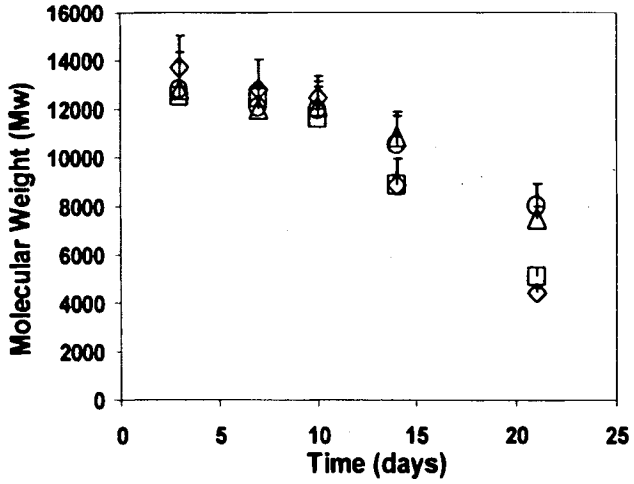

Fig. 3. Molecular weight of PLGA solutions as a function of depot-bath contact time, measured by size exclusion chromatography: NMP depot (◇), triacetin depot (□), ethyl benzoate depot (△), and benzyl benzoate depot (○), n = 3.

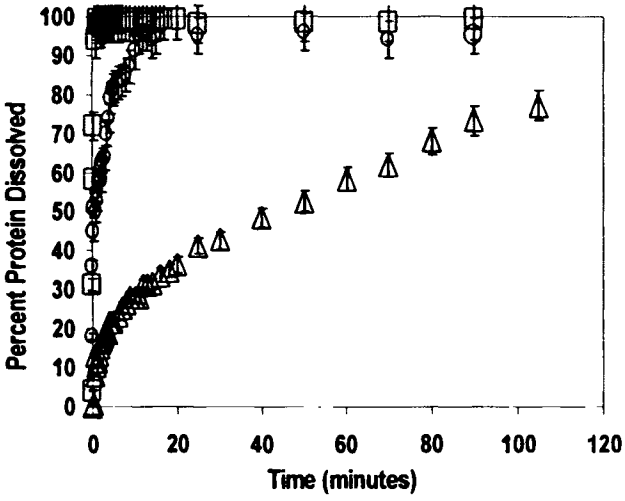

Fig. 4. Protein particle dissolution rates in aqueous buffer: lyophilized protein (□), hGH-Zn complex (△), and densified particle (○), n = 5.

dissolution of the protein particle into water was prolonged by only a few minutes.

Figure 5 illustrates the *in vivo* release profiles of benzyl benzoate depots loaded with either raw hGH complexed with two concentrations of zinc or with densified hGH/stearic acid particles. As expected, the slower dissolving 15 mM-zinc acetate/hGH particles showed a reduction in initial hGH released into the rats. Furthermore, when the concentration of zinc in the particle was doubled to 30 mM, the burst effect was all but eliminated. Surprisingly, the greatest reduction in initial burst occurred with the depots formulated with the densified hGH particles. Although the reduction in burst for the hGH/Zinc particles is explained by the dissolution rate experiment (Fig. 4), the data for the densified particles, which show a relatively high dissolution rate, do not predict the obtained result. Clearly there are other factors at work in this system. One such factor is the amount of water absorbed into the polymer solution depot. In our previous work (15), the initial rate of protein release was found to correlate well with the bulk water absorption rate of the depot.

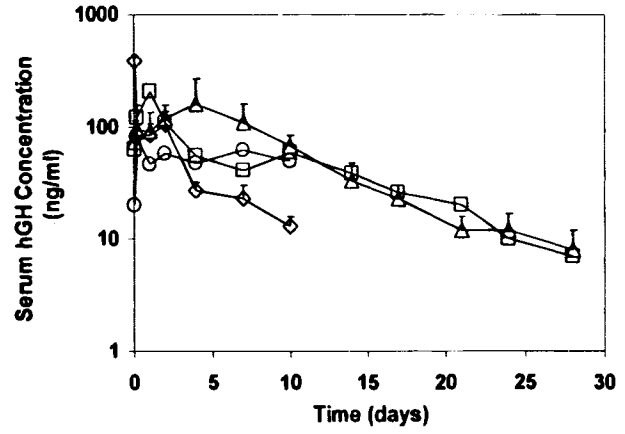

Fig. 5. The effect of altering the protein particle formulation on the hGH release in rats receiving a benzyl benzoate depot injection with: lyophilized hGH (◇), 15 mM zinc complexed with hGH (□), 30 mM zinc complexed with hGH (○), or densified hGH particle (△), n = 4.

Figure 6 shows that the water content in the depot with densified particles is lower than in the depot with the lyophilized protein. It seems reasonable that the reduced burst effect observed with the densified protein particle is a result of this reduction in the water absorption rate. The lower water absorption is most likely the result of lowering of the surface-to-volume ratio of the particle and/or the addition of stearic acid to the particle. Therefore, in addition to the phase inversion dynamics, there are two competing factors that govern burst in PLGA solution depots, namely the protein particle dissolution rate and the amount of bulk water present in the system. The role of the latter two factors becomes apparent in the slower phase-inverting systems. This suggests an approach for formulating an optimal polymer solution sustained-delivery system for therapeutic proteins.

CONCLUSIONS

Therapeutic levels of hGH, delivered from a benzyl benzoate/PLGA solution depot, were maintained in normal rats for a period of 28 days with little or no drug burst on injection. As was the case with previous *in vitro* experimentation, lowering the solvent/non-solvent affinity of the PLGA solution slowed the rate of phase inversion, producing a depot morphology favorable to a more uniform release rate. The use of an alkyl benzoate in the PLGA solution substantially reduced injection burst and zinc complexation and particle densification both helped to eliminate the remaining protein burst effect. Particle compression is preferable to zinc complexation because it is potentially more applicable to a wide range of drug formulations.

The onset of biodegradation was observed at 2 weeks after injection into an aqueous environment for all four model depots. However, the extent of the biodegradation was slowed as the aqueous affinity of the solvent was reduced. It is concluded that the release of protein from the slow phase-inverting depot models predominantly occurs via diffusion through the polymer matrix. Clearly it is possible to engineer protein release in these systems by adjusting the thermodynamic and mass transfer

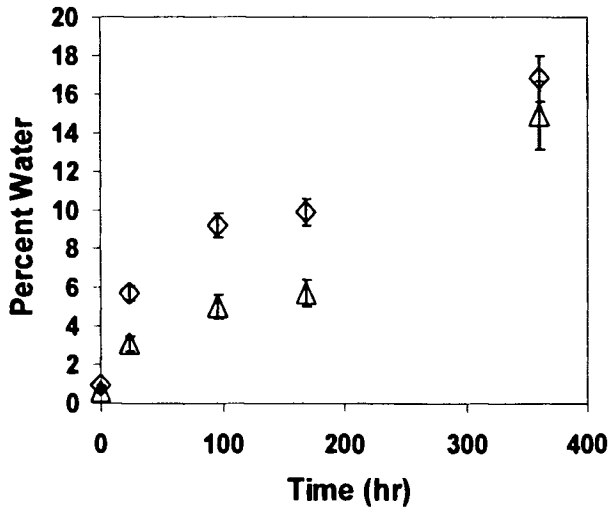

Fig. 6. Impact of particle densification on the water absorption in the benzyl benzoate depot with: lyophilized protein (◇), densified protein particles (△), n = 3.

properties of the polymer solution and suspended protein formulations.

ACKNOWLEDGMENTS

This work has been supported under grants from the National Science Foundation (CTS 97-31509) and ALZA Corporation.

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