A Potential Approach for Decreasing the Burst Effect of Protein from PLGA Microspheres

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ABSTRACT: A central issue in controlled delivery of therapeutics from biodegradable microspheres is the immediate burst of drug release upon injection. This burst is often observed with microsphere systems made by the double emulsion (w/o/w) technique, and may be prevented by improving the drug distribution throughout the polymer matrix. To this end, protein and polymer (poly-lactide-*co*-glycolide or PLGA) were dissolved within the same solvent system, and micron-sized microspheres were created from this solution by spontaneous emulsification. Improved protein loading was achieved by ion-pairing the protein with charged surfactants to increase solubility in the single-phase solvent system. Both *in vitro* and *in vivo* results showed a much diminished burst: compared to microspheres made by double emulsion, it was reduced over 10-fold. J Pharm Sci 92:1582–1591, 2003

Keywords: r-met-HuGdNF; PLGA microspheres; spontaneous emulsification; burst effect; proteins

INTRODUCTION

The most widely used delivery form of protein and peptide drugs is as injectables. As these therapeutics have short half-lives due to proteolysis and rapid clearance from the bloodstream,[1] patients often require daily injections. Encapsulation of proteins within biodegradable polymers has been shown to enhance the half-life *in vitro*[2,3] and *in vivo*.[3–5] PLGA is often used in such applications because it is safe. The most common technique for protein encapsulation in PLGA microspheres is the double-emulsion (DE) method.[4,6] Because it is difficult to entrap hydrophilic molecules within a hydrophobic polymer, this technique relies on high energy mixing. To this aim, an initial water-in-oil (w/o) emulsion of an aqueous protein solution and an organic polymer solution is formed, and then a second emulsion, (w/o/w), is formed by dispersion of the first in an aqueous phase.

Typical release kinetics from DE microspheres exhibit an initial drug burst upon immersion in release medium either *in vitro* or *in vivo*. The loss

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over the initial 24-h period can be over 50% of the total loaded drug.[7] This is usually a distinct drawback in terms of efficient use of therapeutic and a desirable release profile. The immediate release is thought to be due to protein either associated with the surface of the particle or situated in pockets close to the surface and easily accessible once the particle is hydrated.[8,9] In the DE process, the distribution of protein within the particles is determined by the homogenization and/or sonication of the aqueous phase within the organic phase. Because the emulsion is created by physical mixing, the distribution of protein within the particles is not homogeneous.

A spontaneous emulsification (SE) solvent diffusion technique[2] utilizes a single-phase solvent system in which both protein and polymer are soluble and avoids the need for high energy mixing as the particles form spontaneously. This may aid in improving protein stability by minimizing aggregation due to the aqueous/organic interface.[3,10–12] Published methods for fabrication of SE particles describe procedures for encapsulating substances within nanospheres.[2,13–16] There are, however, inherent difficulties in working with nanoparticles. Because of the large surface to volume ratio, the burst is usually high, drug loadings are low, and polymer degradation occurs more rapidly. Also, the smaller particles are difficult to recover and tend to aggregate. There are several potential advantages for using the SE method for encapsulation. As the protein is soluble in the same phase as the polymer, its homogeneous distribution within the microspheres is expected. This may lead to a minimal initial burst of protein upon hydration of the particles and release, which is closely tied with polymer degradation. In scaling up for manufacturing, there should theoretically be few problems because the process is driven by thermodynamics.[15,17] There is also the added possibility of designing a continuous manufacturing scheme as opposed to a batch process.

Our objective in this study was to encapsulate large, hydrophilic proteins within micron-sized particles. Three model proteins—recombinant methionyl human Glial-cell line derived neurotrophic factor (r-met-HuGdNF), bovine serum albumin (BSA), and hen egg-white lysozyme—were tested within this system. Exogenous r-met-HuGdNF is a particularly relevant controlled release drug delivery candidate because when injected into rats through the intramuscular route it has an extremely short half-life.

MATERIALS AND METHODS

Materials

R-met-HuGdNF was manufactured at Amgen, Inc. (Thousand Oaks, CA). Monoclonal anti-r-met-HuGdNF antibody and biotinylated goat polyclonal anti-r-met-HuGdNF were purchased from R&D Systems (Minneapolis, MN). Chicken egg-white lysozyme (95% purity), BSA, fluorescein isothiocyanate-BSA (FITC-BSA), sodium dodecyl sulfate (SDS), docusate sodium (AOT), and taurocholic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxy methyl polyethylene glycol (CM-PEG), 3400 MW was purchased from Shearwater Corp. (Huntsville, AL). Streptavidin-horseradish peroxidase was obtained from Jackson Immuno Research (West Grove, PA), biotin from Pierce (Rockford, IL), goat serum from Gibco (Carlsbad, CA), and 2,2′-Azinobis[3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt (ABTS) from Kirkegaard & Perry Laboratories (Gaithersburg, MD). PLGA (50:50, RG502H, inherent viscosity 0.2 dL/g in $CHCl_3$ at 30°C, MW 9300 Da) was purchased from Boehringer Ingelheim (Montvale, NJ). PVA (88% hydrolyzed, 25,000 Da) was obtained from Polysciences (Warrington, PA). The Lowry protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA), the bicinchoninic acid (BCA) assay kit from Pierce (Rockford, IL), and the 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) assay kit from Molecular Probes (Eugene, OR). All other chemicals were reagent grade.

Solubility of r-met-HuGdNF:Surfactant Complex in the Single-Phase Solvent System

One hundred microliters of a 1-mM r-met-HuGdNF solution or r-met-HuGdNF (1 mM):surfactant complex mixture was added to 2.5 mL of the single-phase solvent system and vortexed. The solvents tested included methylene chloride, chloroform, and ethyl acetate, and the cosolvents included acetone, dioxane, and trifluoroethanol (TFE). Turbidity measurements (OD 600 nm) were then made on the mixture. A low turbidity indicated higher protein solubility.

Protein Partitioning between the Aqueous Phase and Methylene Chloride

Protein partitioning experiments were conducted following an approach similar to that of Bromberg

and Klibanov.[18] One milliliter of methylene chloride was first added to each 18 × 100 mm glass test tube. An equal volume of aqueous solution containing protein (1 mM) with or without surfactant was then added to the test tube, and the tube was capped and vortexed. The mixture was then incubated at room temperature overnight to allow partitioning to reach equilibrium. One-half milliliter of the organic phase was removed, transferred to a clean test tube, and placed under vacuum to evaporate to dryness. To the remaining residue was added 250 μL of 0.1 N HCl, and the tube was placed in a sonicating water bath for 30 s. The BCA assay was then performed on the solution to determine protein content.

Quantitation of r-met-HuGdNF by Enzyme-Linked Immunoassay (ELISA)

Standard sandwich ELISA tests were performed using a Kinetic Microplate Reader, Model Vmax (Molecular Devices Corp., Sunnyvale, CA). Ninety-six-well plates were coated with monoclonal anti-r-met-HuGdNF antibody (3 μg/mL) and blocked with 2% BSA blocking buffer. Following sample addition (50 μL diluted with 20% normal goat serum in Dulbecco's phosphate-buffered saline), biotinylated goat polyclonal anti-r-met-HuGdNF (50 μL solution at 0.1 μg/mL in Dulbecco's phosphate-buffered saline/0.5% Tween-20) was added. After incubation for 2 h at room temperature, streptavidin-horseradish peroxidase (50 μL at 0.1 μg/mL in Dulbecco's phosphate-buffered saline/0.5% Tween-20) was added followed by ABTS substrate. After a 20-min incubation at room temperature, optical density was measured at 405 and 490 nm for a dual wavelength kinetic reading. R-met-HuGDNF concentrations and coefficient of variation were calculated for each unknown sample from a five-parameter curve fit of the plate standard dilutions. Standards ranged from 0.03 to 2 ng/mL.

Preparation of r-met-HuGdNF-Containing Microspheres by the DE Technique

Protein microspheres were prepared by the double-emulsion technique.[3] To 250 μL of aqueous 1 mM r-met-HuGdNF solution was added 7 mL of solution of 29 mg/mL PLGA in methylene chloride, and the mixture was sonicated on ice for 10 s with a Vibracell (Danbury, CT) sonicator using a microtip probe with an output of 3 and 50% duty. This primary emulsion was then added to 100 mL of aqueous solution of 1% (w/v) PVA. The second emulsion was formed by homogenization for 1 min at 3,000 rpm on a Silverson (East Longmeadow, MA) homogenizer using a 5/8″ micromixing assembly with a general-purpose disintegrating head. The resultant water-in-oil-in-water (w/o/w) emulsion was stirred continuously for 3 h at room temperature to promote solvent evaporation. The hardened microspheres were centrifuged, washed three times with water, freeze dried, and stored over desiccant at −20°C.

Preparation of Microspheres by the SE Technique

Protein-containing microspheres were prepared by a modified SE method.[2] Cosolvent and organic solvent were added to the polymer, and then the aqueous protein solution was added. This mixture was vortexed and poured into a larger volume of a PVA solution. The resultant mixture was stirred continuously for solvent evaporation. The hardened microspheres were then centrifuged, washed three times with water, freeze dried, and stored over desiccant at −20°C.

Optimal conditions for manufacturing the r-met-HuGdNF microspheres were found to be: addition of 150 μL of r-met-HuGdNF:surfactant complex [1:30 (mol:mol)] to a solution of 100 mg of polymer dissolved in 2.5 mL of single-phase solvent (1:4 methylene chloride:trifluoroethanol). After mixing, this mixture was added directly to 100 mL of 5% chilled solution of PVA, stirred, washed, and freeze dried.

Protein Loading and Spatial Distribution within the Microspheres

Ten milligrams of dried microspheres were suspended in 0.1 N NaOH/0.5% SDS (3 mL) and incubated overnight at 37°C. Under these conditions, the polymer is completely hydrolyzed. Protein content was then determined using the Lowry assay.

Microspheres containing FITC-BSA were imaged using a Bio-Rad MRC 1200 confocal microscope with an excitation wavelength of 488 nm and emission wavelength of 522 nm.

Particle Size Distribution and Morphology

The particle size distribution was measured using a Beckman-Coulter Counter Multisizer II (Miami,

FL). A 100-μm orifice was used and at least 50,000 particles were counted for each sample.

Scanning electron micrographs were obtained using a JEOL JSM-6320 FV microscope at 0.8 kV. Microspheres were mounted in the powder form and were not coated.

In Vitro Studies of Protein Release from Microspheres

Ten milligrams of dried microspheres were suspended, in triplicate, in round-bottom, 1.8-mL cryovials each containing 1 mL of 10 mM citrate buffer, pH 5.0. Sample tubes were incubated at 37°C with rotation on a Labquake shaker/rotator. To sample the released protein, tubes were centrifuged and the supernatant was removed and frozen at −80°C. The sample tubes were replenished with 1 mL of buffer, vortexed to resuspend the microspheres, and placed back in the incubator. Protein concentrations were determined using either the BCA or CBQCA assay.

In Vivo Studies of Protein Release from Microspheres

Male Sprague-Dawley rats of age 40–60 days and weighing approximately 230 g were selected for this study. Fifteen rats were separated into three equal groups injected subcutaneously either (a) daily with soluble r-met-HuGdNF (0.7 mg/day for a total of 9.8 mg over 14 days), (b) once with r-met-HuGdNF-containing microspheres made by DE (containing 9.8 mg of the protein), or (c) once with r-met-HuGdNF-containing microspheres made by SE (containing 9.8 mg of the protein). For the DE and SE microspheres, the actual r-met-HuGdNF loading was 4%, and the average diameter of the particles was 14.6 ± 2.7 μm (DE) and 14.4 ± 1.9 μm (SE). Prior to injection, 0.45 mL of blood was obtained from the tail vein and subsequently, blood samples were taken at 1, 2, 4, 8, and 12 h postinjection and then daily for 13 days. Following sampling, all blood samples were kept at room temperature for approximately 30 min and then centrifuged at 11,500 rpm for 10 min at 2–8°C. The separated plasma was removed and stored in fresh vials at −80°C until assayed by ELISA.

The relative bioavailability was estimated by quantifying the area under the curves (AUCs) using the trapezoidal rule over 14 days of the DE and SE microspheres and comparing them to the AUC of the subcutaneous injection.

RESULTS AND DISCUSSION

To encapsulate r-met-HuGdNF within micron-sized particles using the SE technique, a solvent system in which both the protein and polymer were soluble was selected. The solubility of the protein within the solvent system was studied, and experiments were conducted to optimize the size of, and the protein loading within, the microspheres.

Stability of r-met-HuGdNF at 37°C

The integrity of r-met-HuGdNF was assessed by ELISA after incubating the protein in 10 mM citrate buffer, pH 5.0 at 37°C for over 40 days; the test conditions were chosen considering that r-met-HuGdNF is not stable in phosphate-buffered saline. Results showed good relative stability (the average level of $94 \pm 9\%$) with a slight decrease at days 4 and 40 (80 and 86%, respectively). Although this test does not address the stability of r-met-HuGdNF in biological fluids, it indicates that physiologic temperature does not significantly damage the protein.

Protein Solubilization in the Single-Phase Solvent System

To achieve protein encapsulation within the SE microspheres, a single-phase solvent system had to be selected in which both the protein and polymer were soluble. Systems consisting of aqueous citrate buffer, organic solvent, and a cosolvent were tested by measuring the turbidity of the protein- and polymer-containing solutions (see Materials and Methods). A clear or slightly turbid solution gave indication of protein and polymer solubility over a turbid or cloudy solution. Of those considered, TFE mixed with any of the organic solvents tested (methylene chloride, chloroform, and ethyl acetate) yielded higher protein solubility than any other solvent/cosolvent combination. Thus, it became the cosolvent of choice.

Solubility of the protein in the single-phase solution is critical for achieving high loadings within the microparticles and high encapsulation efficiencies. It also allows for a more uniform distribution of protein within the polymer microspheres, which in turn, leads to a minimization of the burst effect. To further increase the solubility of the protein in the single-phase solvent system, the former was complexed with an ionic surfactant

before encapsulation. This approach is actively studied for drug delivery across membranes such as in the lungs, gastrointestinal tract, and blood–brain barrier.[18,19] Here, improvement of the solubility of the model protein lysozyme and of r-met-HuGdNF in organic solvents and in a single-phase solvent system upon complexation with a charged surfactant was studied.

Both proteins have high isoelectric points (10.5 and 11.0, respectively) and thus had a net positive charge in the pH 4.5–5 buffer systems selected. Accordingly, negatively charged and relatively nontoxic surfactants were chosen for the study: CM-PEG, cholic acid, SDS, AOT, and taurocholic acid; the first two carry weakly acidic carboxylic groups, while the rest carry strongly acidic sulfonic groups.

The solubilization of lysozyme in methylene chloride was assessed by measuring the ability of the lysozyme:surfactant complex to partition from an aqueous into an organic phase (see Materials and Methods). Because lysozyme has a net charge of +10 in the selected pH 4.5 buffer system (calculated based on the amino acid composition), the molar ratio of protein to surfactant tested was 1:10. The results of these experiments (Figure 1) indicate that both the nature of the ionizable group and hydrophobicity of the surfactant affect the partitioning of the protein. AOT, which combines a sulfonate head and a large hydrophobic moiety built of two separate chains, was found to be the most effective achieving 7.5 times (0.05% relative to protein in aqueous phase) the solubility obtained complexing the protein with SDS (0.0007% relative to protein in aqueous phase). Overall, the solubility of lysozyme in organic solvents can be dramatically increased when it is complexed with a surfactant.[18]

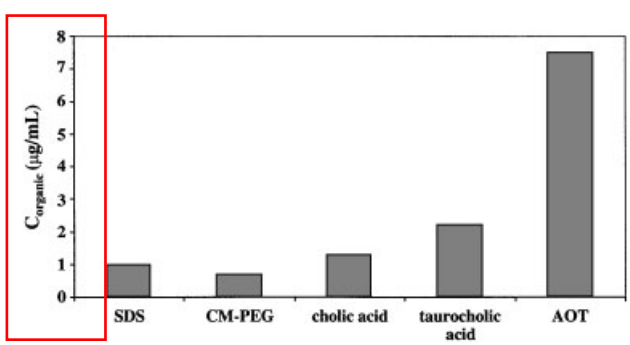

Figure 1. Effect of surfactant on the partitioning of the lysozyme:surfactant complex (1:10 mM) between the aqueous phase (10 mM citrate buffer, pH 4.5) and the organic methylene chloride phase. $C_{organic}$ represents the concentration of protein detected in the organic phase.

Attempts were made to solubilize r-met-HuGdNF (1 mM):AOT and r-met-HuGdNF (1 mM):SDS complexes in water-immiscible organic solvents. However, under the conditions tested, no significant improvement of the solubility was achieved. The solubility of r-met-HuGdNF in the single phase solvent system containing methylene chloride:TFE (1:4 v/v) was also assessed using the turbidimetric procedure. The lowest turbidity, i.e., highest solubility, is achieved at pH 2.5, in the presence of AOT (Figure 2).

Preparation of Microspheres by the SE Technique

The effects of solvent and cosolvent composition and volume, PVA concentration in the outer aqueous phase, and polymer concentration on particle size of SE microspheres were examined with the same solvents and cosolvents as listed above.

Previous studies have shown that increasing the PVA concentration of the outer aqueous phase causes an increase in nanoparticle size,[20] presumably because of a lowered rate of solvent diffusion due to a higher viscosity of the aqueous medium. We found herein that, with all other conditions being constant, the average particle size doubled with a 5-fold increase in PVA concentration (to 5%).

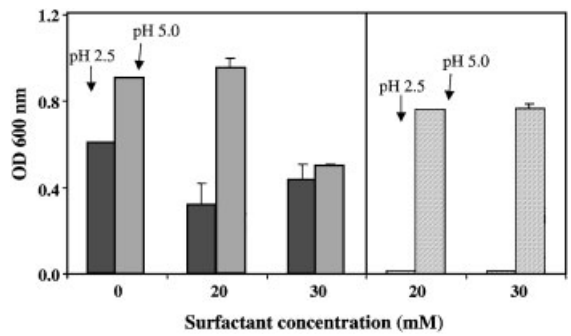

Figure 2. Effect of surfactant concentration and pH of the aqueous phase on the solubility of r-met-HuGdNF (1 mM):surfactant complex in the single phase solvent system (methylene chloride:TFE, 1:4 v/v). An increase in turbidity or optical density (OD) indicates a lack of solubility. The bars in the left panel represent r-met-HuGdNF (1 mM) complexed with SDS; the dark bars represent aqueous phase pH 2.5 and the light bars represent aqueous phase pH 5.0. The bars in the right panel represent AOT; the dark bars represent aqueous pH 2.5 and the light bars an aqueous pH of 5.0.

This observed effect for microspheres is thus similar to that seen for smaller particles.

Experiments were conducted to assess the effect of volumes and combinations of solvents and cosolvents on particle size, and the results are shown in Figure 3. With the system of chloroform and acetone, at a constant volume of the former, there is an optimal volume of acetone that yields particles of maximal size. For all other solvent and cosolvent systems, upon increasing cosolvent volume the trend is toward smaller particles.

The combined effects of the organic solvent, cosolvent, and polymer concentration on the size of particles obtained were examined by varying the organic solvents (methylene chloride, chloroform, and ethyl acetate) and the cosolvents (acetone and TFE). In each experiment 100 mg of polymer was used, and thus changing the volume of the cosolvent added altered the polymer concentration. These findings are consistent with those of previous studies.[16] Raising the cosolvent volume probably increases the driving force for its diffusion from the organic-rich phase to the aqueous phase.[13] However, while the choice of cosolvent has an effect on particle size, the main criterion for its selection was the solubility of the protein in the single-phase system.

For the various cosolvents tested, the volume of cosolvent required to achieve particles of a particular size range was different (Figure 3). Thus, while the rate of diffusion of cosolvent out of the organic phase is important in predicting the size of the particles, so are the characteristics of the organic solvent.

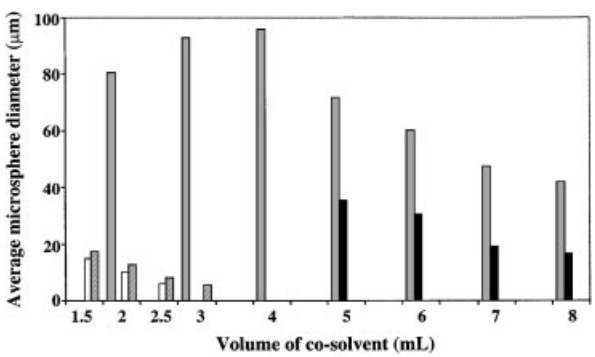

Figure 3. Effect of solvent:cosolvent system on the diameter of SE microspheres. Solid dark columns: methylene chloride:acetone; light gray columns: chloroform:acetone; open columns: ethyl acetate:acetone; gray columns: methylene chloride:TFE. The volume of cosolvent used is indicated on the x-axis. All other conditions were kept constant, including 0.5 mL of organic solvent, 100 mg PLGA, 100 μL lysozyme in aqueous solution, and 100 mL 5% PVA external aqueous phase.

r-met-HuGdNF Containing Microspheres

By combining the results from the protein solubility and microsphere size optimization studies, microspheres containing r-met-HuGdNF were made with the SE method. The protein was complexed with AOT and encapsulated within PLGA microspheres under various experimental conditions. From the results obtained, the optimal composition of components is 150 μL of aqueous solution containing r-met-HuGdNF (1 mM) and AOT surfactant (30 mM), 2.5 mL of the single-phase solvent system (methylene chloride:TFE, 1:4 v/v) in which 100 mg of PLGA is dissolved, and 100 mL of 5% aqueous PVA solution. These conditions produced microspheres with a mean diameter of 14.1 μm and an r-met-HuGdNF load of 4%.

To assess the effect of surfactant on the initial protein burst, SE microspheres were also prepared in the absence of AOT. They gave a 2-h burst value of 1.7% of total loaded protein, while SE microspheres containing r-met-HuGdNF complexed with AOT yielded a 2-h burst of less than one-fourth of that (data not shown).

Agglomeration and Morphology of, and Protein Distribution within, the SE Microspheres

Drug-loaded particles frequently tend to aggregate. However, this has not been a problem with the particles made by SE.[13] The microspheres made in this study by SE behaved similarly: when reconstituted to 200 mg/mL in 10% saline, they passed easily through a 21-gauge syringe needle.

SEM studies revealed that the particles are smooth and spherical (Figure 4b). The SEM studies also showed that the larger microspheres may be more porous than the smaller ones (Figure 4a). As discussed above, the porous internal architecture may be caused by phase separation between various components of the system.

The spatial distribution of the protein within the SE particles was examined using confocal microscopy, with FITC-BSA as the model protein and AOT as the surfactant. Representative results are depicted in Figure 4c. In the solid annulus of the particle (there is a pore in the center of the

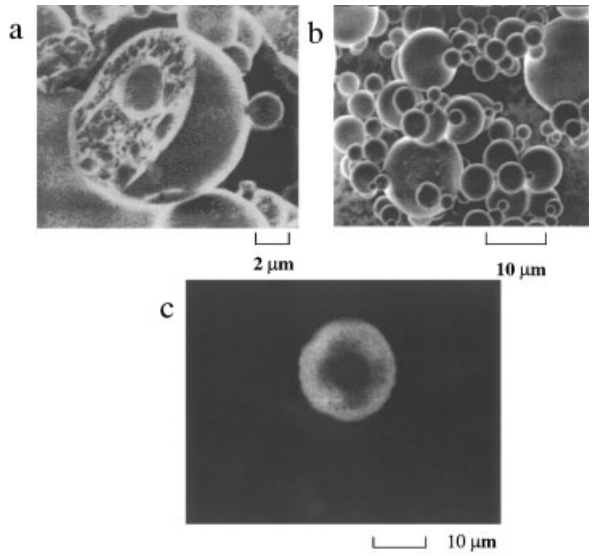

Figure 4. Images of PLGA microspheres made by SE. Scanning electron micrographs: (a) fractured microsphere revealing an internal porous structure—magnification 3,000×, (b) Intact microspheres—magnification 5,000×. Confocal microscopy image: (c) cross-sectional view of an SE microsphere containing FITC-BSA:AOT.

particle), the distribution of the fluorescent protein is homogeneous which may explain the undetectable burst of protein release exhibited by these particles.

Process Scale-Up

In view of the potential therapeutic application of r-met-HuGdNF-containing microspheres, we considered the feasibility of scale-up for the microsphere manufacturing process. The latter was repeated at scales of 1×, 10×, and 40×, and the microspheres obtained were analyzed for mean diameter, protein loading, and 2-h burst (Table 1). Regardless of the scale, the characteristics measured were essentially identical suggesting that the process is governed by thermodynamics. Thus, the scalability and ease of manufacturing of this process makes it a potentially attractive alternative to a DE-based process.

In Vitro Release of r-met-HuGdNF

The ability of r-met-HuGdNF-containing microspheres to release the protein over a sustained period of time is critical. Hence, a comparison was carried out between microspheres made by a standard DE method encapsulating the protein and the SE method encapsulating r-met-HuGdNF:AOT complex. The two types of microspheres had an equal protein loading of 4% and were of the average diameters of 17.7 ± 2.7 and 14.1 ± 2.6 μm, respectively.

The results obtained from the *in vitro* release experiments (Figure 5) indicate that although both types of particles released r-met-HuGdNF for over 40 days, there were significant differences. The SE microspheres showed little release of protein (less than 10% of the total amount of protein loaded) over the first 5 days, followed by sustained release. After 55 days, over 75% of the total protein loaded was released (data not shown). The DE microspheres exhibited similar behavior in protein release with the exception of a large initial burst of almost 20% of the total protein loaded within the first 2 h of incubation. After 55 days, approximately 90% of the total protein loaded was released (data not shown).

An initial burst of protein, which can range from 10–90% depending on the drug and carrier formulation, is commonly seen with particles made using various processing techniques including DE. Nanoparticles made by SE can also exhibit a large initial burst.[21] We hypothesize that the lack of initial burst observed with the SE microspheres prepared in this study is due to the careful mixing of polymer and protein within the single-phase solution. In contrast to the DE process, where polymer and protein are mixed together physically, the SE process allows coexistence of both species in one solution.

Table 1. Scale-up of SE Method of Encapsulating r-met-HuGdNF within PLGA Microspheres

Scale	Mass of Polymer (g)	Mean Microsphere Diameter (μm)	Protein Load (%, w/w)	2-Hour Burst Relative to Total Protein Loaded (%)
1	0.1	14.1	3.9	1.70
10	1	14.6	4.1	—
40	4	14.4	4.0	1.67

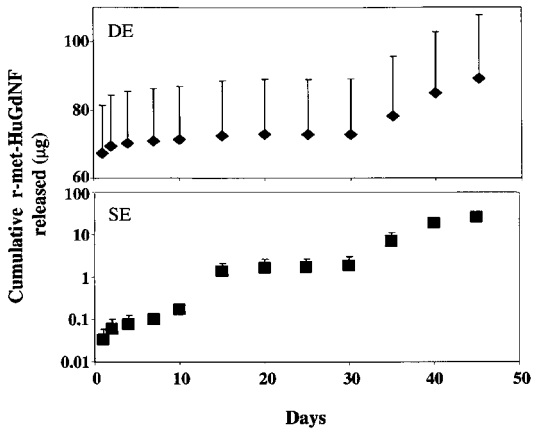

Figure 5. Cumulative *in vitro* release of r-met-HuGdNF from the DE (◆) and SE (■) microsphere formulations as determined by ELISA. Please note the cumulative release for the DE microspheres are plotted on a linear scale and a log scale for the SE microspheres.

In Vivo Serum Levels

Both DE and SE microspheres containing r-met-HuGdNF were tested in rats to assess whether the release profiles observed *in vitro* translated to *in vivo* conditions. Three groups of animals were injected with equal amounts of r-met-HuGdNF. The first group was given daily injections of the protein; this was performed to benchmark previous data and served as a positive control for the ELISA assay. (A therapeutic concentration of r-met-HuGdNF has yet to be determined.) The second group received one injection of r-met-HuGdNF encapsulated within PLGA microspheres made by DE, and the last group received a single injection of r-met-HuGdNF encapsulated within PLGA microspheres made by SE. The microspheres for the last two groups were fabricated under scaled-up conditions and were of equivalent size (average particle diameter) and protein loading. The animals were bled daily, and the blood samples were analyzed for r-met-HuGdNF.

Over 14 days, r-met-HuGdNF release was detected continuously from both types of microspheres (Figure 6). The DE microspheres exhibited a large initial burst followed by sustained release. In contrast, the SE microspheres showed no initial burst and exhibited a sustained release over the entire course of the experiment. In addition, both microsphere formulations appeared to be still releasing protein at the end of the 14-day experiment.

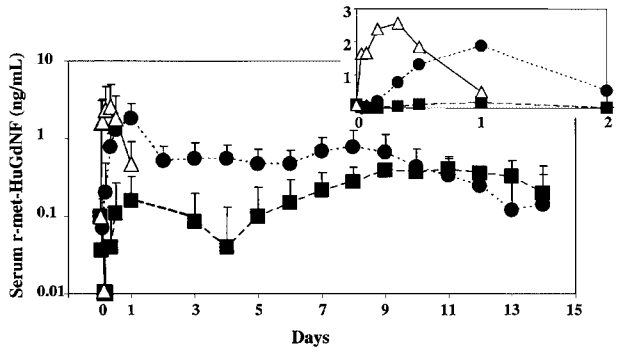

Figure 6. Serum r-met-HuGdNF present in rats dosed on a daily basis with r-met-HuGdNF in solution (△), dosed once with DE microspheres (●), or dosed once with SE microspheres (■).

The SE microspheres exhibited a significantly smaller initial burst than the DE ones. Over the initial 24-h postinjection period, the burst from the DE microspheres was 12-fold higher than from the SE ones. The latter displayed a lag period where little to no release of protein occurred, presumably due to an insufficient polymer degradation to give rise to protein release.

Although the microspheres were still releasing protein after 14 days, area under the curve (AUC) values were calculated to estimate the bioavailability. Because the microspheres were not depleted of protein at day 14, these calculations likely underestimated the relative bioavailability. Compared to daily r-met-HuGdNF injection, the AUC of the DE microspheres was 35% and that of the SE microspheres was 13%.

The results of this study are encouraging, and indicate the feasibility of the SE microspheres as a sustained release device.

CONCLUSIONS

The major objective of this study was to develop a microparticle-based therapeutic modality releasing r-met-HuGdNF in a sustained manner without an initial burst. To this end, r-met-HuGdNF-containing PLGA microspheres were prepared using an optimized SE method. This method relies on the solubility of the protein in an aqueous/organic single-phase solvent system. Protein solubility in the mixed solvent system considered was further improved by increasing its hydrophobicity upon complexation with ionic surfactants.

The SE method has several advantages: (1) the dissolution of both polymer and protein in the

same solvent system leads to a homogeneous distribution of protein within the polymer phase, (2) the lack of high energy mixing in the process of microsphere preparation, and (3) the ease of process scale-up. Critical factors affecting microsphere size were identified to include the choice of organic solvent and the choice and volume of cosolvent. Protein loading was greatly affected by the PVA concentration in the outer aqueous phase, the protein concentration in aqueous solution, and the protein solubility in the single-phase solvent. Enhanced drug solubility was achieved by increasing its hydrophobicity upon complexation with ionic surfactants.

By altering the fabrication method to obtain a more homogeneous protein distribution, the burst effect in r-met-HuGdNF-containing PLGA microspheres was eliminated as demonstrated by *in vitro* and *in vivo* experiments. It is hypothesized that release of protein from the SE microspheres is closely linked with polymer degradation. Thus, there is a time lag before onset of protein release. As this spontaneous emulsification method affects the matrix of the microsphere, the selection of polymer for particular release profiles or time periods will require reevaluation.

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