AAPS PharmSciTech **2003; 4 (2) Article 28 (http://www.pharmscitech.org).**

Preparation and Characterization of Poly(D,L-lactide-co-glycolide) Microspheres for Controlled Release of Human Growth Hormone

Submitted: March 18, 2003; Accepted: April 30, 2003

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ABSTRACT

The purpose of this research was to assess the physicochemical properties of a controlled release formulation of recombinant human growth hormone (rHGH) encapsulated in poly(D,L-lactide-co-glycolide) (PLGA) composite microspheres. rHGH was loaded in poly(acryloyl hydroxyethyl) starch (acHES) microparticles, and then the protein-containing microparticles were encapsulated in the PLGA matrix by a solvent extraction/evaporation method. rHGH-loaded PLGA microspheres were also prepared using mannitol without the starch hydrogel microparticle microspheres for comparison. The detection of secondary structure changes in protein was investigated by using a Fourier Transfer Infrared (FTIR) technique. The composite microspheres were spherical in shape (44.6 ± 2.47 μm), and the PLGA-mannitol microspheres were 39.7 ± 2.50 μm. Drug-loading efficiency varied from 93.2% to 104%. The composite microspheres showed higher overall drug release than the PLGA/mannitol microspheres. FTIR analyses indicated good stability and structural integrity of HGH localized in the microspheres. The PLGA-acHES composite microsphere system could be useful for the controlled delivery of protein drugs.

KEYWORDS: Microspheres, human growth hormone, protein delivery, composite microspheres

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INTRODUCTION

Biodegradable microspheres have been extensively investigated as delivery systems for biologically active peptides and proteins.[1,2] Sustained-release characteristics of microspheres reduce the need for frequent administrations and enhance patient compliance by maintaining in vivo drug levels in the therapeutic range.[3] Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) are the most widely used and well characterized polymers for biodegradable microspheres.[4]

Several factors, however, have limited the development of sustained-release formulations of protein therapeutics. One is the need to stabilize the protein for long periods in an aqueous environment at physiological conditions. In contrast to lower molecular weight drugs, proteins often have large globular structure and exhibit secondary, tertiary or, in some cases, quaternary structure that is necessary for biological activity. In addition, protein instability has been observed during the preparation of protein-loaded microspheres.[5,6] Usually, an aqueous protein solution is dispersed in an organic polymer solution by using a homogenizer or sonicator to create a water-in-oil (w/o) emulsion. The exposure of proteins to organic solvent or aqueous/organic interface might have adverse effects on their stability.

Human growth hormone (HGH), a single polypeptide chain of 191 amino acid residues with a molecular mass of 22 kDA, is a somatotropic hormone secreted from the anterior pituitary gland. HGH therapy was developed in the early 1950s and was used successfully to treat growth hormone deficiency (GHD) in hypopituitary dwarfism.[7] Several manufacturers have received approval to market HGH in a variety of indications,[8] including pediatric GHD, adult GHD,[9] chronic renal insufficiency,[10] Turner's syndrome,[11] and cachexia,

secondary to AIDS.[12] Treatment is by frequent injection (usually daily or every other day), and doses are in the range of 0.1 to 0.35 mg/kg/week.[13]

It is important when producing microencapsulated formulations of therapeutic proteins, that the physical, chemical, and biological properties of the protein remain intact during encapsulation. It is particularly important, during these processes, to preserve protein structure and bioactivity and not impart any immunogenicity. In fact, antibody responses can lead to safety concerns and, if neutralizing, can limit the efficacy of subsequent treatment. To overcome these problems, one approach is physical encapsulation of protein-loaded hydrophilic particles or hydrogels into a PLGA matrix. For example, heterogeneous structured microspheres were prepared by fabrication of PLGA with hydrophilic particles such as agarose hydrogels,[14] PVA,[15] or poly(acryloyl hydroxyethyl) starch (acHES) microspheres.[16] These heterogeneous composite systems were designed to stabilize entrapped protein drugs and to improve drug release characteristics. However, in the preparation processes of these heterogeneously combined microspheres, protein drugs were exposed to large amounts of organic solvent and multiple freezing and thawing or heating-cooling processes during protein loading on the primary hydrophilic particles.

In this study, hydrophilic starch-based hydrogel particles containing rHGH were prepared by a simple swelling procedure. Then, using a solvent extraction/evaporation method, the rHGH-loaded hydrogel particles were encapsulated in PLGA microspheres to form the hydrogel-PLGA combined composite microspheres. Using the same solvent extraction/evaporation method, rHGH-loaded PLGA microspheres were also prepared using mannitol instead of starch hydrogel microspheres. Mannitol has already demonstrated its effectiveness in preserving the native structure of rHGH in PLGA microspheres.[17] Hence, the aim of this work was to assess the physicochemical characteristics and in vitro protein release of both microsphere formulations and to establish poly(acryloyl hydroxyethyl starch)-PLGA (acHES-PLGA) composite microspheres as a novel protein-delivery system in comparison with the mannitol-containing PLGA microspheres. The physical integrity of rHGH in both microsphere batches was assessed using FTIR analysis.

MATERIALS AND METHODS

Materials

PLGA (50:50, M_w 7831, M_n 4544) with free carboxyl end groups was purchased from Boehringer Ingelheim (Boehringer Ingelheim, Germany, RG502H). Hydroxyethyl starch (Hetastarch [HES]) was obtained from Dupont Pharmaceuticals (Wilmington, DE), and acryloyl chloride was purchased from Aldrich Chemicals Company, Inc (Milwaukee, WI). rHGH (Somatropin freeze-dried, 86% GH 14% sodium phosphate tribasic) was obtained from Dong-A Pharm Co, Ltd (Kyunggi, Korea). Polyvinyl alcohol (PVA, M_w 30 000-70 000) was obtained from Sigma Chemical Co (St Louis, MO). A micro-BCA total protein assay kit was obtained from Pierce Biotechnology (Rockford, IL). Mannitol was obtained from Fisher Scientific (Nepean, Ontario, Canada).

Preparation of Microspheres

Preparation of PLGA-acHES Composite Microspheres

Acrylic acid ester of hydroxyethyl starch (acHES) was prepared as described previously.[14] The PLGA-acHES composite microspheres were prepared by a modified solvent extraction/evaporation method with 10% target loading of rHGH. Briefly, 58.1 mg rHGH powder was dissolved in 0.40 mL of 0.1 M phosphate buffered saline (PBS) (pH 7.4). The protein solution was added to acHES particles (10% of total polymer weight), and the particles were allowed to swell for 5 minutes with vortex mixing at room temperature.

A 30% (wt/wt) PLGA methylene chloride solution was added to the swollen acHES particles and vortexed for 3 minutes at room temperature to form a (protein in hydrogel)/(polymer in solvent) dispersion. This primary dispersion was then added to precooled (4°C) 100 mL 6% PVA solution and stirred by a Silverson mixer (Silverson, Chesham Bucks, UK) at 2500 rpm for 1 minute. The resulting secondary suspension was transferred to 1 L deionized water and stirred gently for 3 hours at room temperature to remove the organic solvent and solidify the polymer. The microspheres were filtered and freeze-dried.

Preparation of Mannitol PLGA Microspheres

Similarly, Mannitol PLGA microspheres were prepared by the modified solvent extraction/evaporation method with 10% target loading of rHGH. A primary dispersion was prepared by mixing the protein solution con-

taining 10% mannitol with 30% PLGA solution and then the emulsion was added to 6% PVA solution while stirring at 200 rpm. The resultant suspension was transferred to 1 L deionized water and stirred gently for 3 hours at room temperature to remove the organic solvent and solidify the polymer. The microspheres were filtered and freeze-dried and stored at 4°C.

Microsphere Characterization

The morphology and size of the microspheres were analyzed by scanning electron microscopy (SEM) (Hitachi Model S800, Japan) and laser light diffraction (Malvern Instrument, Malvern, UK). To determine drug content, triplicate samples of 5 mg of microspheres were dissolved in 0.5 mL 1M NaOH by overnight rotation; then the solution was neutralized with 0.5 mL 1M HCl. The content of rHGH in the samples was determined by Micro-BCA protein assay.[18]

In Vitro Release

The in vitro HGH release was determined by suspending 15 mg microspheres in 1 mL of PBS (pH 7.4) and gently rotating at 37°C. At regular intervals, samples were centrifuged, and the supernatant was removed for Micro-BCA protein assay.[19] Fresh replacement medium was added to resuspend the microspheres. The analysis was performed in triplicate.

Dry and Rehydrated Protein FTIR Analysis

The secondary structure of rHGH was investigated either in the rHGH native protein and freeze-dried powder or after entrapment in PLGA with mannitol and acHES-PLGA composite microspheres. Infrared spectra were obtained by using a Bio-Rad Excalibur FTS 3000 MX spectrometer (BIO-RAD Labs, Hercules, CA) equipped with a Deuterated Triglycine Sulfate (DTGS) KBr detector, with a 0.25 cm^{-1} maximum resolution and signal-to-noise ratio 25 000:1. Analyses on dried polymer and microspheres were performed on samples prepared by mixing an amount equal or correspondent to 1 mg of rHGH to 200 mg of KBr and annealed into disks. This process does not modify the spectrum profile of dry proteins, as already reported elsewhere.[20] A background spectrum consisting of blank KBr was previously collected. For all spectra, 256 consecutive scans were collected in a single beam mode with a 2 cm^{-1} resolution. A reference spectrum of PLGA polymer was recorded under identical conditions, and the protein spectra were obtained by subtraction of the reference spectrum. Spectra of native protein solution, dissolved freeze-dried powder, and rehydrated microspheres were recorded in a CaF_2 windows cell with a 6-μm spacer. In brief, microspheres were incubated in PBS 0.1 M for 4 hours at 37°C and immediately analyzed according to the method already employed elsewhere.[21] Background and reference spectra consisting in air, buffer, and rehydrated blank microspheres, respectively, were recorded and properly subtracted from the sample spectra to eliminate vapor, buffer, and polymer contributions.

The obtained curves were employed in order to compare the amide I region profiles of the samples with the standard rHGH profile. The evaluation of the protein secondary structure retention was accomplished by applying an 11-point Savitzky-Golay smoothing function to eliminate noise and operating the second derivative transformation on the subtracted spectra. These curves were imported using SYSTAT's Peakfit, version 4.11, software (SYSTAT Software Inc, Richmond, CA), and fitting was performed applying a 2-point baseline, in order to assess the percentage of retention of the dominant α-helix band in the amide I region. Secondary structure retention of rHGH was expressed as a percentage of the normalized area of the peak at ca 1655 cm^{-1} with respect to protein standard. The standard consisted in the native rHGH solution.

RESULTS AND DISCUSSION

Characterization of Microspheres

To stabilize the protein during the microsphere encapsulation process and within the microspheres after hydration, the protein was formulated with the acHES particles. The acHES particles for protein entrapment possess a submicrometer mean particle size of 0.14 μm, a low bulk density of 0.05 g/cc, and a high specific surface area as described previously.[1] In addition, the acHES hydrogel particles showed fast and good swelling property. The hydrated particles showed approximately an 11-fold larger particle diameter and were 10.5-fold heavier than the dry particles. These results suggest that the hydrophilic starch-based hydrogel could imbibe inside a large amount of aqueous drug solution and protect the drug from degradation caused by solvent and polymer interactions during microsphere preparation and drug release.

On the other hand, the encapsulation in PLGA microspheres was performed with aqueous protein solution containing mannitol as an alternate means of stabilizing

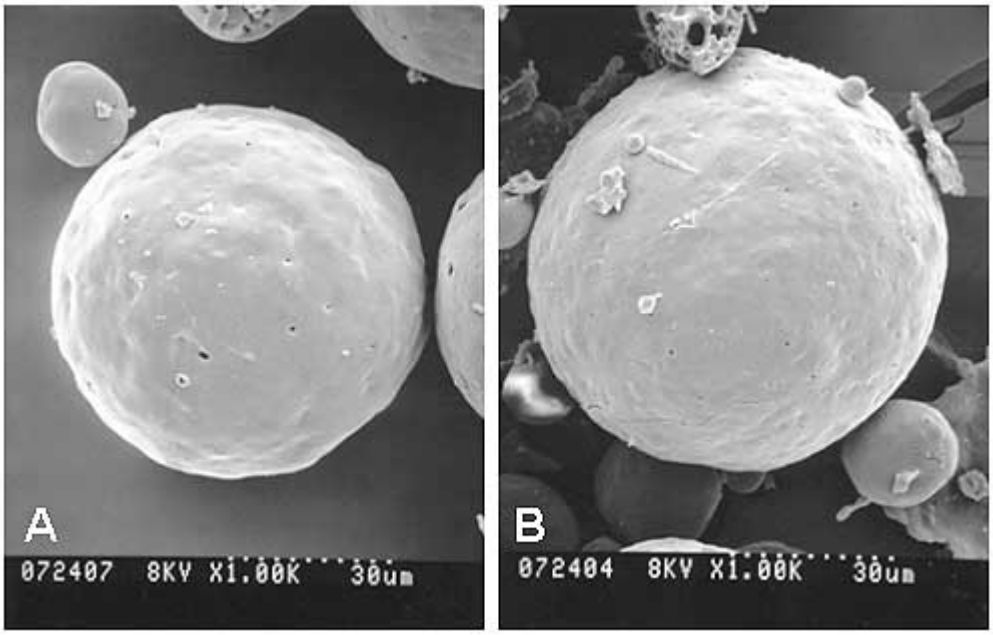

Figure 1. SEM pictures of (A) PLGA-mannitol microspheres and (B) rHGH-loaded acHES-PLGA microspheres.

Table 1. Characterization of rHGH-Loaded Microspheres*

Microspheres	Target Loading (%)	Encapsulation Efficiency (%) ± SD	Average Particle Size (μm) ± SD
PLGA-acHES	10	103.9 ± 0.88	44.6 ± 2.47
PLGA with mannitol	10	93.2 ± 0.94	39.7 ± 2.50

rHGH during microparticle preparation. Previous studies with sugars and proteins demonstrated that sugars cause preferential hydration of proteins, resulting in stabilization of the compact native state.[22] The formation of a hydration layer around the compact protein may reduce the protein-organic solvent interactions preventing protein denaturation. SEMs of rHGH-loaded acHES-PLGA composite microspheres are shown in **Figure 1**. **Figure 1B** shows the spherical shape and relatively smooth surface of the PLGA-acHES composite microspheres. **Figure 1A** shows the conventional PLGA-mannitol microspheres, which are spherical in shape and have a smooth surface with small pores.

As shown in **Table 1**, the average particle size of the composite microsphere was 44.6 ± 2.47 μm. The conventional PLGA-mannitol microspheres showed an average particle size of 39.7 ± 2.50 μm. rHGH was encapsulated successfully in the composite and PLGA-mannitol microspheres with 93.2% to 104% drug-loading efficiency. The protein incorporation efficiency increased with increasing PLGA polymer concentration in the disperse phase and PVA in the continuous phase as described previously.[1] Higher viscosity, achieved by increasing polymer and PVA concentration, could minimize diffusion of protein from the disperse phase to the continuous phase during the fabrication of microspheres and may also have resulted in more condensed PLGA matrices around entrapped aqueous protein droplets.

In Vitro Release

Drug-loaded PLGA-acHES composite microspheres exhibited a high burst effect with 50% HGH release after 1 day compared with the PLGA with mannitol microspheres (**Figure 2**). In general, the release of rHGH from PLGA-acHES composite and PLGA-mannitol microspheres occurs by 2 mechanisms. The strong burst effect observed for PLGA-acHES compos

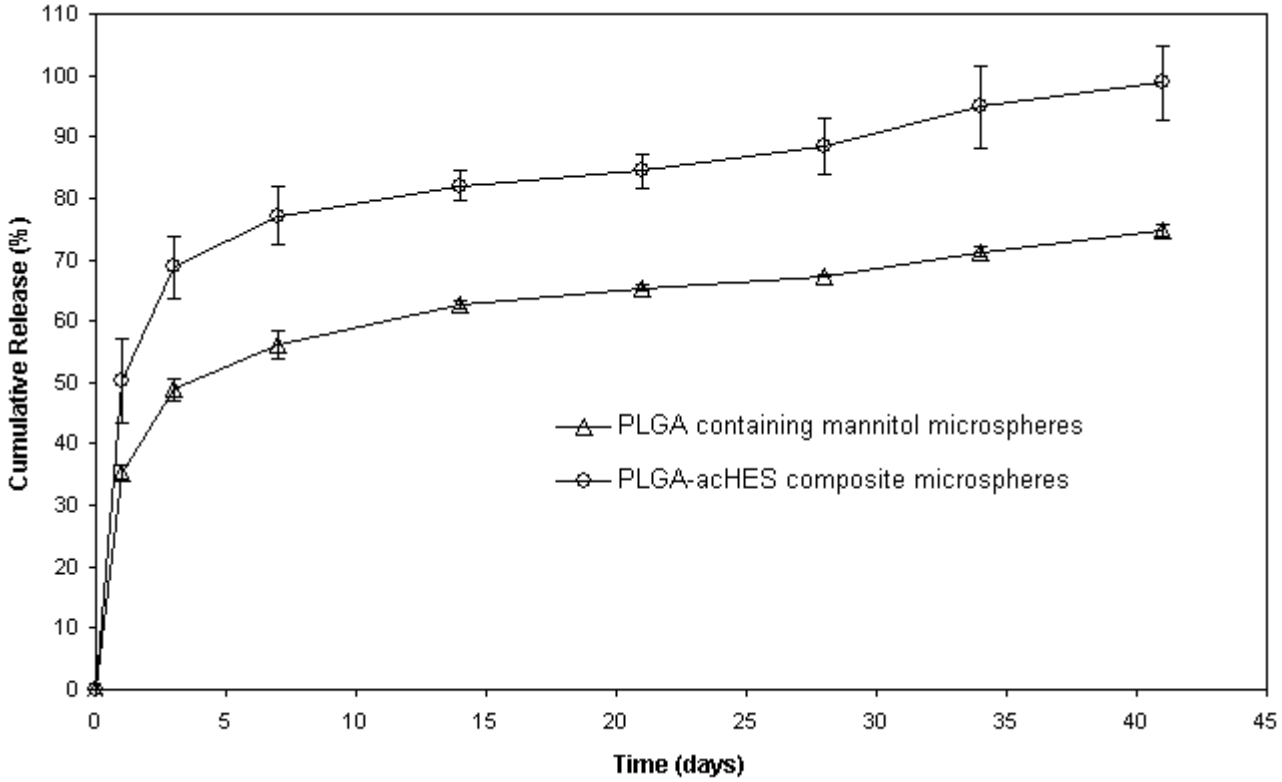

Figure 2. Release profile of rHGH-loaded acHES-PLGA microspheres and PLGA-mannitol microspheres. All measurements were performed in triplicate.

ite microspheres is due to diffusion of rHGH from acHES hydrogel particles located near the microsphere surface, through the channels and inner pores formed by solvent removal during the microspheres solidification process. The second mechanism involves the degradation and solubilization of the PLGA matrix. Compared with the composite microspheres, as shown in **Figure 2**, the conventional PLGA-mannitol microspheres showed about 15% less initial release followed by a progressive release for 41 days. As shown by mass balance investigation at the end of 41 days (**Figure 3**), 83% of rHGH was released from PLGA-acHES composite microspheres. Compared with the PLGA-mannitol microspheres, dissolution of PLGA domains in the composite microspheres could expose the entrapped rHGH-containing acHES hydrogel particles to the release media, and the exposed hydrogel could release more rHGH molecules with little or no interaction with the PLGA polymer. As a result, the composite microspheres showed more favorable in vitro release than the conventional PLGA-mannitol microspheres for rHGH drug delivery.

Secondary Structure Evaluation by FTIR

The use of FTIR technique for the detection of secondary structure changes in proteins, in particular for rHGH, has been investigated.[23-25] The bands at around 1656 cm^{-1} and at 1631 and 1695 cm^{-1} in the amide I region (1600-1700 cm^{-1}) have been identified as carbonyl stretching of α-helices and β-sheets, respectively, which characterize the secondary structure of the protein.[25] Several other peaks contribute to the broad amide I band, and these modes have been associated with the vibration frequencies of the disordered internal structure elements, such as random coils, extended chains, and β-turns.[26] In particular, the relative high α-helix content of rHGH is important in the study of the encapsulation procedure effect on the structure of the protein. In fact, the evaluation of any change caused by the process of preparation of rHGH-loaded microspheres on the α-helix band gives fundamental information not only on the retention of the original structure, but also on the relative activity associated with it.

Analyses were performed on every sample in the dry and rehydrated state. Such analyses are required since the protein profile in the dry state may not be predictive of that obtained after rehydration. In fact, according to

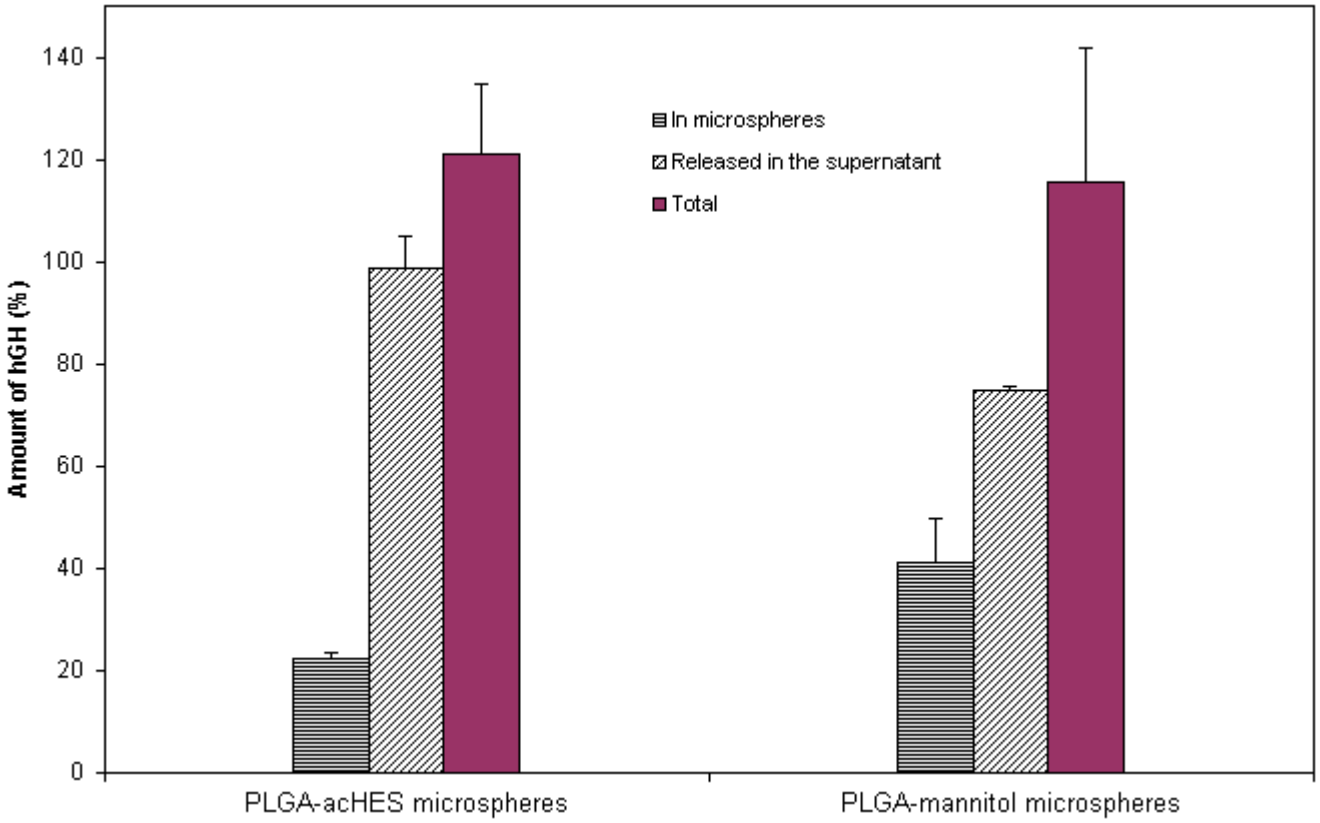

Figure 3. Mass balance of rHGH-loaded acHES-PLGA microspheres and rHGH-loaded PLGA-mannitol microspheres at day 41.

the chemical-physical properties of the molecule, many factors, such as excipients, pH, and aggregation may cause nonreversible unfolding of the protein. Predicting how these factors affect the protein behavior is particularly difficult.[21]

Therefore, scans of dry and rehydrated samples were performed under identical conditions. Spectra of dry rHGH protein and microspheres were obtained, as already mentioned in the experimental section, after subtraction of the PLGA reference spectrum as shown in **Figure 4**. **Figure 4A** shows the minimal absorbance in the amide I region of the PLGA employed in this study. The amide I (1600-1700 cm^{-1}) and amide II (1500-1600 cm^{-1}) regions are highlighted in **Figure 4B**. The flat region between 1700 and 1730 cm^{-1} demonstrates the fulfilling of the subtraction criteria reported elsewhere.[21] Similar treatment was employed for rehydrated microspheres analysis. In this case, rehydrated blank microspheres spectrum was employed as a reference. The resulting profiles are shown in **Figures 5** and **6**. α-Helix band retention evaluation was accomplished by comparison of relative percentage areas of the peaks of the rehydrated samples at ca 1655 cm^{-1} that can be deduced by curve-fitting of the second derivative spectra in the range 1600-1700 cm^{-1}.

Figure 5 shows the second derivative profiles and the calculated components in the amide I region for dry rHGH and dry rHGH-loaded microspheres having a correlation of >0.992. Matching closely the results already published,[26] α-helix and β-sheets components resulted at ca 1655 and 1631-1695 cm^{-1}.

Table 2 lists the calculated normalized percentage areas of dry samples obtained for α-helix and β-sheet components. The comparison of the obtained profiles with the profile of the native protein shows the broadening effect as a result of the induced structural changes and solid-state aggregation. This effect is less dramatic for rHGH entrapped in microspheres. Additionally, the calculated amide I α-helix content was comparable for all samples and a higher percentage value (33%) was found for the composite microspheres, whereas the β-sheet contribution increased for the microspheres (21% for the composite) compared with the freeze-dried powder. The main contribution

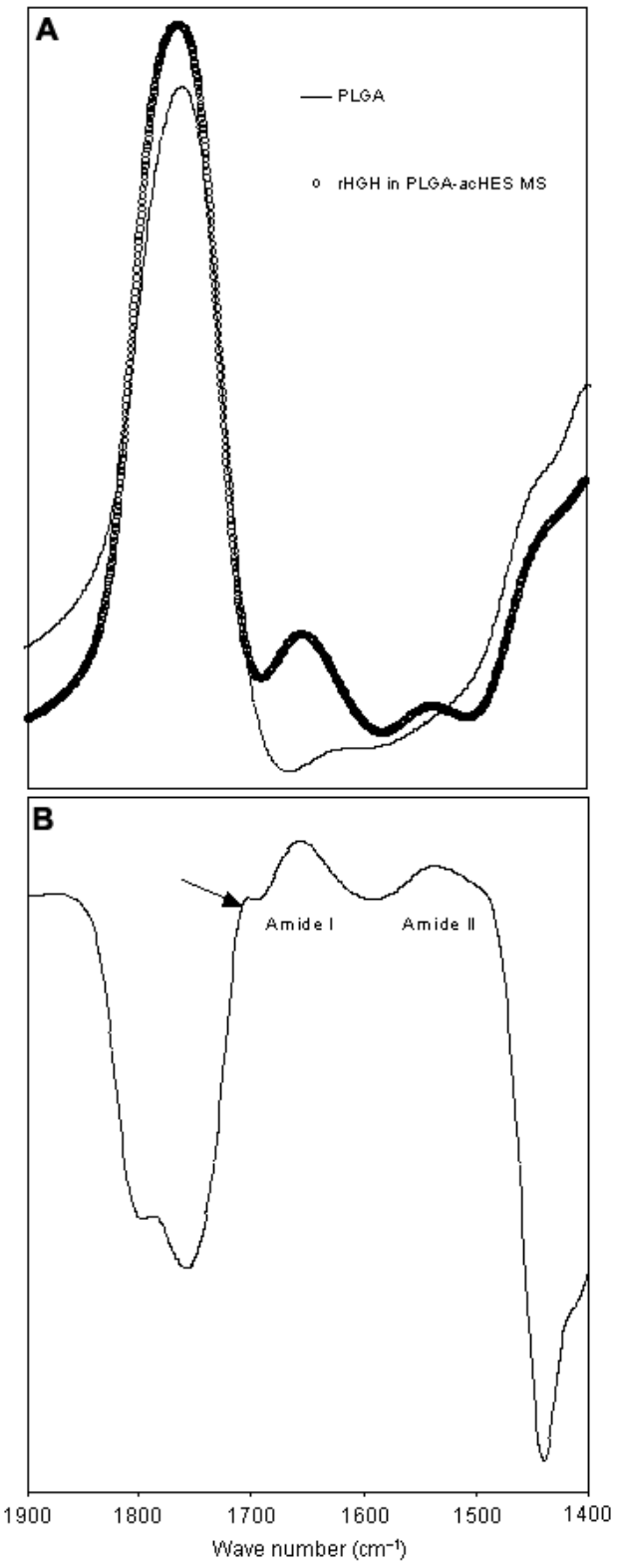

Figure 4. (A) Infrared spectra of rHGH in acHES-PLGA microspheres and PLGA microspheres without the protein. (B) The profile resulting from the subtraction of the PLGA background. The flat region between 1700 and 1730 cm^{-1} indicated by the arrow shows the criterion for subtraction. The amide I and amide II regions are indicated.

was found for unordered structures like coils, turns and extended chains.

On the other hand, the profile obtained upon rehydration of the dry samples (**Figure 6**) showed an increase in the α-helix component due to partial refolding of the protein in solution. Percentage areas of α-helix (**Table 3**) were 47% for composite microspheres, 24% for microspheres with mannitol, and 38% for the lyophilized protein. The corresponding retention of the α-helix band at 1655 cm^{-1} was close to 80% for the composite batch, only 40% for PLGA-mannitol microspheres, and 63% for freeze-dried rHGH as compared with the standard band area. In addition, although β-sheets content remained almost the same, the high presence of coils, turns, and extended chains was now reduced, and the lowest contribution was found for the composite microspheres with only 33% of the total. The low α-helix value for PLGA-mannitol microspheres may be correlated with the presence of nonnative aggregates of the protein as shown by the 26% β-sheets contribution to the total amide I band. Such high β-sheet is usually found to be representative of protein aggregation.[21] Besides, the components at 1610-1615 cm^{-1} and 1695 cm^{-1} represent intermolecular β-sheets aggregates. These bands are more evident in the rehydrated freeze-dried powder in comparison with composite microspheres, whereas in the rehydrated microspheres with mannitol only the band at 1695 cm^{-1} is visable. However, the large band at 1625-1627 cm^{-1} may be the result of the overlap of intra- and intermolecular β-sheets aggregation. Additional research is warranted to assess the storage stability of the encapsulated protein as nonnative proteins often have poor storage stability in the dried state. The addition of a stabilizing sugar such as sucrose to improve the retention of the native protein structure should also be considered.

These findings infer that rHGH entrapment in acHES-PLGA composite microspheres caused much less modification of the protein secondary structure during the preparation process than was observed with PLGA-mannitol microspheres and freeze-dried powder. The starch environment seems to exert an effective protective action on the entrapped rHGH, perhaps by avoiding direct contact with the surrounding dichloromethane phase during the microsphere formation process.

CONCLUSION

A novel biodegradable microsphere system has been developed for controlled rHGH delivery. The composite microspheres of a starch-based polymer and PLGA

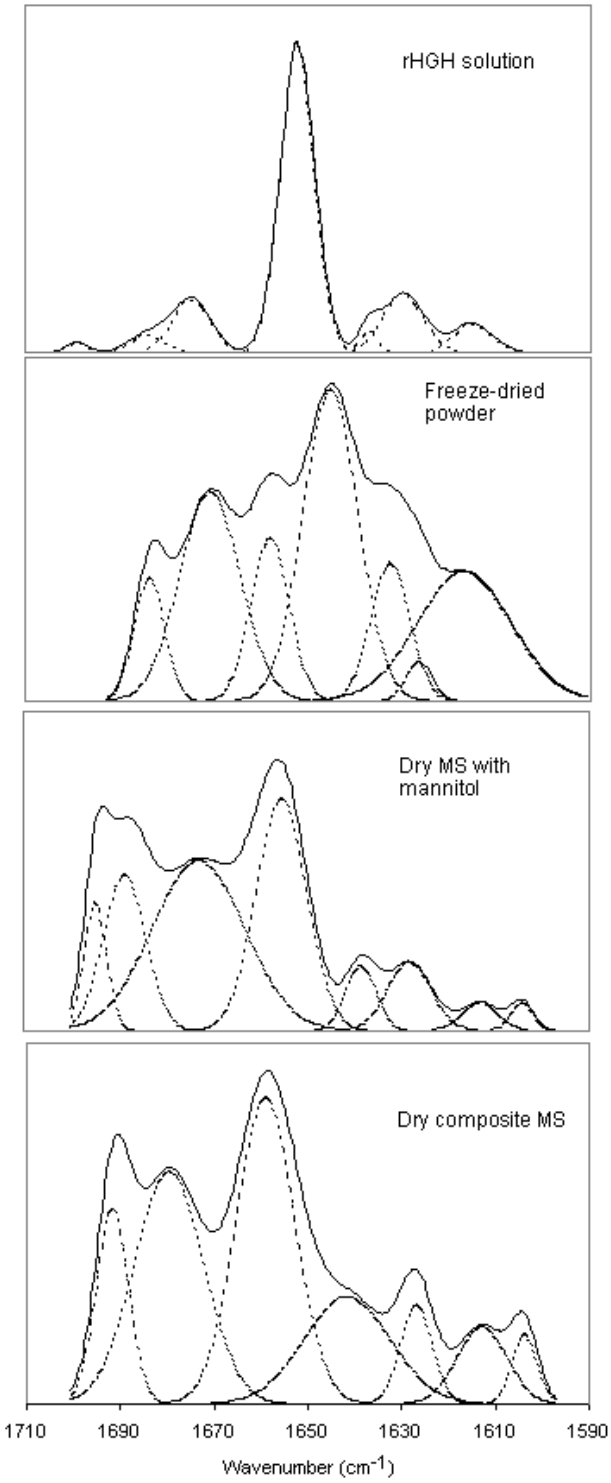

Figure 5. Second derivative amide I profiles curve-fitting for native, freeze-dried protein, dry rHGH encapsulated in microspheres with mannitol and dry rHGH entrapped in the acHES-PLGA composite microspheres. The band at ca 1656 cm^{-1} is the main rHGH secondary structure. Ratio of rHGH-microspheres was 0.093:1 for PLGA-mannitol and 0.103:1 for the acHES-PLGA microspheres sample. The dashed lines represent the calculated components from the fitting.

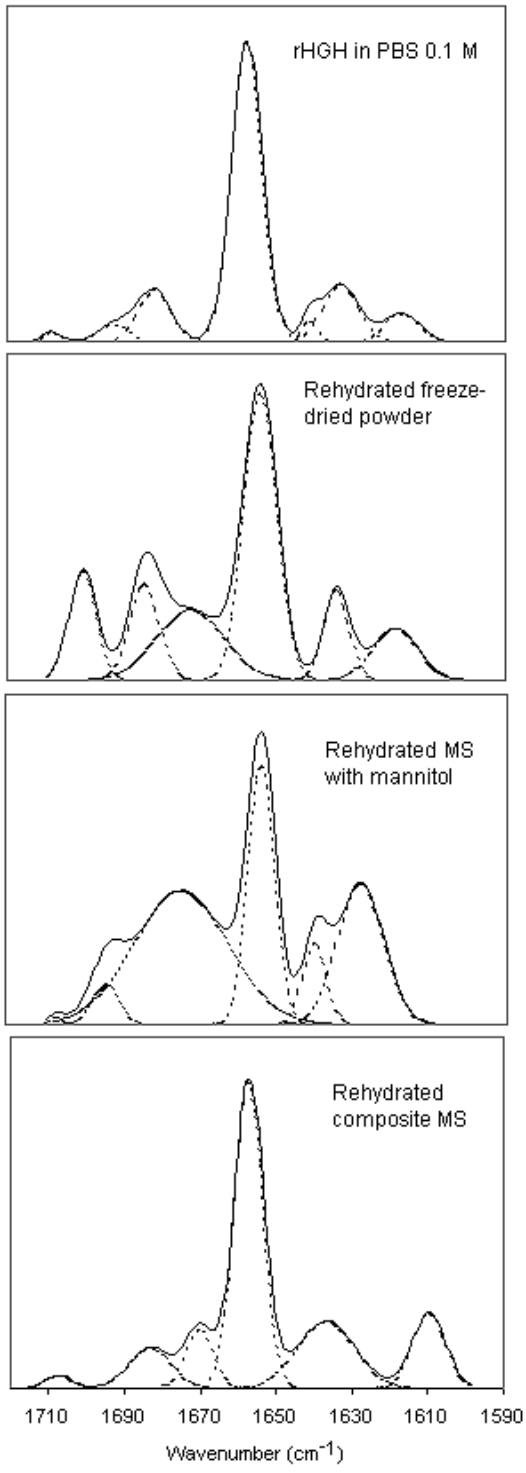

Figure 6. Second derivative spectra curve-fitting in the amide I region of native rHGH solution pH 7.4, rehydrated rHGH freeze-dried powder in PBS pH 7.4, rehydrated rHGH in microspheres with mannitol, and rehydrated rHGH in acHES-PLGA composite microspheres. The dashed lines represent the calculated components from the fitting.

Table 2. Calculated Data for α-Helix and β-Sheets Components in the Amide I Region for Standard, Freeze-Dried and encapsulated rHGH Dry Samples*

Secondary structure content	α-Helix	β-Sheet	Other†
	% Normalized Area ± SD	% Normalized Area ± SD	% Normalized Area ± SD
Standard rhGH in solution	61	14	25
rhGH dry	29 ± 2	10 ± 1	61 ± 2
Dry MS with mannitol	27 ± 1	14 ± 1	59 ± 2
Dry composite MS	33 ± 1	21 ± 1	46 ± 1

*rHGH indicates recombinant human growth hormone; and MS, microspheres.
† Includes random coils, turns, extended chains.

Table 3. Percentage of α-Helix Structure and β-Sheet After Rehydration of rHGH Free and Entrapped in Microspheres with Mannitol and Composite Microspheres*

Secondary structure content	α-Helix	β-Sheet	Other†	
	% Normalized Area ± SD	% Normalized Area ± SD	% Normalized Area ± SD	% α-Helix Retention* ± SD
Standard	61	14	25	–
Rehydrated freeze-dried powder	38 ± 1	10 ± 1	52 ± 2	63 ± 2
Rehydrated MS with mannitol	24 ± 1	26 ± 2	50 ± 2	40 ± 2
Rehydrated composite MS	47 ± 1	20 ± 1	33 ± 1	77 ± 2

*Retention of α-helix was calculated with respect to the normalized peak area. Increase in percentage of the β-sheets component is evident with respect to the standard. rHGH indicates recombinant human growth hormone; and MS, microspheres.
†Includes random coils, turns, extended chains

have been successfully formulated with spherical morphology, high protein incorporation efficiency, and good stability. The system possesses sustained rHGH release and rHGH protein stabilization characteristics. Noninvasive assessment of protein secondary structure in dried and rehydrated microspheres was accomplished rapidly by FTIR spectroscopy. The protein was effectively protected by the starch environment when entrapped in acHES-PLGA composite microspheres. The structure of the protein in dried microspheres may be predictive of storage stability of the protein. AcHES-PLGA microspheres are biodegradable and may potentially be useful for the in vivo delivery of rHGH.

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