Encapsulation of Protein Nanoparticles Into Uniform-sized Microspheres Formed in a Spinning Oil Film

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ABSTRACT

A new spinning oil film (SOF) solid-in-oil-in-oil emulsion process was developed to produce uniform-sized protein-loaded biodegradable microspheres. A thin SOF on a cylindrical rotor was used to shear droplets from a nozzle tip to control droplet size. The resulting microspheres with low polydispersity (6%) produced a low burst (6%-11%) release even at high loadings (13%-18% encapsulated solids, 8%-12% protein). The SOF process had a high yield and did not require the presence of water, which can cause protein denaturation, or surfactants, which may be unwanted in the final product. Amorphous protein and crystalline excipient solids were encapsulated into 3 different polymers, giving a homogenous drug distribution throughout the microspheres, and an essentially complete protein encapsulation efficiency (average = 99%). In contrast, large burst release was observed for polydisperse microspheres produced by a conventional emulsification technique, particularly for microspheres smaller than 25 μm in diameter, which gave 93% burst at 15% loading. The uniform encapsulation of high loadings of proteins into microspheres with low polydispersity in an anhydrous process is of practical interest in the development of controlled-release protein therapeutics.

KEYWORDS: microsphere size control, monodisperse emulsions, spray freezing into liquid process, bovine serum albumin, solid-in-oil-in-oil processing, PLGA, initial burst.

INTRODUCTION

Biodegradable microspheres have shown significant potential for the delivery of peptide and protein drugs and vaccines.[1] Lupron Depot consists of a prostate cancer fighting polypeptide drug (molecular weight [MW] ~1.27 kDa) that is administered monthly or once every 3 to 4 months from poly-D,L-lactide-co-glycolide (PLGA) and polylactic acid (PLA) microspheres, respectively.[2] Recombinant human growth hormone (~22 kDa) in PLGA microspheres has been delivered in its active form over several weeks in animals and man (Nutropin Depot).[3,4] Immunization therapies, which often require the repeated or sustained presence of an antigen to trigger an immune reaction, have used PLGA microspheres to provoke stronger responses to diptheria and tetanus toxoids, among others.[1] To further advance protein and peptide therapeutics, key challenges in depot delivery are to achieve higher loadings and lower burst release in the first 24 hours, and to provide greater protein stability within the microsphere.

An ideal biodegradable microsphere formulation would consist of a free-flowing powder of uniform-sized microspheres less than 125 μm in diameter and with a high drug loading.[5,6] In addition, the drug must be released in its active form with an optimized release profile that could be pulsatile for some vaccines, or continuous for most other protein and peptide therapeutics.[5] The manufacturing method should produce such microspheres in a process that is reproducible, scalable, and benign to the often delicate protein molecule, with a high encapsulation efficiency.[5,6]

Biodegradable microspheres generally are made from a polymer-organic solvent solution in which the drug is dispersed either as a solid (solid-in-oil [s/o] suspension) or as droplets (water-in-oil [w/o] emulsion). Many fragile protein molecules unfold at the large w/o emulsion interface, thus s/o suspensions are generally considered more benign.[5,7] Once a drug is dispersed in the dissolved polymer, droplets are formed and the solvent is extracted, leaving hardened, drug-loaded microspheres. When these droplets are created by emulsifying in water (eg, the solid-in-oil-in-water [s/o/w] technique), the protein product can leak out into the bulk water phase resulting in low encapsulation efficiencies.[6,8,9] In addition, the drug can become partially wetted and rendered unstable in the organic solvent.[9] These pitfalls are overcome by using nonaqueous techniques such as the Prolease spray-freeze process or solid-in-oil-in-oil (s/o/o) emulsions.[5]

Recent work has shown that solid protein nanoparticles produced by the spray freezing into liquid (SFL) process

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could be encapsulated uniformly into microspheres by an s/o/o process.[10] As expected, this nonaqueous process was shown to be benign, with monomer losses of 1.3% in the SFL process and 2.6% in the remaining polymeric encapsulation process. The SFL protein powders were dispersed into the polymer microspheres to give homogeneous dispersions and uniform microsphere interiors. This approach enabled the production of microspheres with high encapsulation efficiencies and very low burst.[10]

Now that protein has been dispersed uniformly in a benign nonaqueous process, the next step is to control microsphere diameter and polydispersity, as these factors affect several aspects of product effectiveness. For example, to inject microspheres through a syringe, the upper size limit is approximately 125 μm.[6] Macrophages can engulf particles <10 μm in diameter, and flocculation as well as the burst effect is worse for smaller microspheres.[1,5] Water and buffer exchange to the interior of the microspheres and protein diffusion also depend upon the microsphere size.[11] For polydisperse microspheres, differences in diffusion rates may produce undesirable variation in microsphere degradation rates, drug stability, and drug release profiles.[11] Therefore an ideal depot formulation would target an average microsphere size within the 10 to 125 μm range and would minimize polydispersity.

Although microsphere size plays a significant role in drug delivery, few processes have shown adequate control over this parameter. The emulsion-based processes discussed above use shear from an impeller or stir-bar to form droplets, and these techniques invariably lead to microspheres with a broad size distribution.[12] Berkland et al[11,13,14] used sonication and 2 fluid nozzles to produce highly monodisperse o/w PLGA microspheres of approximately 5- to 500-μm size and loaded these microspheres with dichloromethane-soluble drugs to study release properties. A nozzle with sonication was also used in the development stages of the ProLease process, but air atomization, which usually leads to polydispersity, was used at the manufacturing scale.[5] Umbanhowar et al[15] developed a technique in which a nozzle was submerged in a surfactant-containing fluid stream to produce monodisperse o/w and w/o emulsions from water, hexadecane, silicone oil, and liquid crystal. These monodisperse liquid droplets were made to target sizes as small as 2μm and as large as 200 μm. However, this technique did not consider encapsulation of solids and was not applied toward drug delivery systems. New processes would be desirable to achieve better control over size distribution in protein-loaded polymeric microspheres prepared by nonaqueous techniques.

The objective of this study was to develop a novel spinning oil film (SOF) s/o/o process for the encapsulation of SFL protein nanoparticles into uniform-sized PLGA microspheres. To place the SOF results in perspective, results are presented first for polydisperse microspheres produced from s/o/o emulsions formed with an impeller. These microspheres were size selected with sieves, and the burst effect was shown to range from 2% to 100%, depending on the microsphere size and loading. In this new SOF process, uniform microspheres were made by shearing droplets from a nozzle tip with a SOF. Microspheres with high drug and excipient loadings (13%-18% encapsulated solids by weight) were produced with a low burst of 6% to 11%. The new SOF method provided high encapsulation efficiency and allowed flexibility in polymer and excipient choice and the amount of solid protein loading. Confocal fluorescence microscopy is used to show that the protein may be loaded uniformly throughout the microspheres. The ability to achieve high loading and low burst in uniform microspheres is of great interest in advancing controlled-release protein therapeutics.

Materials and Methods

Materials

PLGA 50:50 (Resomer RG502H, inherent viscosity of 0.2 dL/g, Boehringer Ingelheim, Ingelheim am Rhein, Germany), fraction V bovine serum albumin (BSA, MW ~66 kDa), trehalose (Tre) and magnesium hydroxide (all from Spectrum Chemical Manufacturing, Gardena, CA) were used in the preparation of protein-loaded microspheres. Paraffin oil (saybolt viscosity of 345-355 at 100°F, Mallinkrodt Baker, Phillipsburg, NJ), cottonseed oil and sorbitan trioleate (both from Spectrum Chemical), acetonitrile and hexanes (both high-performance liquid chromatography [HPLC] grade, EMD Chemicals, Darmstadt, Germany) were used as received. Sodium chloride and mono- and di-basic potassium phosphate salts (all from EMD Chemicals), polyvinyl alcohol (*United States Pharmacopeia* [*USP*], 85%-89% hydrolyzed), and methylparaben (from Spectrum Chemical) were used to prepare buffers. Dimethyl sulfoxide (DMSO) and trifluoroacetic acid (TFA) (both from Spectrum Chemical) were used in loading assays.

Block copolymers have been synthesized with polyethylene oxide (PEO) and poly(lactic acid).[16] The copolymers, PLGA-F127-PLGA and PLGA-PEO-PLGA were prepared by melt polymerization, where F127 (Pluronic block copolymer surfactant, BASF, Mount Olive, NJ) and PEO (Sigma-Aldrich, St Louis, MO) were used as initiators for polymerizations of d,l-lactide/glycolide, catalyzed by stannous octoate. The MW contributions of the constituent parts are (LA/GA-F127-GA/LA = 31.9K/28.5K-12.7K-28.5K/31.9K) and (LA/GA-PEO-GA/LA = 7.6K/1.9K-10K-1.9K/7.6K) for these 2 block copolymers. Glycolide and d,l-lactide (both from Polysciences, Warrington, PA)

were used to synthesize the PLGA component of the block copolymers.

Protein Preparation

Details of the SFL process and its effect on protein stability have been previously documented.[10,17,18] Briefly, BSA crystals were dissolved into deionized water to give a concentration of 10 mg/mL. The protein solution was delivered at 5000 psi through a 4-inch, 65-μm inner diameter (ID) nozzle, which was submerged below the surface of liquid N_2. Nozzle-induced shear appeared to have minimal effect on stability in previous studies.[10,17,18] The frozen slurries were spread onto glass or stainless steel trays in thin layers (<0.75 inch) and stored at −70°C. Lyophilization was conducted in a Virtis Advantage Lyophilizer (The Virtis Company, Gardiner, NY) with a condenser temperature of −67°C. The trays were transferred from the −70°C freezer to precooled −40°C lyophilizer shelves, then dried at −25°C and 100 mTorr for 24 hours, ramped over 12 hours to 25°C, and dried at 25°C and 100 mTorr for 24 hours. Two formulations of SFL-BSA were used for SFL in this study. The first formulation contained only protein at 10 mg/mL and water, and these powders had specific surface areas of 100 ± 10 m^2/g and D_{v50} of 0.35 ± 0.05 μm. The second formulation contained BSA at 8 mg/mL and trehalose as a lyoprotectant at 2 mg/mL. This batch had a specific surface area of 50 m^2/g, and a D_{v50} of 3.6 μm. These 2 formulations are referred to as SFL-BSA and SFL-BSA/Tre in this article.

Encapsulation

Two s/o/o encapsulation processes were used in this study that were adapted from previous reports.[10,19-22] These 2 processes, depicted in Figure 1, differ in the mechanism of droplet formation. The first used an impeller, which gave rise to a broad size distribution, and the second employed an oil-coated rotor to shear droplets from a nozzle tip, resulting in a narrower, more reproducible size distribution.

In each process, protein powders and basic salts were suspended into acetonitrile at a solid:solvent ratio of approximately 1:75 (wt/vol). This dilute suspension was then sonicated in an ice bath using a Branson Sonifer 450 sonicating probe (Branson Ultrasonics, Danbury, CT) with a 102 converter and tip operated in pulse mode (cycled at 0.5 seconds on, 0.5 seconds off) at 35 W. Approximately 1 to 2 mL of a PLGA solution (50% [wt/vol] in acetonitrile) was added by pipetting into the sonicated protein suspensions. The suspensions were shaken and sonicated for 20 seconds. They were thickened by evaporating excess solvent under a N_2 purge stream, and solvent loss was monitored by weighing the vials periodically. The target mass of solvent in the polymer-protein mixtures was calculated as 2 times the SFL-protein mass plus 1.15 times the PLGA mass plus the mass of basic salt; this ratio allowed suspensions of similar and acceptable viscosities to be formed at various protein and excipient loadings. An attempt was not made to scale up this process; other shear processes could be considered, such as homogenizers.

In the first process, microspheres were produced from emulsion droplets formed with an impeller. Protein-PLGA suspensions (1-2 mL) were dispersed into approximately 15 mL of paraffin oil containing 0.5% Span 85 and mixed to form droplets with an impeller (1 cm corrugated cylindrical impeller, ~600 rpm). This emulsion was then decanted into a glass jar containing approximately 200 mL of chilled cottonseed oil (~5°C-10°C) containing 0.5% wt/vol Span 85, and the container was capped and inverted approximately 10 times to mix the microspheres into the cottonseed oil. Then the mixture was frozen quickly by placing it into a freezer at −70°C.

The s/o/o droplets were next hardened using a "suspended droplet" hardening procedure. In the freezer, the cottonseed oil converted to a waxy state, and the protein-PLGA droplets remained suspended throughout this wax. Once the oil was completely frozen (after ~15 minutes), the container was moved to a refrigerator at 4°C to allow the oil

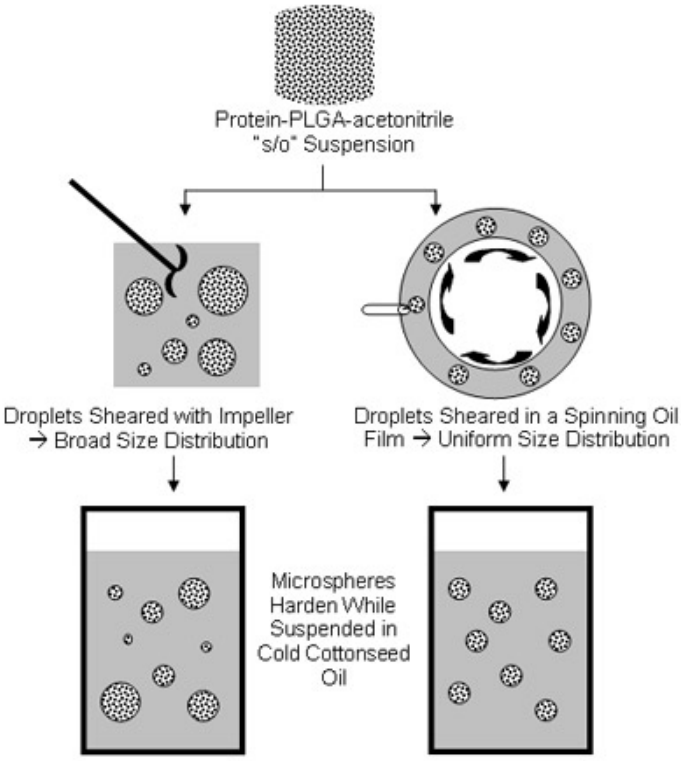

Figure 1. Flowchart of a solid-in-oil-in-oil microsphere production method with suspended droplet hardening using impeller atomization or droplet shearing in a spinning oil film.

to slowly melt during which time the acetonitrile was extracted into the oil. On the next day, the oil/microsphere mixture was placed at room temperature, and the microspheres were allowed to settle. The oil phase was then decanted and replaced with fresh cottonseed oil for a second extraction, following the same freeze and thaw protocol.

The impeller-dispersed microspheres were then separated from the oil according to size using a series of sieves: 100, 150, 300, and 500 mesh, or approximately 150-, 100-, 50-, and 25-μm cutoffs, respectively (Cellector Tissue Sieve Kits and Screens, Thermo EC, Waltham, MA). Particles of less than 25 μm were collected on a 0.22-μm cellulose acetate membrane (Whatman International, Ltd, Middlesex, UK). Microspheres retained on the sieves or membranes were washed with hexanes, lyophilized in a Labconco Freeze Dryer (Labconco Corp, Kansas City, MO) 5 for 24 hours, and weighed to assess yields in the various size classes.

An SOF technique was developed, as depicted in Figure 2, to produce microspheres with a more uniform size. This technique employed an overhead mounted electric motor to turn an upside-down glass Buchner funnel (Sigma-Aldrich, St Louis, MO) of 40 mm outer diameter (OD) at approximately 150 rpm. The shape of the glass Buchner funnel was useful. The thin stem was fixed into the electric motor chuck, and the large cylindrical bowl on the other end provided a smooth surface to shear polymer droplets. Room temperature cottonseed oil was fed by a peristaltic pump at approximately 30 mL/min and allowed to drip onto the thin upper portion of the spinning Buchner funnel. The oil flowed down the outer wall of the spinning funnel in a smooth sheet of approximately 2 mm thickness; upon flowing off the open end of the funnel, the oil was collected in a chilled 1-L beaker, the bottom of which was coated with a frozen cottonseed oil layer of approximately 1-inch thickness. Protein-PLGA suspensions in acetonitrile were fed at 300 to 500 psi (~0.25 mL/min) through a 120-μm ID by 6-inch long polyetheretherketone (PEEK) nozzle, the tip of which was submerged in the oil film in the radial direction normal to the smooth surface of the spinning glass funnel. The depth of the PEEK nozzle in the oil film was controlled by fixing the nozzle in a spring-loaded arm and using an adjustment screw that rested against the spinning glass cylinder. Thus the PEEK nozzle moved in and out to match any imperfections in the glass cylinder shape and kept a constant depth of submersion of approximately 1 mm. The droplets formed on the tip of the nozzle and were swept away by the shear forces created by the moving oil film. The droplets ran down the funnel along with the cottonseed oil, making approximately 5 revolutions before dropping off, and were collected in the beaker. Droplet coalescence in the beaker was minimal as the beaker contents were not stirred. Once all of the PLGA droplets were collected, the beaker was placed in the −70°C freezer, and the acetonitrile was extracted by the suspended droplet-hardening procedure outlined above. Upon hardening, the microspheres were collected on a 25-μm steel mesh, washed with hexanes, and lyophilized for 24 hours.

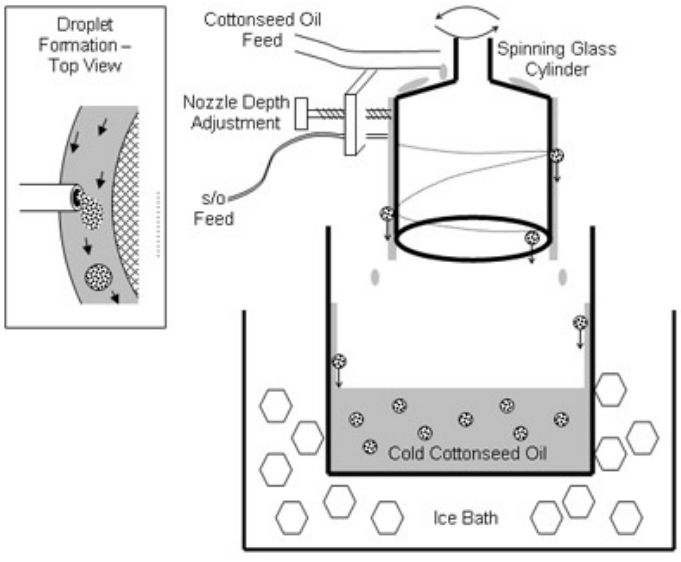

Figure 2. Schematic of an apparatus for the production of microspheres in a spinning oil film with a narrow size distribution.

The extraction capacity limit of cottonseed oil was tested to economize its use. Though pure acetonitrile is soluble up to approximately 10% in cottonseed oil, much leaner mixtures were required for microsphere hardening. When PLGA-acetonitrile mixtures were added at volumetric ratios exceeding 1.7:100, microsphere fusion and agglomeration resulted. Therefore the volumetric ratios of s/o suspension to cottonseed oil were kept below 1:100 in the impeller method, and the s/o to cottonseed oil feed-rate ratios were kept below 1:100 in the SOF method.

Protein Release

Approximately 10 to 15 mg of microspheres were weighed and added to 1.6 mL Biostor conical vials (National Scientific Supply, Claremont, CA). An amount of 1 mL of pH 7.4 release buffer (50 mM phosphate salts, 0.1% wt/vol methylparaben and 0.1% wt/vol PVA) was added to each tube. Samples were placed on a Lab-line Environ shaker (LR Environmental Equipment, Los Angeles, CA) at 37°C and 140 rpm during release. At various times, 0.8 mL of release buffer was removed from the conical tubes for protein concentration measurement and replaced with 0.8 mL of fresh buffer. Protein concentration measurements were performed using a bicinchoninic acid (BCA) micro-assay kit (BCA-1, Sigma, St Louis, MO), using 6 replicate measurements of each tube. Release studies were run on 2 samples from each microsphere batch.

Protein Loading and Encapsulation Efficiency Measurement

Approximately 10 to 100 mg of microspheres, depending on loading, were weighed and added to 5-mL glass test tubes. A volume of 1.8 mL of DMSO containing 10% TFA was added to each vial to dissolve both the polymer and protein. After approximately 1 hour at room temperature, the liquids were mixed by imbibing and expelling the solution through a glass Pasteur pipette several times. Care was taken to avoid contacting the liquid with plastic parts or parafilm, thus a vortex was not used. The high boiling point of DMSO (189°C) prevented significant solvent evaporation from altering the results. The dissolved protein and polymer were then passed through a 0.1-μm polyvinylidene fluoride (PVDF) syringe filter (Whatman International, Middlesex, UK, 6784-2501) to remove basic salts or other solids. Each solution (0.3 mL, n = 6) was added to a UV-transparent 96-well plate (Falcon-353261, BD, Franklin Lakes, NJ,) along with standards, and the absorbance was measured at 280 nm in a μQuant plate reader (Bio-tek Instruments, Winooski, VT). Absorbance at 320 nm was also measured to indicate possible light scattering due to particulates, if present.

Scanning Electron Microscopy

Microsphere samples were analyzed using a LEO 1530 scanning electron microscope (SEM) (Leo Electron Microscopy, Thornwood, NY). Some samples were analyzed as manufactured; others were submerged in release buffer and removed at various time points, and lyophilized for more than 1 week. Cross-sectioned microspheres were prepared by dispersing them on a glass microscope slide and chopping more than 50 times with a razor prior to mounting for analysis. Metal stubs were coated with double-sided adhesive tape, and microsphere powders were dispersed on the sticky surface. All samples were coated with 30 nm Cr to avoid sample charging during analysis.

Light Microscopy

Microspheres were dispersed into a drop of paraffin oil on a glass slide, allowed to settle, and imaged at original magnification ×10 with an Axioskop 2 (Carl Zeiss International, Oberkochen, Germany).

Confocal Microscopy

Confocal microscopy was performed with a Leica TCS 4D microscope (Leica Microsystems, Bannockburn, IL) at original magnification ×40 using immersion oil both on the objective and to wet the dry microspheres. A krypton-argon laser provided excitation light at 488 nm, and a long-pass 515 nm filter was used for the emitted light. Confocal imaging used the natural fluorescence of BSA; no dyes were used.

Particle Size Analysis

Protein samples were sonicated in approximately 20 mL of acetonitrile, and then suspended in 450 mL of acetonitrile for measurement using a Malvern Mastersizer-S (Malvern Instruments, Worcestershire, UK). Sonication of the Mastersizer solution cell was used to break up aggregated protein particles. The measured sizes were compared with SEM photomicrographs to verify accuracy. Microsphere batch sizes were too small to use the sonicated Malvern Mastersizer cell for analyses. The nonsonicated mini-cell, which uses a small stir bar to disperse small samples, was unable to give sufficient microsphere dispersion for accurate analysis. The use of probe sonication or addition of surfactants or salts to the analysis buffer might alleviate these issues in future studies. In this study, however, microsphere size statistics were calculated from analyses of photomicrograph images. SEM and light microscope images were enlarged and printed at 8.5 × 11 inches, then 50 to 200 microspheres were measured and the sizes were entered into a spreadsheet. Volume-based size statistics were obtained by calculating the volumes ($4/3\pi r^3$) of each measured microsphere and the total sample volume (sum of all microsphere volumes), and by finding the radii below which 10% (D_{v10}), 50% (D_{v50}), and 90% (D_{v90}) of the total sample volume resided. Polydispersity was calculated as the standard deviation of all measured microsphere radii divided by the average radius.[15]

Surface Area Measurement

A Quantachrome Nova 2000 (Quantachrome, Boynton Beach, FL) was used to measure surface areas of the lyophilized samples at 77 K with N_2 as the adsorbate. All samples were degassed for at least 12 hours under vacuum at room temperature prior to measurement. The Brunauer, Emmett, and Teller (BET) equation was used to fit adsorption data over the relative pressure range of 0.05 to 0.30.

RESULTS AND DISCUSSION

Determination of Protein Loading in the Microspheres

To verify the efficiency of protein encapsulation, the actual loading was measured and compared with the theoretical loading. To measure the actual loading, protein is usually extracted from microspheres by dissolving the polymer in an organic solvent such as dichloromethane or acetonitrile, after which the protein solid is collected by centrifugation or extracted into an aqueous phase and quantified.[10,20,21]

Protein recovery is usually incomplete in these assays, and the actual loading is underestimated.[10] Another popular approach is to hydrolyze the microspheres in a strong base until all of the polymer and protein fragments dissolve. The solution is then analyzed for protein content. Such procedures prescribe hydrolysis times of approximately 24 hours at sodium hydroxide concentrations of 0.1 to 1 N. Some of the microsphere samples still contained solids after 2 days when treated with sodium hydroxide. Therefore, a method was developed to employ a strong solvent to dissolve both the polymer and protein within an hour, allowing for rapid measurement of protein loadings at 280 nm with a 96-well plate reader.

Mixtures of DMSO and TFA were effective at dissolving both PLGA and BSA. The solubility of BSA without PLGA at various solvent ratios (Figure 3A) shows a maximum of over 15 mg/mL in both 10% and 15% TFA/DMSO, thus protein concentrations needed for accurate analysis were easily obtained. Figure 3B shows the spectrophotometric characteristics of PLGA and BSA dissolved in 10% TFA/DMSO. PLGA had no significant absorbance up to 100 mg/mL, while BSA showed a linear increase in absorbance with a broad working range of approximately 0.5 to 3 mg/mL.

This technique was used to measure the protein loadings of the impeller dispersed and sieved microspheres (Figure 3C) and the SOF microspheres (Figure 3D). Encapsulation efficiencies (percentage measured/theoretical loadings) of the sieved microspheres were 82% to 109%, average = 92%, while those for the SOF microspheres were 81% to 111%, average = 99%. When insoluble particles such as $Mg(OH)_2$ were present, filtration was necessary, but the protein did not appear to be lost to the membrane filters. Absorbance at 320 nm showed that light scattering due to particles was negligible.

Variances in the measured encapsulation efficiencies were attributed mostly to the spectrophotometric assay, while protein loss to the oil phase during emulsification was not indicated. Instead of quartz cuvettes, 96-well plates were used for these analyses to improve throughput, but with a modest increase in uncertainty. Assay uncertainty was thought to arise from 2 sources: imperfections of the 96-well plates, and imperfections of sample preparation (weighing and pipetting errors). Imperfections arising in the 96-well plates caused a typical variance of 6% to 7%. This was routinely seen when sampling polymer-protein suspensions from a single well-mixed test tube and measuring the aliquots in a single row (6 wells) of a single plate. The net assay uncertainty (combination of sample preparation and 96-well plate imperfections) was slightly higher. For example, a 10% to 15% variance was seen when a single batch of microspheres was separated into 5 different test tubes, each dissolved in TFA/DMSO, and measured using 5 rows (6 wells per row) on a single 96-well plate. These net uncertainties of 10% to 15% were considered assay variance.

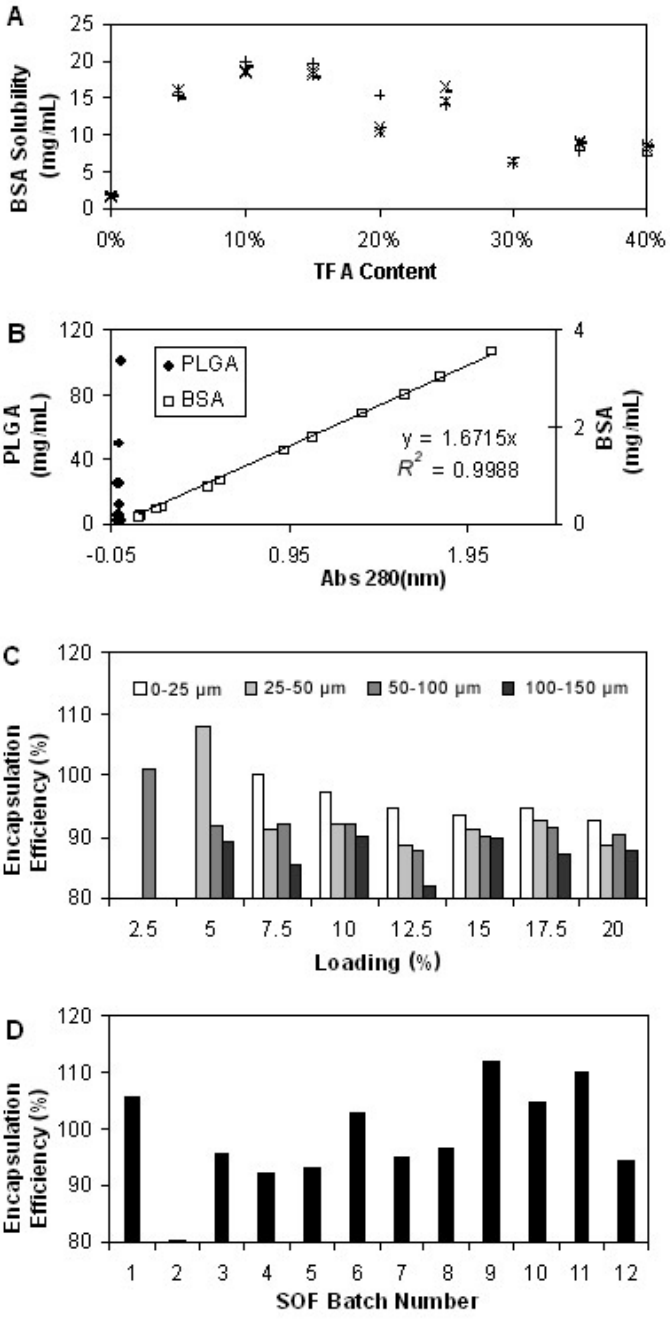

Figure 3. Loading assay based on mixture of 10% TFA in DMSO to dissolve both polymer and protein. (A) protein solubility in TFA/DMSO (the various symbols indicate n = 4 samples measured); (B) molar absorptivities at 280 nm for BSA and PLG; (C) impeller-dispersed and sieved microsphere samples; and (D) SOF-microsphere samples. The 12 batches of SOF microspheres were made from PLGA (Batches 1-4), PLGA-PEO-PLGA (Batches 5-8), and PLGA-F127-PLGA block-copolymers (Batches 9-12).

Next, the method of preparation of the feedstock was considered as a source of variance. Imperfections in the production of the feedstock resulting from small batch sizes could affect the measurement of encapsulation efficiency. For example, if the target loading was 8% (eg, 80 mg protein + 920 mg polymer) but actual delivered masses were different (due to difficulties accurately dispensing the viscous polymer for instance), these errors could alter the measured encapsulation efficiency. Therefore, the loadings of the protein suspension feedstocks were measured and compared with the theoretical loadings. A portion of each suspension feedstock was spread as a thin film on a glass slide, the solvent was evaporated in a 60°C incubator, and the loading of the dried film was measured. The recoveries of protein from the dried films averaged 101% ± 9% when compared with the theoretical loadings. Thus the feedstock compositions met the target loadings, and complete protein recovery was verified using the DMSO/TFA assay.

Impeller Dispersed and Sieved Microsphere Characteristics

Among the continuous phase oils used in s/o/o processes, paraffin and cottonseed oils are widely used. To test its solubility, acetonitrile was added in 50-μL aliquots to 50 mL of both of these oils and the mixtures were shaken and observed for phase separation or droplet formation. Acetonitrile was soluble in paraffin oil up to approximately 1%, and soluble in cottonseed oil up to approximately 10%. Thus cottonseed oil is better able to extract acetonitrile than paraffin oil.

This solubility difference was exploited in the impeller atomization process. It was found that when the s/o suspension was added to a small volume of paraffin oil and emulsified, no agglomeration occurred, while if the same was done in cottonseed oil, agglomeration problems were severe. On the other hand, paraffin oil, being a poor solvent extractor, made hardening of the microspheres slow and difficult. Thus by first forming the droplets in paraffin oil and next adding the emulsion to cottonseed oil for solvent extraction, an easy and effective s/o/o process was obtained. This complexity was completely alleviated in the SOF method because no mixing was required.

Figure 4 shows SEM photomicrographs of the sieved microspheres produced by the impeller atomization technique and hardened using the suspended droplet method. The sieves provided effective cutoff of large microspheres (ie, microspheres larger than 100 μm are not present in the 50-100 μm size class), but some smaller microspheres are seen to persist in the large size class fractions as satellites around the larger microspheres. The microspheres in these images appear to be mostly discrete spheres, and few examples exist of double-lobed microspheres that are the product of fusion.

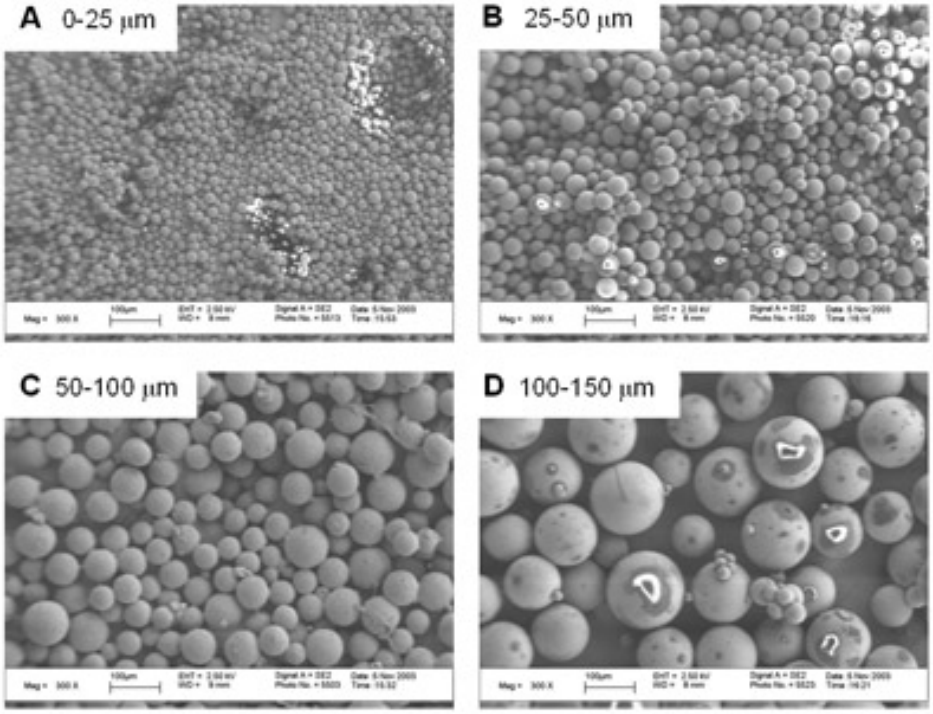

Figure 4. BSA-loaded PLGA microspheres produced by the suspended-droplet s/o/o process using an impeller to generate a broad size distribution. Microspheres from a single polydisperse emulsion were separated with sieves into (A) 0 to 25 μm, (B) 25 to 50 μm, (C) 50 to 100 μm, and (D) 100 to 150 μm size classes. Scale bars indicate 100 μm.

Figure 5 indicates the mass recovery of these polydisperse microsphere batches in the various size classes. The dominant size class is from 50 to 100 μm in all cases, and the frequency in each of the size classes is similar for all 8 batches as well. Adding up the recovered microsphere masses from all size classes, the emulsion yields were between 75% and 95% in these 8 batches. To put these findings in perspective, suppose specifications were set for microsphere size to be between 50 and 100 μm in a drug product. Due to the inability to produce uniform sizes, this impeller atomization scheme would only be able to provide approximately 45% yield at best.

The impeller-made PLGA microspheres exhibited a characteristic triphasic release pattern (Figure 6A), that is, an initial burst phase, a lag phase, and final release phase.[3,23,24] The lag and secondary release phases can be useful for pulsatile immunization applications. The burst phase can be used to rapidly increase systemic levels during early treatment in some cases, but burst has been difficult to control in most reported studies and could cause toxicity during the delivery of more potent molecules.[10,24-26]

The burst release is most rapid in the first few (<5) hours and is practically complete after 24 hours (Figure 6B, 5% loading). The burst in these samples is more severe for microspheres of smaller size. Figure 7 shows the effect of microsphere size on the degree of burst over a wide range of loadings. The burst effect can be affected by various factors, such as internal microsphere morphology, loading, and microsphere skin porosity, so efforts were made to rule out effects other than microsphere size. Cross-sectional SEM and confocal microscopy showed similar dense and homogeneous interior morphologies for all size classes and loadings. Furthermore, the various size fractions shown at each of the 8 loadings were separated via sieves from a single batch for direct comparison. The burst is at its lowest

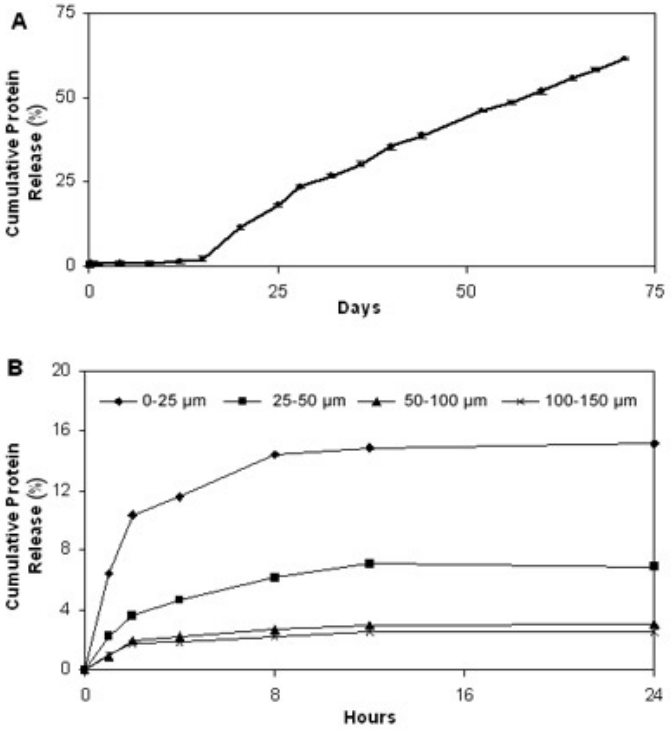

Figure 6. Release behavior of 5% loaded microspheres is shown for (A) nonsieved impeller-made microspheres over 75 days, and (B) sieved, impeller-made microspheres over the first 24 hours.

(~2%) at the minimum loading and maximum microsphere size, and at its highest (~100%) when the loading is high and the microspheres are small. It is clear that larger microspheres are desirable to prevent burst at high loadings, and small "satellite" microspheres in a polydisperse population could contribute to a high and unwanted burst. Microsphere size was previously shown to impact longer-term release through competition between drug diffusion and acid catalyzed polymer hydrolysis.[11] This study focused on shorter-term release only.

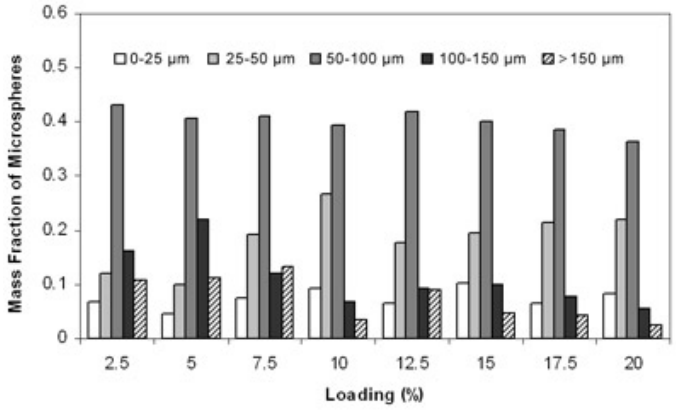

Figure 5. The mass fraction in each size class is shown for 8 impeller-made microsphere batches of varying loadings after sieving and drying.

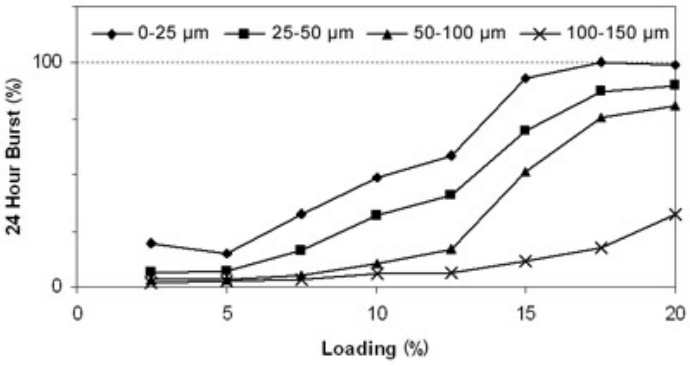

Figure 7. The amount of protein released in 24 hours is shown as a function of loading and size class.

The interiors of microspheres were studied via SEM. Figure 8 shows a progressive change in the interiors of microspheres that were loaded with 5% and 10% SFL-BSA. At first, the interiors appeared dense and pore free (Figure 8A). Over the first few hours of hydration, pores near the microsphere surface started to form and encroached on the more interior regions as time progressed (Figure 8B-8F). Comparing microspheres of similar size, the specimens with 10% loading had more pores at greater depths than those with 5% loading after 9 hours (Figures 8D and 8B, respectively) and 24 hours (Figures 8E and 8C, respectively). Greater water penetration is consistent with the greater fraction of protein, the hydrophilic component. Comparing microspheres with equal loadings and submersion times, the microsphere having a diameter of approximately 100 μm had a nonporous core (Figure 8F), while one with a approximately 50 μm diameter was porous throughout (Figure 8E). A small subset of cross-sectioned microspheres is shown here for brevity, but every size class was similarly analyzed at 5%, 10%, 15%, and 20% loadings to verify these trends. Interior pore formation was enhanced at higher loadings, and the deeply buried cores of large microspheres were generally protected from the initial influx of water, especially at lower loadings, whereas the cores of smaller microspheres were more accessible to water.

Microspheres Formed in the Spinning Oil Film

Microspheres were made by the SOF technique and hardened using the suspended droplet method. This new method was characterized according to its ability to control microsphere size and burst and to prevent microsphere aggregation and coalescence, and its suitability for general use in s/o/o processing was evaluated. The protein formulation

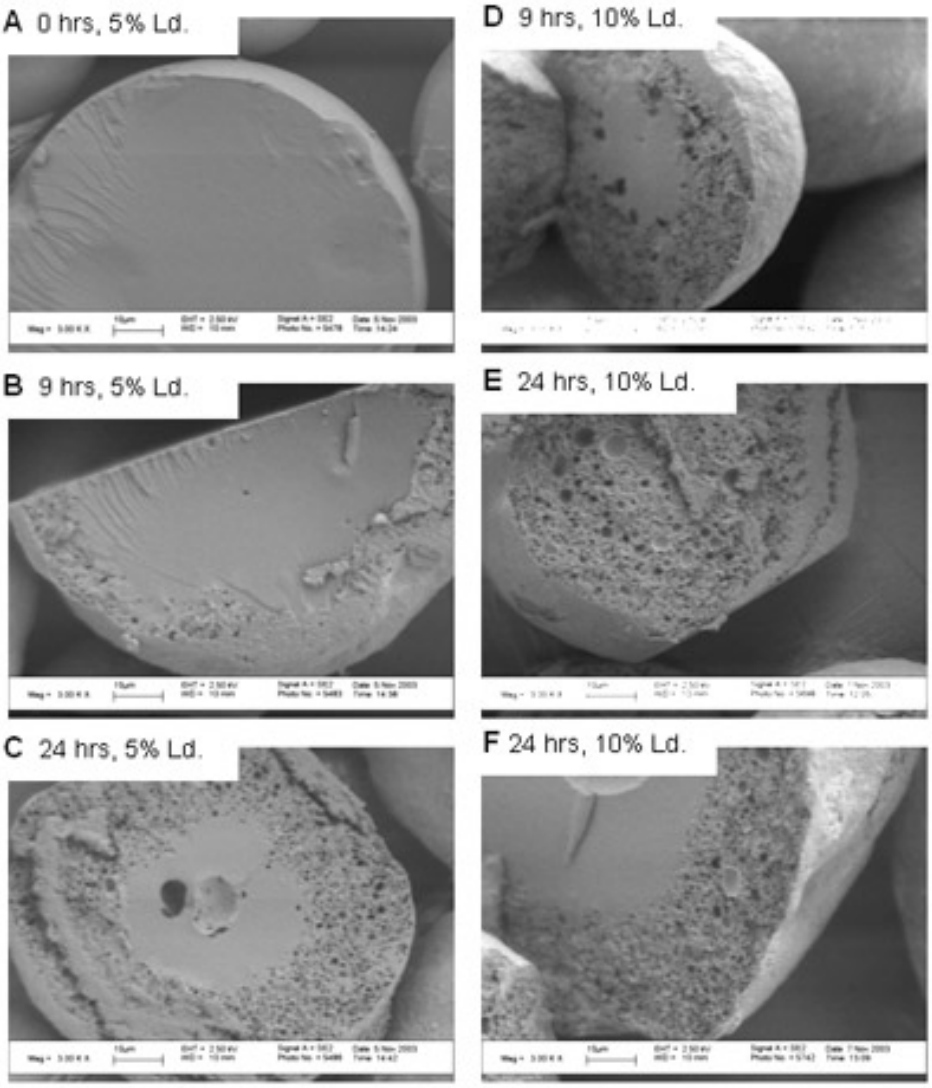

Figure 8. SEM images of cross-sectioned PLGA microspheres formed by emulsification with an impeller for various submersion times in water and loadings as indicated. Scale bars indicate 10 μm.

used in SOF experiments, SFL-BSA/Tre, was encapsulated at 10% and 15% net loadings (8:2 and 12:3 protein/trehalose, respectively).

Figures 9A and 9B show SEM photomicrographs of protein-loaded PLGA microspheres produced by the SOF technique and the impeller atomization technique without sieving, respectively. The SOF technique clearly produced microspheres with a much tighter size distribution. The size distribution parameters (D_{v10} = 113, D_{v50} = 123, D_{v90} = 133, polydispersity = 6% for the SOF microspheres, D_{v10} = 24, D_{v50} = 55, D_{v90} = 106, polydispersity = 74% for the impeller-dispersed microspheres) convey the striking difference in polydispersities. This dramatic improvement in size uniformity is expected to carry over into other product uniformity measures such as burst, extended release, and protein stability. Furthermore, the yield is nearly 100% for the SOF microspheres, whereas about half the size distribution is unusable for the impeller method.

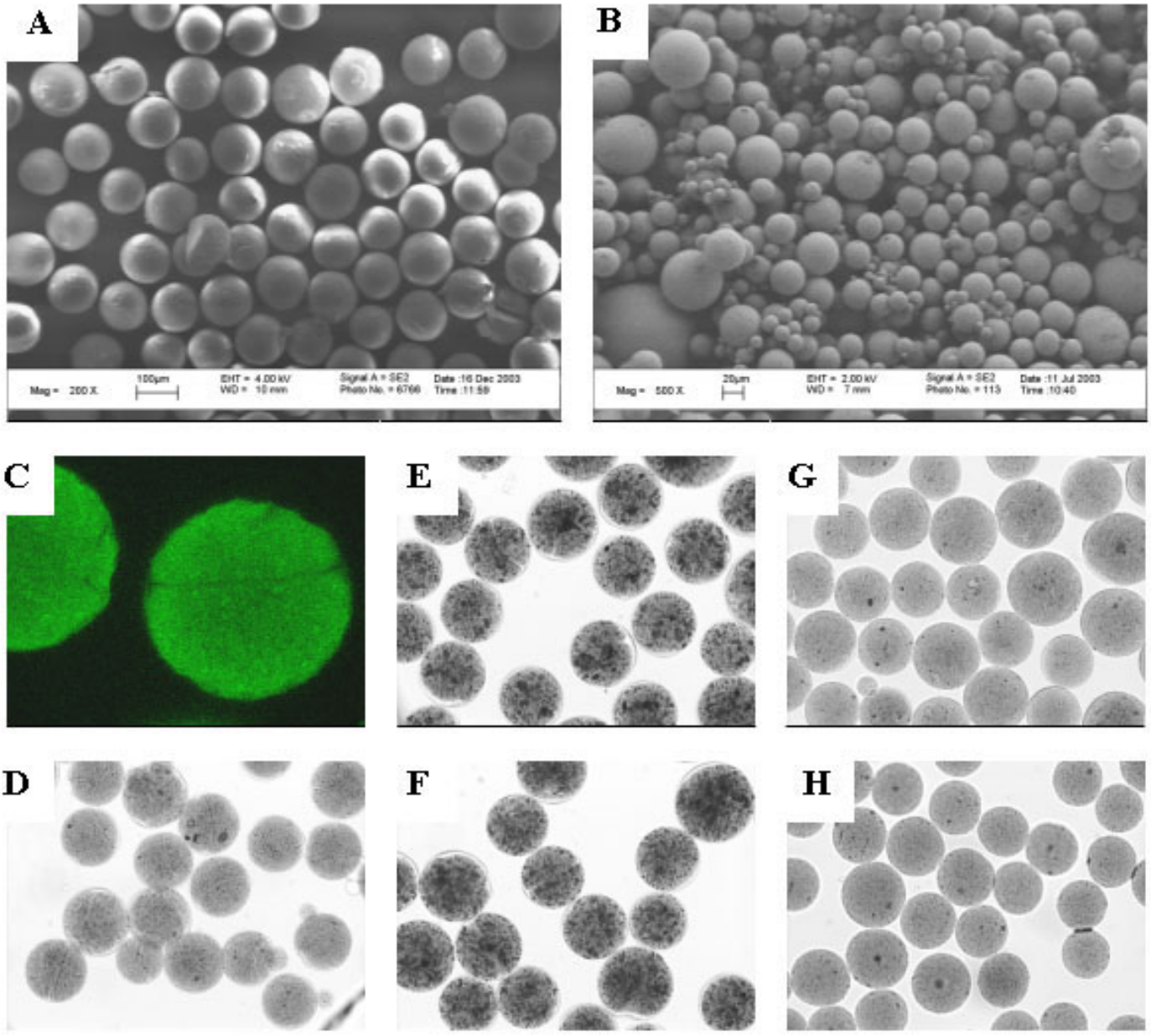

Figure 9. Microspheres with 10% loading of SFL particles in various polymers. SEM photomicrographs show (A) SOF (scale bar, 100 μm) BSA/Tre in PLGA and (B) impeller-sheared (scale bar, 20 μm) BSA in PLGA. (C) Confocal microscopy of fluorescent SOF-encapsulated SFL-BSA/Tre nanoparticles in PLGA microspheres. Light microscopy images show SOF BSA/Tre-loaded PLGA microspheres with (D) no base, (E) 3% $Mg(OH)_2$, and (F) 3% $ZnCO_3$ co-encapsulated (basic crystals are evident as dark spots in E and F). Also shown are SFL-BSA/Tre-loaded microspheres in (G) PLGA-PEO-PLGA and (H) PLGA-F127-PLGA block copolymers.

A previous study demonstrated that uniform distribution of SFL protein nanoparticles throughout impeller-made PLGA and PLA microspheres reduces the burst release.[10] Figure 9C shows a confocal micrograph of 10% SFL-BSA/Tre-loaded PLGA microspheres, where the protein nanoparticles fluoresce (visible as bright green spots). The protein is distributed uniformly throughout the SOF microsphere, as in the earlier study.[10]

In s/o/o processes, the droplets formed contain solids, including the protein and stabilizing excipients, for example buffers. This requirement provided unique challenges for the SOF process; it was necessary to avoid nozzle clogging. The use of ultrafine SFL-protein powders, a solids sonication step, and the use of an in-line 25-μm steel-mesh filter and a 120-μm nozzle were helpful in this regard. Postproduction inspection of the in-line filter revealed no noticeable buildup of particulates, but even one large particle would have been sufficient to clog the nozzle, thus the filter was indispensable.

The suitability of the SOF technique for general s/o/o processing was tested by incorporating 2 established and potentially challenging solid excipients. As a control, Figure 9D shows an image of 10% SFL-BSA/Tre-loaded PLGA microspheres as observed with a light microscope. $Mg(OH)_2$ and $ZnCO_3$, 2 common basic additives, are powders consisting of crystals that range in size from submicron to a few microns, thus their impact on fluid flow and droplet formation at the nozzle tip was checked. Figures 9E and 9F show 10% loaded microspheres with $Mg(OH)_2$ and $ZnCO_3$ added at a loading of 3%. Relative to the base-free sample (Figure 9D), the additives (visible as dark spots) were incorporated throughout the microsphere without a negative effect on the microsphere size distribution. Nine 1-mL batches containing these basic additives were processed with similar size results. Therefore it is reasonable to conclude that the SOF process is likely to be a precise and reliable solids microencapsulation method.

Recent studies have employed the use of various block copolymers to make microspheres more hydrophilic, thus improving swelling and imparting a hydrogel-like interior molecular structure. By improving the permeation of the polymer to proteins and polymer hydrolysis products, linear release profiles and improved protein stabilization have been obtained.[27]Figures 9G and 9H show photomicrographs of 10% SFL-BSA/Tre-loaded PLGA-PEO-PLGA and PLGA-F127-PLGA microspheres, respectively, that were made with the SOF technique. Again, discrete microspheres were obtained with a uniform size. These findings demonstrate that the SOF technique, while capitalizing on the advantages of anhydrous processing, is suitable for the production of uniform microspheres with several key types of polymers of interest in controlled release.

Figure 10 compares the 24-hour burst of PLGA microspheres made by the SOF and impeller-atomization techniques. The crystalline excipient (5% $Mg[OH]_2$) increased burst when added to the impeller-dispersed microspheres because it was added at the expense of PLGA, thus concentrating the protein into less polymer. In addition, the basic crystals provide phase boundaries that attract water where protein molecules might diffuse. At each protein loading, the SOF-microspheres gave similar or lower burst relative to the equivalent impeller produced, nonsieved microspheres without excipient, and substantially less burst than those with excipient.

Comparison of Processes for s/o/o Emulsions

In traditional s/o/o emulsion processes, microspheres are hardened while stirring the oil with a stir bar or impeller and stabilizing the emulsion with a surfactant such as a Span 80, Span 85, or soybean lecithin.[19-22] This process was sufficient when working with PLA, but difficulties arose when working with PLGA, particularly R502H, which is more prone to aggregation. Once stirred, a few microspheres agglomerated on the stir bar or impeller, and then this sticky aggregate rapidly consumed all other microspheres in the emulsion. Even at high surfactant concentrations (up to 5%), such instabilities were common. Thus the suspended droplet hardening procedure was developed to prevent aggregation and coalescence of the sticky particles.

The suspended droplet hardening technique prevented microsphere fusion and loss to the vessel by minimizing their mobility. Once microspheres were dispersed into the cottonseed oil either by an impeller or the SOF method, rapid chilling of the vessel at −70°C caused the conversion of cottonseed oil into a wax. Thus the microspheres were

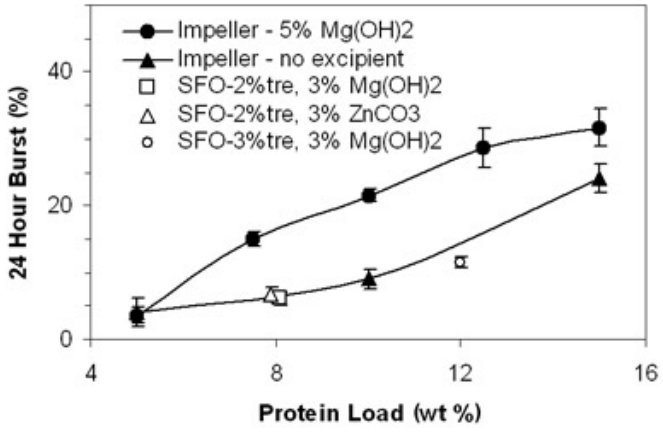

Figure 10. Protein burst-released in the first 24 hours for impeller-dispersed and SOF microspheres of PLGA.

trapped in a dispersed arrangement in the wax during hardening, preventing coalescence. Prechilling of the cottonseed oil to 4°C to 10°C was helpful in facilitating rapid reezing, and prefreezing of a thin layer of oil on the bottom of the vessel partially improved the yield by providing a nonstick floor. Since mixing is not required in the suspended droplet technique, scale-up considerations would likely be limited to heat-transfer. The primary concern is the rapid conversion of the liquid cottonseed oil to a waxy state.

The persistent problem of broad size distributions was overcome by altering the droplet formation process. The SOF technique exploits the same physical principals that Umbanhowar et al used to produce emulsions with uniform droplet size.[15] A droplet forms on the tip of a nozzle and is held in place by surface tension, and the passing fluid, surfactant-free cottonseed oil in this case, produces a drag force on the droplet. This drag force increases as the droplet grows until it becomes greater than the surface tension that holds the droplet in place, and the droplet breaks free. In this manner, the drop diameter can be as small as or smaller than the nozzle diameter. In contrast, droplets formed simply by Rayleigh breakup or dripping under gravity without a drag force would be larger than the nozzle diameter. It was shown in Umbanhowar et al's work that the critical parameters influencing droplet size included the speed of the continuous phase fluid, the nozzle ID, the surface tension of the droplet, and the flow rate of the dispersed phase. Accordingly, simple adjustments to the s/o suspension flow rate and the rotor speed in the SOF process allowed microspheres of the desired size (~125 μm) and narrow size distribution to be produced.

The device described by Umbanhowar et al[15] produced droplets with strikingly low polydispersities (<3%), but it may not be appropriate for microencapsulation owing to sticking and coalescence problems associated with solvent removal. Umbanhowar et al's device acts as a centrifuge; the collection container itself spins, and the continuous phase climbs the vessel walls and becomes a spinning fluid film in which the nozzle is submerged. It was necessary for the present study to provide a moving fluid stream that was independent of the collection vessel (ie, one that would not centrifuge the droplets onto the vessel walls prior to hardening).

Droplet size distributions generally become much tighter as the droplet size is increased, and the very low polydispersities reported by Umbanhowar et al[15] were obtained when droplet sizes were greater than about twice the orifice diameter. The present study used a nozzle with a relatively large orifice, approximately equal to the desired droplet size, to allow for the encapsulation of various coarse solid powders. Very good size uniformity resulted at these proportions, and even lower polydispersities would be expected for formulations that would allow finer nozzles to be used.

CONCLUSIONS

The SOF method led to significant improvement in the control of microsphere size and polydispersity relative to the impeller atomization method, resulting in much larger yields approaching 100% and a large reduction in burst release. The SOF process provided high loadings of uniformly distributed protein solids and basic salt crystals, allowed flexibility in the choice of polymer, and required no surfactants. This improved nonaqueous encapsulation technique shows promise for the development controlled-release microsphere formulations for protein, peptide, and other therapeutic molecules with uniform particle sizes, high encapsulation efficiencies, high yields in the desired microsphere size range, and low burst release.

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