Preparation and *in Vitro/in Vivo* Evaluation of Insulin-Loaded Poly(Acryloyl-Hydroxyethyl Starch)-PLGA Composite Microspheres

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Purpose. The purpose of this study was to develop and evaluate a novel composite microsphere delivery system composed of poly(D,L-lactide-co-glycolide) (PLGA) and poly(acryloyl hydroxyethyl starch) (acryloyl derivatized HES; AcHES) hydrogel using bovine insulin as a model therapeutic protein.

Methods. Insulin was incorporated into the AcHES hydrogel microparticles by a swelling technique, and then the insulin-containing AcHES microparticles were encapsulated in a PLGA matrix using a solvent extraction/evaporation method. The composite microspheres were characterized for loading efficiency, particle size, and *in vitro* protein release. Protein stability was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, high-performance liquid chromatography, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The hydrogel dispersion process was optimized to reduce the burst effect of microspheres and avoid hypoglycemic shock in the animal studies in which the serum glucose and insulin levels as well as animal body weight were monitored using a diabetic animal model.

Results. Both the drug incorporation efficiency and the *in vitro* release profiles were found to depend upon the preparation conditions. Sonication effectively dispersed the hydrogel particles in the PLGA polymer solution, and the higher energy resulted in microspheres with a lower burst and sustained *in vitro* release. Average size of the microspheres was around 22 μm and the size distribution was not influenced by sonication level. High-performance liquid chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis, along with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry showed the retention of insulin stability in the microspheres. Subcutaneous administration of microspheres provided glucose suppression <200 mg/dL for 8~10 days with hyperglycemia recurring by day 16. During the treatment, the time points with higher serum insulin level were consistent with a more significant glucose suppression. The microsphere-treated rats also grew virtually at the same rate as normal control until the insulin level declined and hyperglycemia returned. Multiple dosing given every 10 days demonstrated that the pharmacological effect and serum insulin levels from second or third doses were similar and comparable to that of the first dose.

Conclusion. The AcHES-PLGA composite microsphere system provides satisfactory *in vitro* and *in vivo* sustained release performance for a model protein, insulin, to achieve 10-day glucose suppression.

KEY WORDS: PLGA; hydroxyethyl starch; microsphere; insulin; controlled release.

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INTRODUCTION

In recent years, there has been an increasing interest on the use of poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA) microspheres for the controlled release of proteins/peptides (1–3). The advantages of this drug delivery system include biocompatibility, controlled biodegradability, absorbability and no toxicity of degradation products (4), potential for sustained release, and ease of administration.

Despite the success with small peptides, such as the Luteinizing hormone releasing hormone (LHRH) analogues, PLGA microspheres still have a number of major problems with proteins and polypeptides (5). Structural or conformational changes of proteins during microsphere manufacturing, storage, and release have been reported (6,7). For example, in the microsphere preparation, usually an aqueous protein solution is dispersed in an organic polymer solution by using a homogenizer or sonicator to create a water-in-oil emulsion. The exposure of proteins to organic solvent and high sheer might have adverse effects on the integrity of the proteins (8,9). During drug storage and release, protein unfolding and aggregation often occur because of the interaction of protein molecules with the hydrophobic polymeric surface. Moreover, the low pH generated during polymer erosion could cause chemical degradation of entrapped proteins (10,11). Another major problem with PLGA microspheres is an inconsistent release profile, such as a high initial burst effect within 24 h followed by a plateau and then culminating in incomplete release (12). High initial drug release is not suitable for therapeutic proteins because of the risk of side effects from high serum levels. The fast diffusion of protein molecules located on the surface of internal pores and channels formed by the evaporation of solvent and water during microsphere preparation, particularly in a water-in-oil-in water technique, contributes to the burst release (13).

There have been attempts to improve protein stability and release kinetics of the PLGA system by changing the physicochemical properties of the polymer. For instance, both chemical derivation and physical blending of PLGA with hydrophilic monomers and polymers, such as polyethylene glycol (14), poly(ethylene-co-vinyl acetate; Ref. 15), and polyvinyl alcohol (PVA; Ref. 16) have been reported. Heterogeneously structured microspheres were prepared by a combination of the PLGA matrix with hydrophilic inner microparticles made of agarose (17), PVA (18), or gelatin (19). These heterogeneous composite systems were designed to stabilize entrapped protein drugs and improve release profiles. However, to date there is little information on the *in vitro* and *in vivo* behavior of the composite microspheres and essentially no *in vivo* pharmacological evaluation of therapeutic proteins from these composite systems.

A novel composite microsphere system based on PLGA and poly(acryloyl hydroxyethyl starch) (acryloyl derivatized HES; AcHES) was developed using bovine serum albumin and horseradish peroxidase (20). The composite microspheres showed more favorable and complete *in vitro* release than conventional PLGA microspheres. The composite microspheres also stabilized encapsulated horseradish peroxidase from loss of activity during microsphere preparation and release. In the present study, a model therapeutic protein,

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insulin, was encapsulated into the composite microspheres for pharmacologic assessment. Several research groups have incorporated insulin in the PLGA microspheres and reported stability problems, such as aggregation, degradation and deamidation (21,22). In addition, protein-loaded PLGA microspheres usually exhibit a substantial initial burst effect, which has a potential for toxicity (23). Such a burst can be a serious problem with insulin because of a narrow therapeutic window and the risk of hypoglycemic shock. In our work, insulin content, stability, and *in vitro* release were characterized to optimize appropriate batches for *in vivo* study. Effort was made to develop a convenient and rapid extraction method to isolate insulin form the composite for simultaneous protein content determination and stability assessment. *In vivo* studies were conducted by the subcutaneous administration of the composite microparticles to streptozotocin-induced type I diabetic rats, and sustained pharmacological effect was evaluated with regard to prolonged blood glucose suppression, blood insulin level, as well as animal growth.

MATERIALS AND METHODS

Materials

50:50 PLGA Resomer RG502H (M_w 7831, M_n 4544, RG502H) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Hydroxyethyl starch (Hetastarch, HES, Mn 422 kDa, 0.76 molar substitution of hydroxyethyl groups) was obtained from Dupont Pharmaceutics (Wilmington, DE, USA). Acryloyl chloride was purchased from Aldrich Chemicals Company, Inc. (Milwaukee, WI, USA). Bovine insulin (BI), PVA (M_w 3000–7000), streptozotocin, and Infinity™ glucose reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The other reagents were of analytical grade. Insulin RIA kits were purchased from Linco Research, Inc (St. Charles, MO, USA). Male Sprague–Dawley rats were provided by Harlen (Indianapolis, IN, USA).

Preparation of Insulin-Loaded AcHES-PLGA Composite Microspheres

AcHES was synthesized by esterifying HES with acryloyl chloride (24). AcHES hydrogel microparticles around 0.52 μm were produced by free radical polymerization, and the insulin-loaded composite microspheres were prepared with some minor modification in a previously reported method (20). To prepare a 1.5-g microsphere batch, 150 mg of insulin in 0.75 mL of 30% acetic acid were added to 101 mg of AcHES microparticles, and the particles were allowed to swell for 5 min with vortex mixing. The polymer phase consisted of 1.25 g of PLGA in 2.91 g of methylene chloride (30% w/w). The polymer phase was added to the swollen AcHES particles and either vortexed for 5 min or sonicated (W-370 probe, Ultrasonic, Inc.) for 30 s at a predetermined power setting to form a (insulin in hydrogel)/(PLGA in methylene chloride) dispersion. This primary dispersion was then added to 6% PVA solution and stirred by a Silverson mixer (Chesham, UK) at 3000 rpm for 2 min, then transferred to 1 L of deionized water for solvent extraction and evaporation. These procedures were conducted at ~4°C using an ice bath. Then the temperature was gradually elevated to 39°C to facilitate the removal of methylene chloride. Finally, the microspheres were washed with water and freeze-dried. Blank composite microspheres were fabricated in the same way without insulin. BI029 and BI030 were two repetitive batches of BI022 to provide a sufficient quantity of microspheres for animal study.

Particle Characterization

Particle Size Measurement

The particle size of each batch was measured by laser scattering using a Malvern 2600 sizer (Malvern Instruments PC6300, England). The average particle size was expressed as the volume mean diameter in μm.

Morphology of Microspheres

The surface morphology and internal structure of fractured microspheres were examined by scanning electron microscopy (Hitachi Model S800, Japan) after palladium/gold coating.

Acetonitrile (ACN) Extraction Method for Insulin Content Assay in Composite Microspheres

ACN Extraction and Recovery

Known amounts of insulin (1 or 2 mg) were mixed with blank AcHES-PLGA microspheres (18 or 19 mg) to mimic 5% and 10% loading. The mixture was dissolved in 4 mL of 90% ACN (containing 0.1% trifluoroacetic acid [TFA]) with gentle shaking. Then, 6 mL of 0.1% TFA in water was added to precipitate the polymer. The supernatant was analyzed by high-performance liquid chromatography (HPLC) using a phenomax-C18 column at room temperature with a binary gradient consisting of (A) 0.1% TFA and (B) ACN/water/TFA (90:9.9:0.1). The gradient consisted of 15% B to 75% B in 10 min, followed by equilibration at 15% B for 5 min. The peak elution was monitored at 220 nm. Ten milligrams of each microsphere sample was analyzed using ACN extraction and HPLC described above to determine the loading efficiency.

NaOH Digestion Method

Ten milligrams of microspheres were hydrolyzed in 1 mL of 1 M NaOH with vigorous shaking at room temperature overnight. Insulin standards were also hydrolyzed using the same procedure. After hydrolysis, 1 mL of 1.0 M HCl was added to neutralize the sample solutions. Insulin concentrations were determined by a micro-BCA (Bicinchoniric acid) total protein assay method.

Insulin Integrity Assessment

Electrophoresis

The structural integrity of the bovine insulin extracted from the composite microspheres was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Five milligrams of composite microspheres were boiled with the sample buffer containing 8% SDS and 0.2 M dithiothreitol (DTT) and then loaded onto a 16.5% tris-tricine SDS-PAGE gel after spinning down. The electrophoresis was performed at a constant voltage of 150 V. Pro-

tein bands on the gel were stained with GELCODE® Blue Stain Reagent (Pierce, Rockford, IL, USA).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Ten milligrams of microspheres were mixed with 0.4 mL of 50:50 ACN:H_2O, vortexed and shaken for 30 min, centrifuged, and then the supernatant analyzed by MALDI-TOF MS. Spectra were obtained on a Kratos Kompact SEQ time-of-flight mass spectrometer (Manchester, UK), with α-cyano-4-hydroxycinnamic acid as the matrix.

In Vitro Insulin Release

Twenty milligrams of microspheres were weighed and placed in 1.5-mL centrifuge tubes containing 10 mM glycine buffer (pH 2.8). The tubes were incubated at 37°C. At designated sampling times, the tubes were vortexed before centrifugation at 4000 rpm for 5 min. The supernatant was collected, and the volume of the release medium was restored with fresh glycine buffer. The samples were analyzed by HPLC.

In Vivo Evaluation of Insulin Composite Microspheres

Diabetic Animal Model

Sprague–Dawley male rats were injected intraperitoneally with 75 mg/kg of streptozotocin (40 mg/mL in 50 mM citrate buffer at pH 4.5). After 7 days the animals were anesthetized with ethyl ether after 34 h fasting, and 0.7 mL of blood was collected from the tail vein. Rats with serum glucose levels higher than 500 mg/dL analyzed by Infinity™ glucose reagent were used for the following experiments.

Treatment with Insulin Composite Microspheres

Single Dose Treatment. Eight and six diabetic animals received BI021 and BI022, respectively, at 345 IU insulin/kg (80 IU/rat) via subcutaneous injections at the neck region to simulate a dose of approximately 8 IU/day. The microspheres were suspended in an aqueous solution containing 1% carboxymethylcellulose sodium and 2% mannitol. The diabetic control group consisted of six animals without insulin treatment. At the predetermined time points, 0.7 mL of blood was collected after 3–4 h fasting and the serum was assayed for glucose level (Infinity™ glucose reagent) and insulin content by radioimmunoassay (Linco Research, St. Charles, MO, USA).

Multiple Dosing Treatment. Eight diabetic rats were treated with 26 mg of microspheres (80 IU insulin) every 10 days. Blood was collected every other day until day 36. Control diabetic rats without insulin treatment were included.

Table I. Batch Summary of Insulin Loaded Composite Microspheres

Batch	Batch size (g)	Sonication output level[a]	Loading (%)	Loading efficiency (%)	Burst release (%)	Average size (μm)
BI018	2.0	Vortex	7.65	76.5	69.2	23.8
BI019	2.0	1.7	8.73	87.3	60.1	20.6
BI020	1.5	2.7	9.59	95.9	16.2	24.6
BI021	1.5	3.0	9.94	99.4	5.07	22.1
BI022	1.5	3.3	10.1	101.1	1.86	21.3

[a] Vortexing and sonication time was 5 min and 30 s, respectively.

RESULTS

Particle Characterization

Table I shows that the average particle size of the composite microspheres was 20.6–24.6 μm, suitable for parenteral administration through a 21-gauge needle. The particle sizes of each batch were similar and independent of the sonication power used for preparing the primary dispersion. This is expected because the final particle size depends mainly on the droplet size in the secondary emulsion and solidification rather than the size in the primary emulsion. Additionally, stirring speed, PLGA concentration in the organic phase, and PVA concentration in the aqueous continuous phase were the same for each batch. This result is in agreement with Heya et al. (25).

The microspheres prepared were spherical with relatively smooth surfaces (Fig. 1a). Fig. 1b shows a fractured composite microsphere (BI022), with the insulin loaded

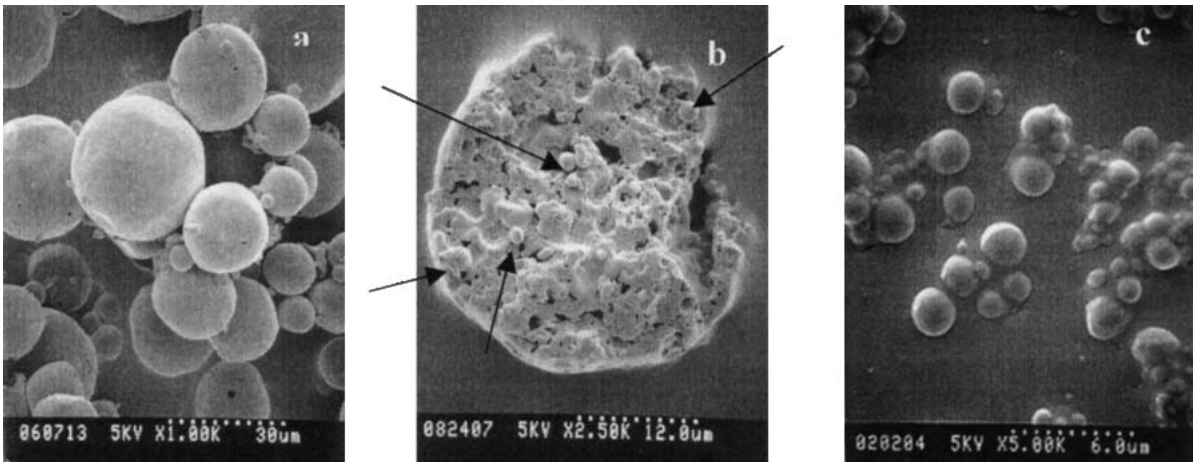

Fig. 1. Scanning electron micrographs of insulin-loaded acryloyl chloride with HES (AcHES)-poly(D,L-lactide-co-glycolide composite microspheres. (a) Interior structure of a fractured microsphere (b, arrows point to embedded AcHES microparticles) and (c) freeze-dried AcHES hydrogel microparticles.

AcHES particles distributed throughout the matrix. The size of the AcHES particles was 0.5–2 μm (Fig. 1c), small enough for multiple particles to be encapsulated in a composite microsphere. The composite reduced the contact of protein molecules with the organic solvent during the emulsifying process, which could contribute to protein denaturation and aggregation.

ACN Extraction Method for Insulin Assay in Composite Microspheres

A typical RP-HPLC chromatogram is shown in Fig. 2, where the insulin sample from composite microspheres had an identical retention time as that of the insulin standard; this provided supportive evidence that insulin did not degrade to products of different chemical nature, such as the A21 desamido-insulin. With the ACN extraction approach, blank microspheres did not show any interference in the HPLC chromatograph, and insulin-spiked samples of blank microspheres had recoveries of 97.3–99.3% in all cases regardless of the type of PLGA or the amount of spiked insulin (5 or 10% w/w). The satisfactory recovery enabled further determination of insulin content in protein-loaded microspheres, which yielded results similar to that from NaOH digestion (detailed data not shown).

Characterization of Insulin Integrity in Composite MS

SDS-PAGE (Fig. 3a) of extracts from composite microspheres which were subjected to DTT reduction shows an insulin band at 3 kDa, corresponding to a mixture of A chain and B chain. No impurity band was found. In the MALDI-TOF mass spectrum (Fig. 3b), insulin extracted from the microspheres displayed an identical monoisotropic peak at MH^+ of 5734 with no evidence of covalent aggregation or degradation peaks. In contrast, forced degradation of insulin by DTT reduction showed no intact insulin peak and only separate peaks at MH^+ of 3401 and 2338 for the A chain and B chain, respectively (spectrum not included). Therefore, insulin fragments could be detected using this extraction and MALDI-TOF MS as a stability-indicating assay. Both the electrophoresis and mass spectrometry provided additional evidence for integrity of bovine insulin in the composite microspheres.

Influence of Microsphere Preparation on Protein Incorporation and Release

Insulin incorporation efficiency was influenced by the preparation method of the primary emulsion. An efficiency of

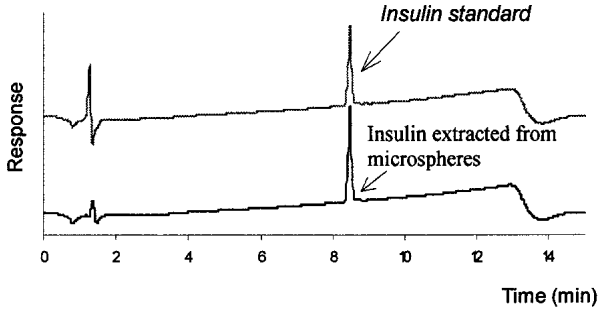

Fig. 2. High-performance liquid chromatography chromatogram of insulin sample isolated from composite microspheres by acetonitrile extraction and intact insulin standard.

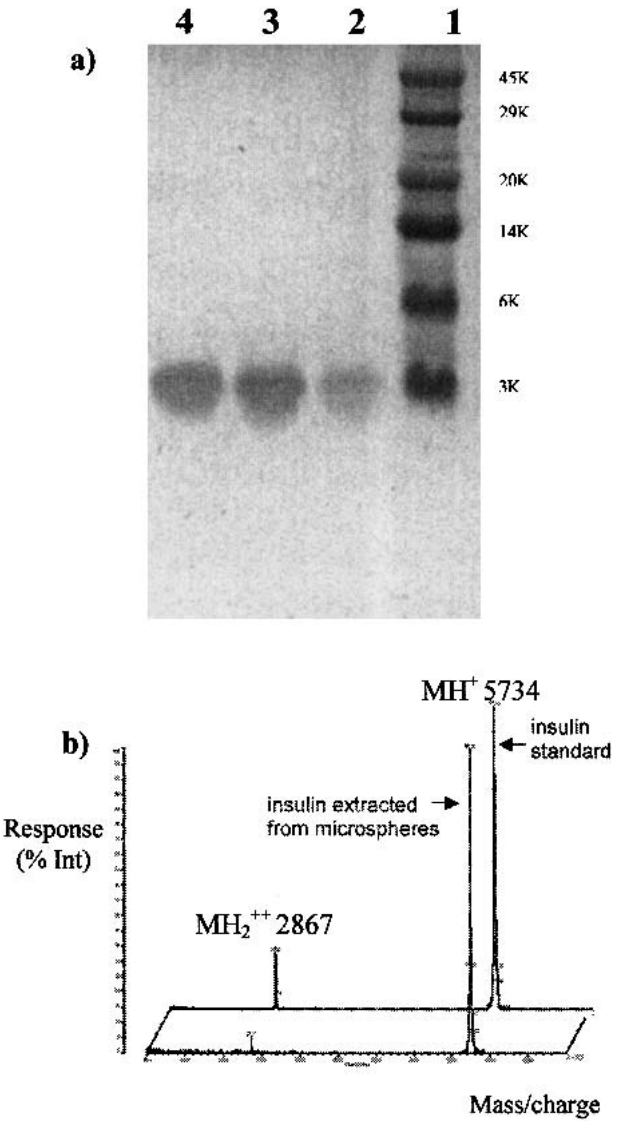

Fig. 3. Characterization of insulin integrity in the composite microspheres (a) sodium dodecyl sulfate polyacrylamide gel electrophoresis with dithiothreitol. Lane 1, Molecular weight marker; lane 2, bovine insulin standard; lane 3, insulin sample from BI021; and lane 4, insulin sample from BI022. (b) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of insulin extracted from composite in comparison to intact standard.

76.5% was obtained by vortexing, whereas sonication yielded efficiencies > 87.3% (Table I). Igartua et al. (26) and Cohen et al. (1) obtained similar results with PLGA microspheres containing either bovine serum albumin or isothiocyanate-labeled bovine serum albumin prepared by a water-in-oil-in-water emulsion. In this study, the loading efficiency also increased slightly with higher sonication power level. This might be because vortex mixing at a lower power output resulted in large inner emulsion droplets, which aided protein escape into the bulk aqueous phase when the secondary emulsion was prepared. At the higher sonication settings to further disperse the hydrogel particles, a more uniform and finer primary emulsion was possible, resulting in a more effective incorporation of the protein.

Because insulin has a narrow therapeutic window, the

burst release from the microspheres has to be low to avoid hypoglycemic shock and fatality in the animals. The sonication reported by Igartua et al. (26) reduced the burst release of albumin by nearly one half. Table I shows that increasing the power level to disperse hydrogel in the polymer phase resulted in a significant decrease in the burst release. For example, BI018, prepared by vortexing, showed around 70% burst. Sonication reduced the burst to 5.07% and 1.86% at sonication output levels of 3.0 and 3.3, respectively.

The burst effect reported by Pean et al. (13) is likely to be ascribed to a honeycomb-like internal structure. Similarly, with insufficient dispersion power, the hydrogel particles may not be separated from each other and were eventually encapsulated in the PLGA matrix as clumps of multiple microparticles, which increased the accessibility of insulin to the release medium through pores and channels formed during preparation of the microspheres. When the hydrogel was suspended in the polymer phase by vortexing or low energy level sonication (level 1.7), the resulting suspension did not appear very white and upon settling, hydrogel clumps were found on the wall of the vial. At sonication levels of 2.7, 3.0, and 3.3, the suspension appeared much whiter with no evidence of clumping, indicating a more uniform dispersion of the hydrogel particles.

Figure 4 shows the release profiles of insulin from four batches of composite microspheres prepared. The vortex batch had a 70% burst with slow subsequent insulin diffusion until erosion of polymer-enhanced drug release after day 7. Because of the limited amount of insulin left within the microspheres, the release soon exhausted and reached a plateau. Three sonicated batches displayed similar release patterns except for the extent of initial burst. There was not much release from day 2 to 7 when protein diffuses through the incompletely hydrated matrix before polymer erosion occurs. The release accelerated during the second week because of polymer degradation and erosion and was even more rapid through the third and fourth weeks before the release plateaued after 30 days. Interestingly, in comparison with some previous reports where insulin release from PLGA microspheres was incomplete (10,27), each batch in this study released almost the entire incorporated drug (>78%). The improved release pattern indicates AcHES hydrogel particles may have a protective function for enhancing the retention of insulin stability and reducing insulin adsorption to PLGA. As well, the appropriate choice of release medium could also contribute to complete drug release.

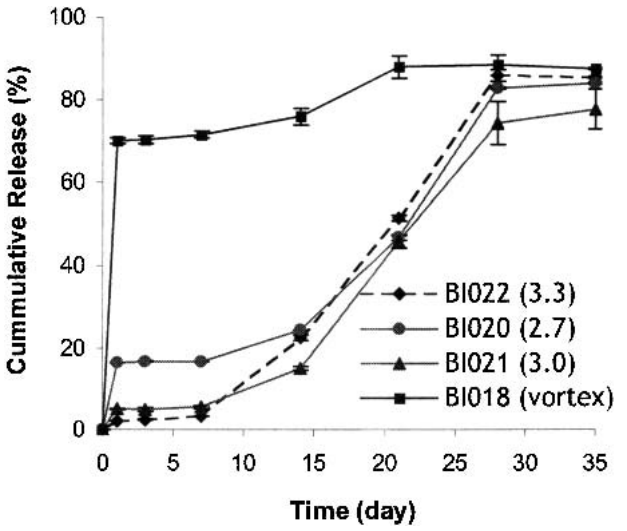

Fig. 4. *In vitro* release of insulin from composite microspheres in glycine buffer at 37°C. Sonication levels are indicated in parentheses (n = 3; mean ± SE).

In Vivo Evaluation of Insulin Composite Microspheres

Single Dose Treatment

Two composite microsphere batches, BI021 and BI022, with 5.0% and 1.9% *in vitro* release on day 1, were selected for *in vivo* study. Fig. 5a and 5b describe the *in vivo* serum glucose profiles obtained upon subcutaneous administration of the two batches to diabetic rats. The rapid and immediate decrease in the serum glucose concentration observed on the very first day with batch BI021 seems in good agreement with its higher initial *in vitro* release rate. On day 2, glucose level elevated to around 200 mg/dL but was still effectively suppressed compared with diabetic animals. The suppression was sustained through day 8 between 70 and 150 mg/dL with the most remarkable reduction seen on day 6. Hyperglycemia recurred after day 10 and returned to the diabetic control level on day 16. With respect to BI022, the suppression of glucose level was more gradual on the first 2 days, most likely resulting from the lower burst. On day 3, glucose fell to

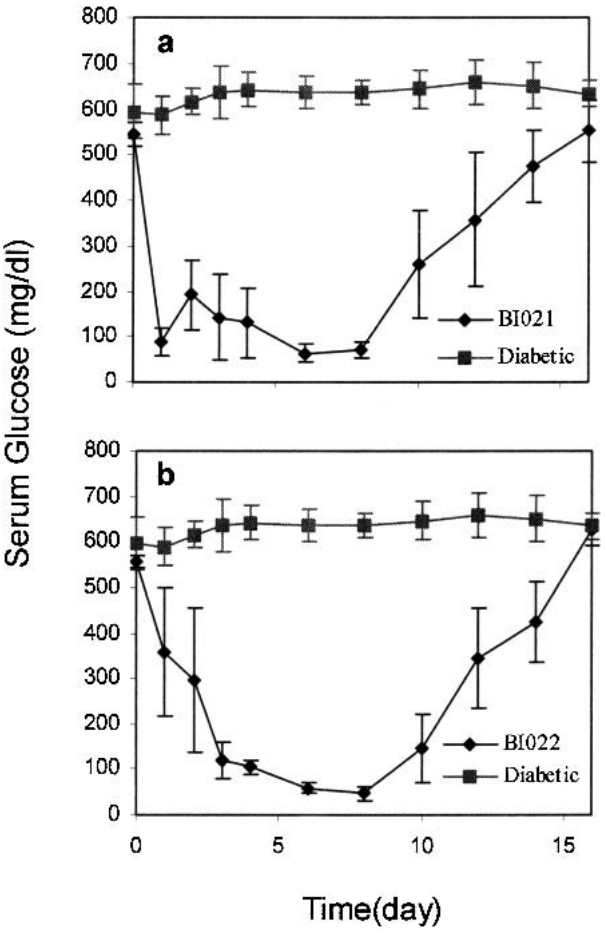

Fig. 5. Serum glucose suppression in diabetic rats treated with insulin loaded composite microsphere batches BI021 (a) and BI022 (b) (n = 6 for diabetic; 8 for BI022; mean ± SE).

around 130 mg/dL, and the suppression was sustained for 10 days. After day 10, the level elevated to that of the diabetic control on day 16.

The serum insulin profiles seen in Fig. 6a and 6b show the insulin level in the two treated groups as well as the diabetic controls. In Fig. 6a, the administration of BI021 gave rise to an immediate peak on day 1, which correlated well with the rapid glucose suppression and the 5% *in vitro* burst release. Although the levels were in the 4–6 ng/mL range between 2 and 8 days, there is some evidence of a triphasic pattern, where the insulin level decreased on the second day and then elevated to a second peak on day 6. The decline of insulin level was gradual, and there was steady insulin release from the composite microsphere up to day 10. In contrast, serum insulin levels of the control group showed a low level with no more than 0.7 ng/mL throughout the study. Treatment with BI022 led to a lower initial insulin level of 3.5 ng/mL within 24 h, which also correlated well with the 1.9% *in vitro* burst of this batch and the gradual suppression of blood glucose seen in Fig. 5b. Again, three phases could be discerned with relatively lower insulin level on days 2 and 3, a peak of 5.8 ng/mL on day 6, and then the gradual decrease to original diabetic level after day 12.

Because a physiologic response to insulin was the growth of the animals and body weight increase, body weight of the BI021- and BI022-treated rats was monitored. The treated diabetic rats grew at a comparable rate as the control group of normal rats until day 10 when hyperglycemia reappeared and loss of body weight reoccurred.

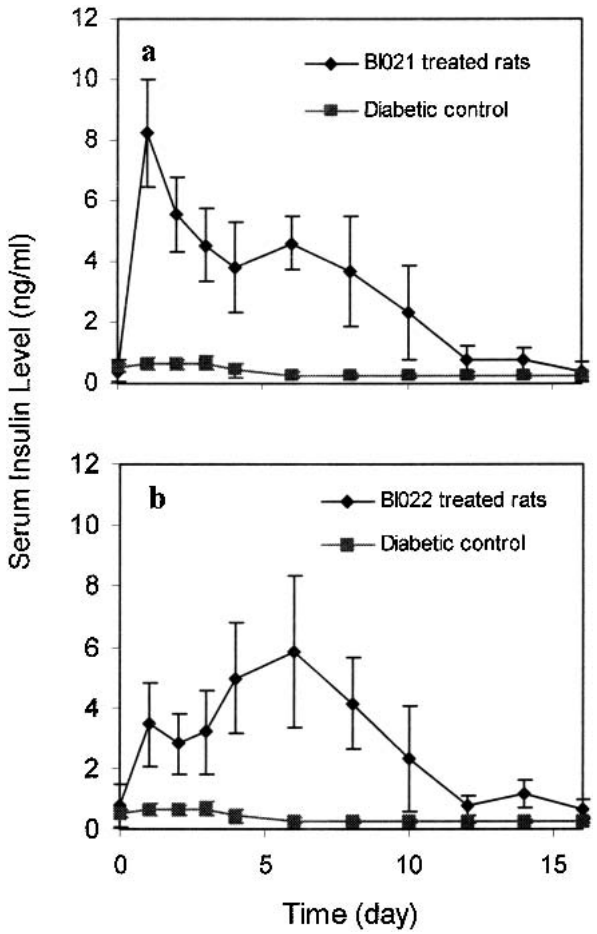

Fig. 6. Serum Insulin level of (a) BI021- and (b) BI022-treated diabetic rats. (n = 6 for diabetic control; 8 for treated rats; mean ± SE).

Multiple Dosing Treatment

The objective of this treatment was to asses a multiple dose regimen for the model protein and evaluate if the composite dosage form would have potential therapeutic application. As seen in Fig. 5b, the blood glucose elevated on day 10 after dosing. Therefore, in this study, rats (n = 8) were treated with microspheres containing 80 IU insulin every 10 days. The profile of glucose shown in Fig. **7** from day 0 to day 10 was similar to that in Fig. 5b with maximum glucose suppression on day 6. The rate of glucose suppression was similar for the first and second doses, but because there were detectable residual levels of serum insulin upon administration of the second dose, the levels were suppressed to below 100 mg/dL faster, that is, 5 days for the first dose but only 3 days for the second dose. The second and third doses both exhibited prolonged and comparable pharmacological effect over 8 days. The result suggests that 10 days could be an appropriate dosing schedule because once the blood glucose elevates beyond normal level, a subsequent dose could take over and keep the glucose level in control. The *in vivo* insulin level (Fig. 8) also displayed comparable pharmacokinetic profiles from the three doses with regard to C_{max}, T_{max}, and area under the curve.

DISCUSSION

Two techniques have been reported for extraction of proteins from PLGA or PLA microparticles. One involves the dissolution of the microspheres in methylene chloride followed by aqueous extraction of the organic phase (1,28). This two-phase extraction was often incomplete, giving rise to an underestimation of the actual protein content (29). A second technique was alkaline hydrolysis of the microparticles, followed by total protein assay (30), a method that accurately determined protein loading but failed to provide any stability information because the extraction process digested the protein. In this study, the ACN extraction method was found to be a rapid and convenient approach for determination of protein content and, simultaneously, assessment of protein degradation to products of different chemical nature by HPLC.

The more homogenous distribution of hydrogel particles throughout the PLGA matrix retarded the initial release of insulin and made the subsequent release dependent on polymer hydration and mass loss. As an alternate to sonication, Rojas et al. (2) used surfactants, such as Tween 20, to reduce the burst. However, such burst suppression was not observed in Rosa et al.'s (31) study where non-ionic surfactants, poloxamer 188, polysorbate 20, and sorbitan monooleate 80, were co-encapsulated in microspheres. Yamaguchi (32) used certain hydrophilic additives, such as glycerol in the S/O (solid in oil) dispersion, i.e., crystalline insulin suspended in dichloromethane, to achieve a heterogeneous internal localization of insulin instead of on the surface of the PLGA microcapsules and thereby achieving a lower burst effect.

Previously in neutral medium, a release pattern with a burst within 1 day and almost no further release afterwards was reported for insulin loaded PLGA microspheres (10).

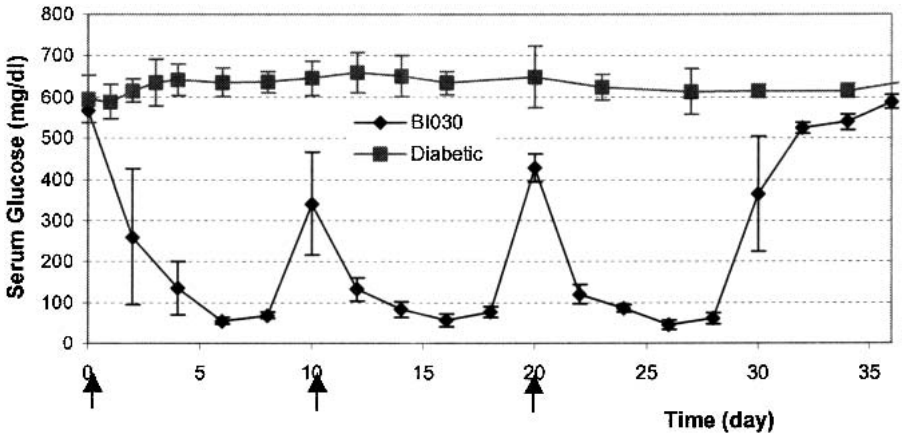

Fig. 7. Blood glucose suppression of multiple dosing treatment of insulin loaded composite microspheres (n = 8, dose 80 IU/rat; mean ± SE).

Considering the limited solubility in PBS (145.8 μg/mL), a neutral pH medium was not desirable to study the release. Moreover, bovine insulin has a higher tendency to self aggregate or "fibrillate" in solution than either human or porcine insulin (33). Additionally, insulin adsorption to PLGA might occur and contribute to the incomplete release. In this study, glycine buffer was used as the *in vitro* release medium because it ensured high solubility (>5 mg/mL) to mimic sink condition, and no insulin precipitation occurred upon incubation in the buffer for 2 weeks (data not shown).

Comparing the *in vitro* release and *in vivo* profiles of the composite microspheres, there appeared to be a lack of time wise point-to-point correlation. The release test medium conditions were based on insulin solubility and fibrillation. However, an *in vivo* system is far more complex and often will not correlate with the *in vitro* release because of the presence of proteolytic enzymes, cellular infiltrates, various cytokines, and pH gradients. Nevertheless, an *in vitro* release test can serve to demonstrate performance and reproducibility in the preparation of microspheres, despite not simulating *in vivo* kinetics. In this study, the *in vitro* release does share some similarity with *in vivo* blood profile. For example, the triphasic pattern, i.e., an initial release followed by a period of relatively slower release and then an accelerated stage, was found both *in vitro* and *in vivo*, albeit the phases occur sooner *in vivo*. Further investigations will have to address whether polymer accumulation *in vivo* gives rise to any adverse effects.

CONCLUSION

Bovine insulin was successfully encapsulated into the composite microspheres with retention of insulin integrity and the microsphere preparation process was optimized to reduce the burst and provide *in vitro*-sustained release. An extraction and HPLC analytical method was developed to simultaneously determine insulin loading and protein stability. The glucose suppression in diabetic rats was prolonged through 810 days with the most remarkable reduction seen on days 6–8. Multiple dosing reflected the repetitive pharmacological efficacy and pharmacokinetic profile of a single dose. The *in vitro* and *in vivo* results suggest that the novel composite microsphere system could be used as a carrier for prolonged delivery of protein drugs.

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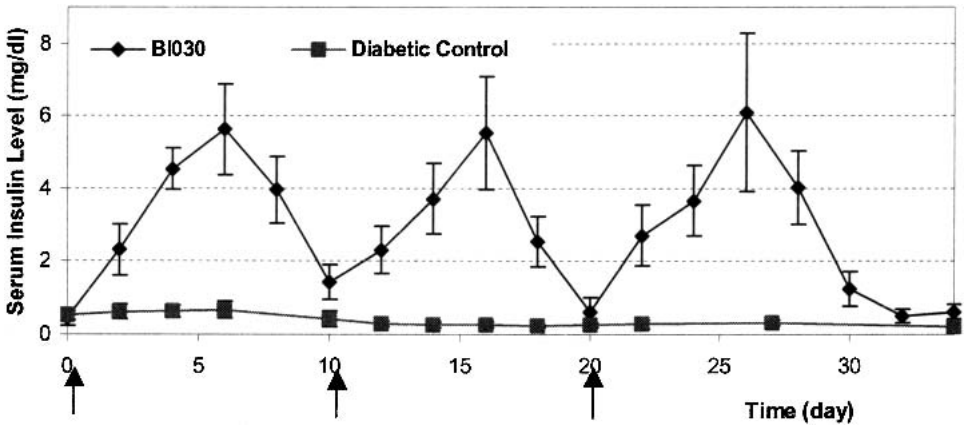

Fig. 8. Serum insulin level of multiple dosing treatment of insulin loaded composite microspheres (n = 8, dose 80 IU/rat; mean ± SE).

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