A Large-Scale Process to Produce Microencapsulated Proteins

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Revised October 1, 1997; accepted November 19, 1997

KEY WORDS: sustained-release, microencapsulation, microspheres, human growth hormone, poly(lactide *co*-glycolide), PLGA

INTRODUCTION

Proteins are a rapidly expanding class of therapeutic agents and, although there are only a few proteins currently marketed, there are hundreds in clinical testing (1,2). Widespread acceptance and use of proteins by patients and the medical community, however, may be limited by several problems with their delivery. Proteins have negligible oral and transdermal bioavailability, which necessitates their administration by injection (3). Moreover, injections are given frequently because the *in vivo* half lives of proteins are generally no more than several hours.

One way to overcome this problem is to develop injectable sustained-release protein formulations (4). These formulations would offer advantages including local delivery to a particular site or body compartment thereby lowering the overall systemic exposure, protecting the drug over an extended period from degradation or elimination, and increased patient comfort, convenience, and compliance. Efforts to develop sustained-release formulations of proteins have focused on incorporating the protein into microspheres made of biodegradable polymers (for reviews, see (4–6)). After subcutaneous or intramuscular injection, the protein is released by diffusion as the polymer degrades and hence the microspheres do not have to be removed. Polymers that are generally used to form such microspheres, the homo- and co-polymers of lactic and glycolic acid (PLGA polymers), degrade by hydrolysis to ultimately give the acid monomers. They are chemically unreactive under the conditions used to prepare the microspheres and therefore do not modify the protein, and are available in a range of molecular weights and ratios of lactic:glycolic acid thus providing a range of variables with which the release rate can be adjusted. In addition, they are non-immunogenic, well tolerated and non-toxic, and are used to make biodegradable sutures, bandages, and bone plates (7–9).

Although PLGA microspheres have been made that deliver small organic molecules and peptides (e.g. steroids (10–12) and the depot formulation of the luteinizing hormone releasing hormone (LHRH) agonist (13,14)), the processes used to make such microspheres (e.g., phase separation, solvent evaporation, and spray-drying (5,6)) are generally not acceptable for proteins. In contrast to lower molecular weight drugs, proteins often have large globular structures with secondary, tertiary, and, in some cases, quaternary structure that is necessary for their biological activity. In addition, proteins have many more labile bonds and chemically reactive groups on their side chains which can undergo oxidation, deamidation, or disulfide reduction or interchange. The organic solvent-aqueous interfaces in the phase-separation and solvent-evaporation processes, the elevated temperature required for microsphere drying in the spray-drying process, and surfactants (which are usually present in all three processes to prevent agglomeration of the microspheres) can cause aggregation, deamidation, oxidation, or denaturation of the protein (6,15–18). In addition to causing loss in bioactivity, such modifications can render the protein immunogenic which can present safety concerns and limit the efficacy of subsequent treatments.

To avoid these problems we have developed a microencapsulation process that is conducted at cryogenic temperatures, employs no water and hence avoids oil-aqueous interfaces, and requires no surfactants (19). It has been used to formulate recombinant human growth hormone (rhGH), a protein that is given by daily injections as a protein solution, into microspheres that release the protein for up to one month (20,21) and clinical evaluation is currently in progress (22). Although useful for producing microspheres for research and early preclinical development, the process that has been used to date is unsuitable for the larger-scale reproducible production necessary for expanded clinical trials and eventual commercial-scale manufacturing. Sonication, which is used to form the microspheres, is a relatively high energy process which limits scalability. In addition, microsphere formation and solvent extraction are performed in open vessels, thus making it difficult to provide the necessary temperature and environmental control at larger scales. Microspheres must be made in an aseptic environment because, unlike protein solutions, microspheres are too large to be filter sterilized. Heat or irradiation can degrade the polymer or the protein (23–27) and affect microsphere performance.

We herein report a process with steam-in-place sterilization that has been used to produce PLGA microsphere batches up to several hundred grams. We show using a battery of assays that measure physical, chemical, and biological properties of the encapsulated protein, as well *in vitro* and *in vivo* performance of the microspheres, that microspheres made by this development-scale process are equivalent to those produced by the research-scale process.

MATERIALS AND METHODS

Microsphere Preparation

Both the research-scale and development-scale processes begin with a lyophilized complex of zinc-rhGH. This complex was made by mixing a solution of rhGH (Nutropin, Genentech, Inc.) with zinc acetate solution at a molar ratio of 6:1 zinc acetate:rhGH to create a zinc-hGH precipitate in which the protein is stabilized and more resistant to denaturation (28). In the research-scale process, the Zn:hGH precipitate was atomized through an ultrasonic nozzle into liquid nitrogen and the frozen droplets were lyophilized using an FTS lyophilizer. In

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0724-8741/98/0200-0357$15.00/0

the development-scale process, filter sterilized zinc acetate and protein solutions were combined in an isolator (LaCahene, Bordeaux, France) and passed through an air atomizer into a closed, sterilized-in-place stainless steel vessel containing liquid nitrogen. The protein powder was recovered in the isolator and lyophilized.

In the research-scale process, the zinc-hGH powder (15% w/v) and zinc carbonate (1% w/v) were added to a solution of the polymer (D,L-PLGA, lactide:glycolide molar ratio of 50:50, Boehringer Ingelheim, RG502H) in dichloromethane (Quantum, Newark, NJ) and sonicated using a Sonics and Materials Vibra-Cell VC600 Sonicator (Figure 1). In the development-scale process, the polymer-protein suspension was prepared in an isolator and added to dispersion tank A. It was then passed into dispersion tank B containing a rotor/stator homogenizer. It was then passed through an in-line high pressure (HP) homogenizer several times. Both tanks are jacketed to provide temperature control to prevent protein denaturation. In both processes, these steps reduced the protein and zinc salt particle size to between 1–3 microns.

In the research-scale process, the polymer-protein suspension was sprayed through a sonicating nozzle (Sonics and Materials probe model CV26 with atomizer model 630-0434) into a vessel containing frozen ethanol (Quantum, Newark, NJ) overlaid with liquid nitrogen. The vessel was then transferred to –80°C and the microspheres hardened as the dichloromethane was extracted by the ethanol. After 3 days, the microsphere suspension was filtered and the microspheres harvested by vacuum funnel filtration at room temperature and dried under vacuum in a lyophilizer. In the development-scale process, the microspheres were formed by passing the homogenized protein-polymer suspension through an air-atomizer. The suspension was atomized using nitrogen gas into a chamber containing liquid nitrogen. This froze the droplets which flowed into a temperature controlled extraction tank containing liquid (rather than frozen) ethanol (at approximately –105°C). Following spraying, the ethanol was warmed to –40°C over several hours with gentle mixing as the dichloromethane was extracted from the microspheres. The microspheres were recovered by filtration and dried using a Nutsche-type filter-dryer vacuum vessel. From the addition of the polymer-protein suspension to tank A through the drying step, the process was conducted entirely in sterilized-in-place stainless steel vessels. In both processes, the microspheres were manually sieved through a 106 μm mesh screen to remove large particles that could interfere with injectability.

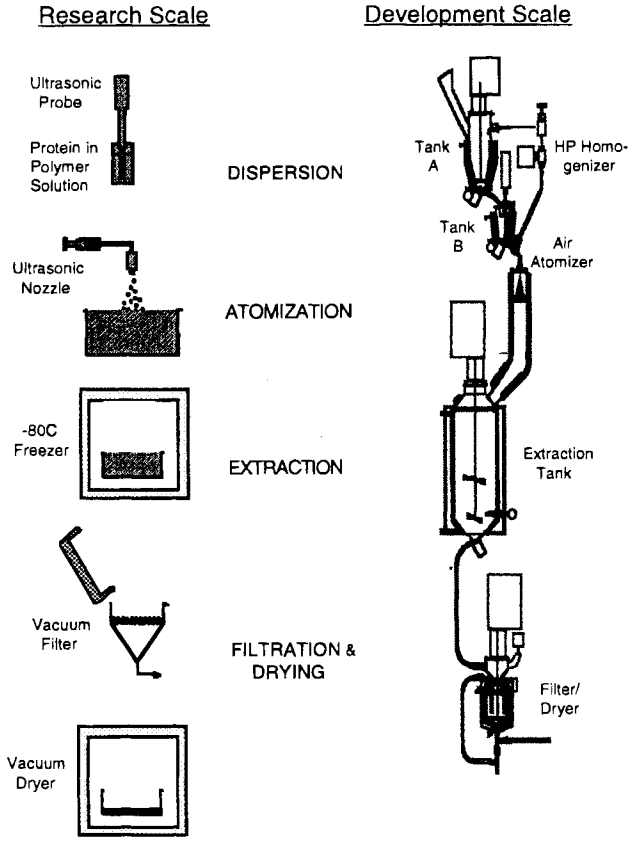

Fig. 1. Diagram of research-scale (left) and development-scale (right) processes to produce PLGA microspheres. The process differ principally in four ways. The development-scale process uses: 1) homogenization rather than sonication to reduce the particle size of the rhGH:Zn complex; 2) air atomization instead of sonication to form the microsphere droplets; 3) mixing with liquid ethanol to extract the polymer solvent (dichloromethane); and, 4) agitated vacuum drying to dry the microspheres.

Microsphere Analysis

Mean particle diameter (by volume) of the microspheres (approximately 10 mgs. in 80 ml of an aqueous electrolyte solution (Isoton)) was determined from the particle size distribution determined using a Coulter Multisizer. Calibration standards were polystyrene microspheres (NIST-traceable). Residual moisture was determined using Karl Fischer titration as described (29). Briefly, approximately 20 mgs of microspheres were added in a closed vessel to a solvent of methanol and pyridine in which the microspheres and encapsulated protein dissolve. The amount of water was determined by coulometric titration. Residual dichloromethane and ethanol were measured by dissolving 10 mgs of microspheres in dioxane, heating to 90°C, followed by analysis with head-space capillary gas chromatography. Zinc content was determined by mixing 10 mgs of microspheres in concentrated sulfuric acid, heating for 10 minutes, adding concentrated nitric acid until the solution became colorless, diluting with water and analyzing by inductively-coupled plasma emission spectroscopy. The glass transition temperature was measured by differential scanning calorimetry (Perkin Elmer Model DSC7). Sterility of the microspheres was assessed using two assays. External sterility was assessed by incubating microspheres (~10 mgs) directly in thioglycollate medium with tryptic soy broth and monitoring growth over 14 days. In addition, contamination within the microspheres was assessed by dissolving the microspheres in DMSO, filtering the solution, and incubating the membranes in the above growth medium. All lots produced were shown to be sterile using both assays.

The rhGH load of the microspheres (weight percent of microspheres as rhGH) was determined by nitrogen analysis. Microsphere aliquots (~5 mg) were combusted at 1000°C in an oxygen atmosphere to produce nitrogen and nitrogen oxides using a CHN elemental analyzer (Control Equipment Corpo-

ration). The gas stream was reduced over copper at 650°C to produce elemental nitrogen which was then quantified using a thermal conductivity detector. The amount of rhGH released from the microsphere *in vitro* was determined by incubating the microspheres (50 mgs) in buffer (50 mM HEPES, pH 7.2, 10 mM KCl, 0.1% NaN_3) at 25°C and determining the amount of protein released after 24 hours and over 28 days by size-exclusion chromatography.

Analyses of rhGH

The protein was recovered from the microspheres and chromatographic and bioactivity assays (using proliferation of a cell line transfected with the hGH receptor) were performed as described (19).

In Vivo Analyses

The microspheres were administered to Sprague-Dawley male rats or juvenile male rhesus monkeys (*Macaca mulatta*) (monkeys were between 1.5 and 3 years old and weighed between 2 to 3 kg). To prevent the formation of antibodies to rhGH in the rats, which would affect the quantitation of the protein in the serum and may affect the pharmacokinetics, the animals were immunosuppressed by intraperitoneal treatment with cyclosporin and hydrocortisone (H. J. Lee, O. L. Johnson, et al., unpublished). Microspheres (50 mg/rat or 50 mg/kg body weight in monkeys) were suspended in an aqueous vehicle as described (20) and injected subcutaneously. Serum hGH concentrations and antibodies to hGH were measured as described (19,21).

RESULTS AND DISCUSSION

The properties of the microspheres made by both processes were analyzed (Table I). The particle diameter and the weight percent of hGH and zinc were equivalent in product made by both methods, and were close to the target value of 15% for the protein and 1% for zinc. The level of dichloromethane in the microspheres produced with the development process were lower than those made using the research process, indicating more efficient extraction. These levels were thirty times lower than the allowable amount for a pharmaceutical product (30). The level of residual ethanol and moisture were lower as well, indicating more complete drying. Minimizing residual solvents and moisture should enhance the product storage stability. The physical properties of the rhGH as analyzed by size exclusion, reverse phase, and ion exchange chromatography (detecting aggregation, oxidation, and deamidation) were similar in protein recovered from microspheres made by both methods. In addition, the bioactivity was equivalent.

The performance of the microspheres was determined by the release rate of the protein, which was measured both *in vitro* and *in vivo*. The amount of protein released *in vitro* on days 1 and over 28 days was similar (Table 1), as was the amount released at intervals between these two timepoints (not shown). Moreover, lots made with the development process exhibited less variability. In addition, the serum profiles in immunosuppressed rats which received a single dose of microspheres were similar as well (Figure 2A). The biological effect of the released was assessed by measuring the IGF-I levels in juvenile rhesus monkeys (Figure 2B). Elevated hGH levels induce elevated levels of IGF-I, which mediates the effect of hGH (31). Microspheres produced by the development-scale process induced IGF-I profiles equivalent to those induced by microspheres made by the research process. The immunogenicity of the released hGH was similar as well; only one of the four monkeys that received microspheres made by the development process produced antibodies to hGH, compared to one of eight monkeys that received microspheres produced with the research process (21).

Process variables in each of the four principal steps of the development process (Figure 1) were investigated to determine

Table 1. Properties of PLGA Microspheres and Encapsulated rhGH Made Using Both the Research and Development-Scale Processes

Attribute	Research-Scale	Development-Scale
Microsphere Diameter (microns)	49.6 ± 5.8	49.8 ± 5.4
Residual Dichloromethane (ppm)	330 ± 210	21 ± 26
Residual Ethanol (%)	0.78 ± 0.85	0.18 ± 0.24
Residual Moisture (%)	1.7 ± 0.7	1.1 ± 0.2
Zinc Content (%)	0.93 ± 0.08	0.96 ± 0.09
Glass Transition Temperature (°C)	40.8 ± 4.4	45.4 ± 1.3
rhGH Load (weight %)	16.0 ± 0.5	14.7 ± 0.4
Initial (day 1) rhGH Release in vitro (%)	42.3 ± 18.3	33 ± 5.1
Extended (day 0–28) Release in vitro (%)	72.8 ± 17.0	65.6 ± 6.1
rhGH Aggregation (% native by SEC)	99.3 ± 0.4	98.4 ± 1.0
rhGH Oxidation (% native by RP-HPLC)	98.5 ± 1.5	99.2 ± 0.4
rhGH Deamidation (% native by IEC)	99.7 ± 0.4	98.1 ± 0.6
rhGH Bioactivity (IU/mg hGH)	2.7 ± 0.2	2.7 ± 0.3

Note: All tests for protein integrity were performed with protein extracted from the microspheres (see Material and Methods). SEC analysis of the rhGH released *in vitro* (at days 14 and 28) showed that the percent native was comparable to that in the extracted protein (data not shown). The particle size distribution of the microspheres produced at the research scale (19) was comparable to those made at the development scale. Reported are the mean ± standard deviations of five lots made at each scale. All lots had an endotoxin level of ≤0.5 eu/mg hGH.

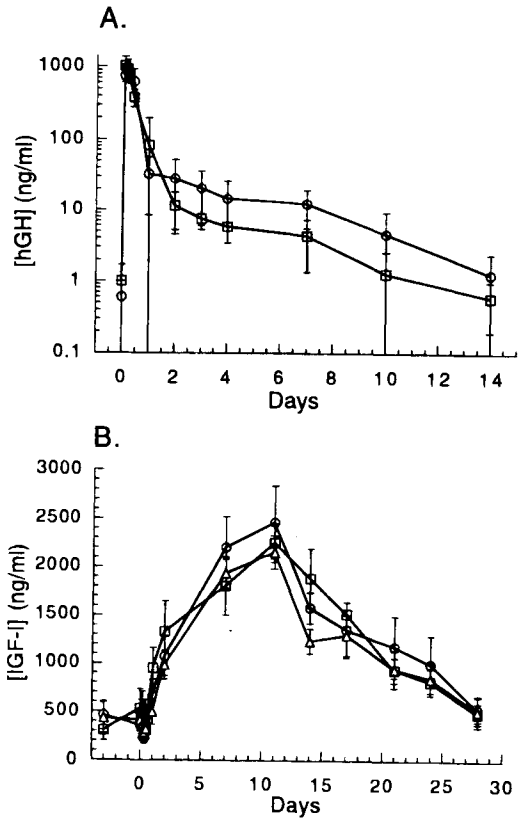

Fig. 2. A Serum rhGH levels in immunosuppressed rats after subcutaneous injection of rhGH containing microspheres produced by the research-scale process (○) and the development-scale process (❑). Each point is the mean (± standard deviation) of five microsphere batches evaluated in three rats. The maximum serum concentration was 830 ± 245 and 1020 ± 365 ng/ml in the animals receiving the research and development scale processes, respectively. B. IGF-I levels (which are induced by hGH) in juvenile rhesus monkeys (n = 4) after receiving a single injection of microspheres made by the research process (○) or the development-scale process (two different batches (❑ and △)).

their impact on the properties or performance of the microspheres. These included: the mixer speed in tank A, the homogenization conditions and temperature and time in tank B (in the dispersion step); the flow rates of the polymer-protein suspension, the gaseous nitrogen, and the liquid nitrogen (in the atomization step); the ethanol temperature, rate of warming and the mixer speed (in the extraction step); and the temperature (in the filtration and drying steps). Variables found to have a significant impact on the microspheres are listed in Table 2. With regard to the dispersion step, it has been long established that the smaller ratio of the size of the protein particles to the microspheres, the lower the initial release (32). The protein particles are reduced to their final size by homogenization and it is therefore important that this process reduce the size to 3 microns or less. In the atomization step, the size of the microspheres is determined by the flow rate of the polymer-protein suspension and the gaseous nitrogen; the higher the polymer or the lower the nitrogen flow rates, the larger the particle size and vice versa. In the extraction step sufficient dichloromethane is removed from the microspheres to fix the polymer network. It is therefore important to maintain the temperature of the

Table 2. Development Process Parameters Important for Producing Microspheres with Acceptable Characteristics and Performance

Process step	Process parameter
Dispersion	Homogenization sufficient to produce protein particles of 1–3 microns
Air Atomization	Flow rates of polymer-protein suspension and nitrogen adjusted to produce microsphere of approximately 50 microns
Extraction	Ethanol temperature below freezing point of methylene chloride (–95°C)
Filtration and Drying	Temperature below glass transition temperature

ethanol below the freezing point of the polymer solution to avoid producing microspheres with a high initial release. Finally, during drying it is important to maintain the temperature below the glass transition temperature of the polymer to prevent agglomeration of the microspheres.

The development-scale and research-scale processes differ in several ways. The research-scale process uses sonic energy both to reduce the particle size in the protein-polymer suspension and to form the droplets that eventually become microspheres. In contrast, the development-scale process uses homogenization to reduce particle size and air atomization to form the microsphere droplets. These methods are less energy intensive with higher throughput than sonication. Another difference is in the solvent extraction step, where the development-scale process uses mixing with liquid ethanol. This allows more precise temperature control and allows the extraction to be completed in less time. In addition, because the microsphere fabrication and solvent extraction steps are done entirely in enclosed vessels, there is more temperature and environmental control (allowing more straightforward aseptic microsphere fabrication) in the development-scale process.

Just as improved delivery systems have enhanced the usefulness of small molecular weight drugs, sustained-release delivery of proteins has the potential to improve and expand the use of protein therapeutics. Although the most readily apparent use of sustained protein delivery is for proteins that are currently administered systemically (e.g., hGH), other applications including local delivery, delivery to sites that cannot be accessed by the blood stream, and vaccines may also represent uses of sustained-release protein formulations. The ability to produce suitable quantities of these improved formulations for clinical development and commercialization will be necessary to enable proteins to become as widespread and commonplace as other classes of drugs.

ACKNOWLEDGMENTS

The authors thank Hye Jung Lee, Paul McGoff, Tracy Olson, Craig Patrizio, Steve Zale, Gary Riley, Tom Last and Jim Wright for helpful discussions, Leonie Bailey, Norman Kim, Margarita Charnis, Rulin Qian, Chichih Wu, Douglas Shepard, and Tony Pinho for technical assistance, and Robert Breyer, Robert Langer, Richard Pops, and Bill Young for support and encouragement.

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