Stable Formulations of Recombinant Human Growth Hormone and Interferon-γ for Microencapsulation in Biodegradable Mircospheres

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Purpose. The successful development of controlled release formulations for proteins requires that the protein not be denatured during the manufacturing process. The major objective was to develop formulations that stabilize two recombinant human proteins, human growth hormone (rhGH) and interferon-γ (rhIFN-γ), at high protein concentrations (>100 mg/mL) in organic solvents commonly used for microencapsulation, methylene chloride and ethyl acetate.

Methods. Several excipients were screened to obtain the maximum solubility of each protein. These formulations (aqueous, lyophilized, milled, spray dried, or isoelectric precipitate) were then rapidly screened by emulsification in the organic solvent followed by recovery into excess buffer. Additional screening was performed with solid protein that was suspended in the organic solvent and then recovered with excess buffer. The recovery of native protein was determined by native size exclusion chromatography (SEC-HPLC) and circular dichroism (CD). The selected formulations were encapsulated in polylactic-coglycolic acid (PLGA) microspheres by either water-in-oil-in-water (W/O/W) or solid-in-oil-in-water (S/O/W) methods. The initial protein released from the microspheres incubated at physiological conditions was analyzed by SEC-HPLC, CD, and biological assays.

Results. The stability of a given formulation in the rapid screening method correlated well with stability during encapsulation in PLGA microspheres. Formulations of rhGH containing Tween 20 or 80 resulted in lower recovery of native protein, while trehalose and mannitol formulations (phosphate buffer, pH 8.0) yielded complete recovery of native rhGH. Other additives such as carboxymethyl cellulose, gelatin, and dextran 70 were not effective stabilizers, and polyethylene glycol provided some stabilization of rhGH. Trehalose/rhGH (1:4 mass ratio) and mannitol/rhGH (1:2 mass ratio) formulations (potassium phosphate buffer, pH 8.0) were lyophilized, reconstituted to 200 and 400 mg/mL rhGH, respectively, and then encapsulated in PLGA microspheres. The protein was released from these microspheres in its native state. Lyophilized formulations of rhGH yielded analogous results indicating the ability of trehalose and mannitol to stabilize the protein. Small solid particles of rhGH generated by spray drying (both air and freeze-drying) formulations containing Tween 20 or PEG were stable in ethyl acetate, but not methylene chloride. Similar results were also obtained with rhIFN-γ (137 mg/mL in succinate buffer, pH 5.0), where both mannitol and trehalose were observed to stabilize the protein during exposure to the organic solvents resulting in the release of native rhIFN-γ from PLGA microspheres.

Conclusions. The rapid screening method allowed the development of stable concentrated protein solutions or solid protein formulations that could be successfully encapsulated in PLGA microspheres. The excipients observed to stabilize these proteins function by preferential hydration of the protein, and in the dry state (e.g., trehalose) may stabilize the protein via water substitution yielding a protective coating around the protein surface. Studies of other proteins should provide further insight into this mechanism of protein stabilization during encapsulation.

KEY WORDS: stability; proteins; microspheres; growth hormone; interferon; drug delivery.

INTRODUCTION

Biodegradable microspheres containing proteins can be produced by several methods including solvent extraction and solvent evaporation techniques (1). These methods usually involve the use of protein in a solid or liquid form mixed with a polymer that is dissolved in an organic solvent (e.g. methylene chloride or ethyl acetate). The protein may contact the organic solvent prior to formation of the microspheres. This solvent-protein interaction may then lead to protein denaturation. Recently, new techniques have emerged that allow the production of protein loaded microspheres under milder encapsulation conditions. For example, one method employs liquid nitrogen and cold ethanol to spray freeze dry the solid protein that has been emulsified with polylactic-coglycolic acid (PLGA) dissolved in methylene chloride (2). Supercritical carbon dioxide has also been used to produce PLGA microspheres by utilizing surfactants to stabilize the polymer phase in the supercritical fluid (3,4). Another method uses hydrophobic ion pairing to make the protein miscible in the organic solvent avoiding aqueous-organic interfaces (5,6). These methods are less common than the traditional solvent evaporation or solvent extraction techniques.

Microspheres produced from these different techniques have been designed to provide a continuous release of protein over time. Typically, the protein is released from PLGA microspheres in three phases: an initial burst, diffusion controlled release, and erosion controlled release (1,7). The initial burst phase is the rapid release (within a few hours) of protein at or near the surface of the microspheres. The diffusion controlled release phase consists of protein diffusion through pores or channels in the microspheres. To obtain a continuous release, the diffusional phase must overlap with the erosion controlled release phase such that new pores or channels are created due to polymer erosion (hydrolysis) allowing continuous diffusion of the entrapped protein out of the microspheres. Some of the major difficulties in producing these microspheres are the minimizing the initial burst phase, maximizing the protein loading within the microspheres, and obtaining a continuous release (e.g. only one phase of release).

To produce continuous release microspheres, we investigated a common method involving a double emulsion process. First, an aqueous protein solution is emulsified with an organic solvent (eg. methylene chloride or ethyl acetate) that is required to dissolve the polymer (e.g. PLGA). This emulsion is then mixed with water containing an emulsifier such as polyvinyl alcohol or polyvinyl pyrrolidone. The water extracts the organic solvent resulting in precipitation of the polymer and subsequent entrapment of the aqueous protein droplets. The theoretical protein loading of microspheres made with this system may be calculated by a simple mass balance as shown in Equation 1:

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$$\text{Protein Loading} = \frac{[\text{protein}]V_a}{[\text{polymer}]V_o + (1 + e)[\text{protein}]V_a} \quad (1)$$

where V_a and V_o are the volumes of the aqueous and organic phases in the first emulsion, [protein] and [polymer] are the concentrations of the protein in the aqueous phase and the concentration of polymer in the organic phase, and e is the mass fraction of excipients to protein (protein concentration/excipient concentration in aqueous phase). For the formation of microspheres, the aqueous to organic phase volume ratio in the first emulsion must be less than 1. In addition, volume ratios approaching or greater than 0.5 usually yield a large initial burst and poor encapsulation of the protein due to the number of aqueous droplets at or near the surface of the microspheres. Low polymer concentrations (e.g., <2 g/10 mL of organic solvent) also result in poor encapsulation of the aqueous protein since the low kinematic viscosity of these solutions allows migration of the aqueous protein droplets out of the organic phase during formation of the second emulsion (1,8).

The double emulsion method for encapsulation of proteins in biodegradable polymers is limited to low protein loadings (<10% w/w) unless concentrated aqueous protein solutions are used in the primary emulsion. We therefore attempted to achieve the maximum possible concentration of two recombinant proteins, rhGH and rhIFN-γ, in aqueous solutions. These concentrated solutions were then incorporated into the microspheres using the double emulsion method.

While high protein concentrations are important to achieve high protein loading in the microspheres, it is also essential to maintain the native conformation of the protein during the encapsulation process. Previous reports in the literature focus on use of denaturing conditions (e.g. SDS-PAGE (9,12)) or assays that are insensitive to conformational changes (e.g. enzymatic activity or antibody-based assays (11,12)) to assess protein stability after encapsulation in biodegradable microspheres. Clearly, these techniques do not provide information on noncovalent aggregate formation or the protein conformation, both of which may make the protein immunogenic and result in a loss of potency. We therefore studied the physical stability of the proteins by two nondenaturing techniques: native size exclusion chromatography and circular dichroism, which measures the secondary and tertiary structure of the protein. In addition, the biological activity of each protein was measured by *in vitro* cell based assays. Through systematic screening of excipients, we were able to select conditions such that the proteins were released from PLGA microspheres in their native, biologically active conformation.

MATERIALS & METHODS

Materials

rhGH was supplied by Genentech Production Recovery Operations and formulated at 10 mg/mL protein in 10 mM ammonium bicarbonate, pH 7. This material was lyophilized to create an excipient and buffer free protein powder. rhIFN-γ was also provided by Genentech Product Recovery Operations and formulated at 1 mg/mL in 5 mM sodium succinate, pH 5.

Reagents were obtained from several suppliers. Sodium phosphate, sodium chloride, potassium chloride, sodium acetate, ammonium acetate, and Tween 20 (low peroxide) were purchased from Mallinckrodt. Trehalose, sodium azide, polyethylene glycol (PEG, 3350 Da), dextran 70 (70 kDa), carboxymethyl cellulose (CMC, sodium salt, low viscosity), Tween 80, and HEPES was obtained from Sigma Chemical Company. Sodium succinate and succinic acid were bought from United States Biochemical Corporation. Acetic acid were supplied by J.T. Baker Corporation. Mannitol was purchased from Aldrich Chemicals. Gelatin (100A, USP) was obtained from Atlantic Gelatin. Sodium dodecyl sulfate was obtained from Pierce Chemical Company. Polyvinyl alcohol (PVA, Airvol 205) was donated by Air Products. Methylene chloride and ethyl acetate were ACS grade from Baxter Healthcare Corporation. PLGA (50:50 lactide:glycolide, 12 kDa; RG502) was purchased from Boehringer Ingelheim.

Preparation of rhGH Formulations

Reconstitution

For development of a stable formulation, rhGH was first lyophilized in ammonium bicarbonate to yield an excipient- and buffer-free protein powder (ammonium and bicarbonate are volatilized in the lyophilization process). This protein powder was reconstituted in the 10 mM sodium phosphate buffer, pH 8.0 with or without excipients and allowed to dissolve. Undissolved protein was removed by centrifugation at 13,000 rpm for 1 min. For initial screening studies, the rhGH concentrations were adjusted to 10 mg/mL in each formulation. To assess solubility in each formulation, solid rhGH was added until solid rhGH was observed to remain suspended in the solution.

Lyophilization

After a stable liquid formulation was developed, the protein was lyophilized in this formulation. For each lyophilization, the rhGH concentration was 10 mg/mL. The residual moisture of these formulations was not determined, but the same lyophilization cycle (frozen to −55°C, primary drying at −10°C and 120 mTorr for 22 hrs, secondary drying at 5°C and 120 mTorr for 12 hrs) was used in each case.

Milling

To obtain an even distribution of the protein throughout the microspheres, lyophilized rhGH was air jet milled. The milling process was performed with a pressure driven impaction mill and resulted in a fine particulate of rhGH.

Isoelectric Precipitation

rhGH lyophilized in ammonium bicarbonate was dissolved in 0.1 M acetate buffer (sodium acetate, pH 8.2 or ammonium acetate, pH 7). The pH of the solution was then adjusted with 0.1 M acetic acid to pH 5.2 resulting in isoelectric precipitation of the protein. To prevent localized precipitation from microscopic pH changes, the pH was adjusted slowly and the solution was maintained well mixed by continuous stirring. The final solutions were centrifuged at 1000 rpm for 5 min to concentrate the solids. The supernatants were then removed. To determine the concentration of the precipitated slurry of rhGH, 100 μL of the slurry was diluted into 10 mL of 5 mM sodium phosphate, pH 8. The resulting solution was measured for protein concen-

tration by absorbance at 278 nm (E = 0.81 $cm^{-1}(mg/mL)^{-1}$) and the protein quality was assessed by native size exclusion HPLC (SEC-HPLC).

Preparation of rhIFN-γ Formulations

The protein in 5 mM sodium succinate, pH 5.0 was concentrated to greater than 100 mg/mL in an Amicon stirred cell containing a 10,000 Da molecular weight cut-off membrane. The protein concentration of the final solution was determined by 100-fold dilution with 5 mM sodium succinate, pH 5.0. The absorbance of the diluted solution was then measured at 280 nm (E = 0.71 $cm^{-1}(mg/mL)^{-1}$). Excipients were added as solids to individual aliquots of the concentrated stock solution. After dissolution of the excipients, the final solutions were filtered with a 0.22 μm filter (low protein binding) to remove any particulates.

Screening of Formulations in Organic Solvents

The effect of organic solvents on protein stability was determined by adding rhGH or rhIFN-γ to a solution of methylene chloride or ethyl acetate. For solid rhGH conditions, the ratio of protein mass (mg) to volume of organic solvent (mL) was 40 mg/mL. For the aqueous protein conditions, 100 μL of protein in a buffered solution was added to 1.0 mL of organic solvent to assess the effects of each buffer system on protein stability. After protein addition, the samples were either sonicated for 30 seconds in a 47 kHz bath sonicator (Cole Parmer, Model 08849-00) or homogenized for 1 min at 8,000 rpm with a microfine homogenizer tip (Virtis) to simulate the primary emulsion step in the microsphere production process. After sonication or homogenization, the rhGH or rhIFN-γ was recovered from the organic solvent by dilution into a 50 fold excess of a stabilizing buffer (rhGH: 5 mM sodium phosphate, pH 8.0; rhIFN-γ: 5 mM sodium succinate, pH 5.0). The amount and quality of the protein recovered in this step was determined by protein concentration measurements (absorbance at 278 nm for rhGH and 280 nm for rhIFN-γ) and native SEC-HPLC. The criterion for a stable formulation was the maximum recovery of monomeric (rhGH) or dimeric (rhIFN-γ) protein without the formation of conformational variants or aggregates larger than dimers. Control samples of the same starting composition (excipients, buffer, and protein concentration) were also analyzed, and these results indicated that the starting material, in each case, did not contain significant amounts of aggregates or conformational variants as determined by native SEC-HPLC.

Size Exclusion HPLC Analysis of Proteins

rhGH

Samples were analyzed by native SEC-HPLC on a Dupont GF250 column and 20 μL of each sample was loaded onto the column. Samples were eluted at 1.0 mL/min with 25 mM sodium phosphate, 100 mM NaCl, pH 7.0. The absorbance at 214 and 280 nm was measured, and standards were included with each set of samples. The total peak area of the standard was used to determine the total amount of protein in each sample (ratio of peak areas and known standard concentration).

rhIFN-γ

Samples were assessed by both native and SDS (denatured) size exclusion chromatography. Native size exclusion chromatography was performed on TSK G3000 SWXL (300 mm × 7.8 mm; TosoHaas) and 20 μL of each sample was loaded onto the column. Samples were eluted at 1.0 mL/min with 10 mM sodium phosphate, 0.45 M potassium chloride, pH 7.0. SDS size exclusion analysis was done using the same type of column. The column was loaded with 200 μL of each sample and eluted at 1.0 mL/min with 0.1% SDS, 0.2 M sodium phosphate, pH 7.0. The absorbance at 214 and 280 nm was measured to assess protein elution. Standards were used to determine the amount of protein in each sample (total peak area ratios).

Circular Dichroism

Circular dichroism (CD) spectra of rhGH and rhIFN-γ were taken with an AVIV/CARY 60 DS spectropolarimeter. Samples were placed in 1.0 cm or 0.1 cm pathlength quartz cells for measurement of near (360–250 nm) and far (250–190 nm) ultraviolet spectra, respectively. The far ultraviolet spectra were taken in 0.2 nm intervals with a bandwidth of 1.5 nm and an averaging time of 3.0 sec. The near ultraviolet spectra were taken in 0.2 nm intervals with a bandwidth of 0.5 nm and an averaging time of 5.0 sec. The average of three spectra were used for both the far and near ultraviolet CD. The averaged spectra were corrected for buffer controls and the mean residue ellipticity was then calculated (mean residue weight: rhGH, 115.8; rhIFN-γ, 117).

Biological Assays

The ability of rhGH to cause receptor dimerization was determined by using an assay based upon the mouse myeloid cell line transfected with the full length hGH receptor[13]. These cells proliferate in response to bioactive hGH (receptor dimerization). In summary, cells (400,000 cells/mL) were added to microtiter plates. The samples and controls (buffer and standards) were then added in triplicate to the plates and allowed to incubate at 37°C for 16–20 hrs. Methyl ^{3}H-thymidine (1 μCi/well) was then added and allowed to incubate for 4 hrs. Incorporation of radiolabelled thymidine was quenched by addition of thymidine to a final concentration 30 mM. Radioactivity in each well was assessed by harvesting the cells. Standard curves were obtained from known concentrations of rhGH and used to calculate the unknown rhGH concentrations with a four-parameter curve fitting program.

The antiviral activity of rhIFN-γ was assessing using A549 (human lung carcinoma) cells (ATCC CCL 185) and encephalomyocarditis virus. The A549 cells were incubated in the presence of rhIFN-γ samples (diluted to 100–500 U/mL in PBS containing 0.5% BSA) for 24 hr at 37°C. The cells were then challenged with virus by incubation at 37°C until >90% lysis of the cells in the control samples (buffer) is achieved (18–24 hr). The cell viability was then determined using crystal violet (0.5% solution). Samples were analyzed in a 96-well microtiter plate format (Falcon, 3075) with serial dilutions of the samples and appropriate controls (buffer and standards). The cell viability was quantitated by optical density measurements (540 nm) of the dry plates (extensively washed to remove unbound dye) with a microtiter plate reader (Flow Titertek Multiscan).

Phase Partitioning of Solid rhGH Formulations

The partitioning of rhGH between the methylene chloride phase and the aqueous phase should define the efficiency of encapsulation of the protein and the initial burst of the protein from the microspheres. Protein that remains well dispersed in the organic phase yields microspheres with a homogeneous encapsulation of the protein, while protein that resides at the water-organic solvent interface results in poor encapsulation and a large initial burst. To measure the partitioning effect, solid formulations of rhGH (20 mg) were added to the methylene chloride (2.5 mL) and sonicated for 30 sec. An equal volume of water was then added to the surface of each solution and the solutions were inverted twice. The solutions were centrifuged at 3500 rpm for 10 to 15 min and the location of the protein was visually observed. The protein either formed a stable layer at the interface or remained somewhat dispersed in the methylene chloride with a layer in the bottom of the tube (methylene chloride phase).

Particle Size Analysis of Solid rhGH

The particle size of the lyophilized rhGH used in the microencapsulation process determines the amount of protein loaded into the microspheres and the release characteristics of the microspheres. Since the microspheres are typically 30 to 100 μm in diameter, the protein dispersion in the homogenization step should be on the order of a few microns. Protein particles which are larger than a few microns will reduce the amount of protein loaded into the microspheres and increase the initial release (burst) of the protein from the microspheres. To assess the size of the rhGH solids, the solid protein was homogenized in methylene chloride without PLGA. After homogenization, the methylene chloride solution was analyzed by the Brinkmann Particle Size Analyzer (Model 2010). Samples were diluted with additional methylene chloride to adjust the concentration to the range required for the analysis.

Microencapsulation of Proteins in PLGA

After a formulation was shown to stabilize the protein against denaturation, it was used in the microencapsulation process. The protein in a formulation which prevented methylene chloride denaturation was used as either a milled solid, homogenized solid, or an aqueous solution. The protein was encapsulated in PLGA using a double emulsion method. The solid (700 mg) or aqueous (2 mL) protein was injected into methylene chloride containing PLGA (0.6 g PLGA/mL solvent; 10 mL). This mixture was homogenized for 30 sec at 7,000 rpm using a Virtis homogenizer with a microfine tip. The resulting emulsion or suspension was then transfered to a stirred tank (900 mL) containing 6% PVA and methylene chloride (4.5 mL). The solution was mixed at 1000 rpm for 1 min. The nascent microspheres in the PVA solution were added to 12 L of distilled water (MilliQ, Millipore Corp). The final bath was gently mixed at 2–8°C and nitrogen was passed over the headspace of the vessel. After 1 hr, the microspheres were filtered with a 150 μm mesh and transferred to an Amicon stirred cell (2 L) containing a 20 μm mesh. The microspheres were then washed with MilliQ water (15 L) and 0.1% Tween 20 (15 L) to reduce agglomeration. The microspheres were dried with nitrogen at 2–8°C for 2 days. The average microsphere size was 30–50 μm in diameter as measured by a Brinkman Particle Size Analyzer.

Protein Loading

The amount of protein in the microspheres (%w/w) was determined by dissolution of the microspheres in 1 N sodium hydroxide. 10 mg of PLGA microspheres was added to 1 mL of sodium hydroxide and agitated overnight in a microcentrifuge tube. The extinction coefficient for each protein was determined by addition of different amounts of protein to 1 N sodium hydroxide and the protein spectra scanned to find the maximum absorptivity between 240 and 400 nm. A plot of the protein concentration versus the absorption at the maximum wavelength allowed calculation of an extinction coefficient for each protein in sodium hydroxide (rhGH: 1.11 $(mg/mL)^{-1}cm^{-1}$ at 294 nm; rhIFN-γ. 0.96 $(mg/mL)^{-1}cm^{-1}$ at 292 nm).

Protein Release

The release of protein from the PLGA microspheres was measured by placing 20–30 mg of PLGA microspheres in microcentrifuge filtration tubes containing 0.22 μm filters. 300 μL of release buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 0.02% Tween 20, 0.02% azide) was then added to suspend the microspheres on the retentate side of the filter. The tubes were sealed with 3 cc vial stoppers and covered with parafilm. The microspheres were then incubated at 37°C. Samples were taken over time by centrifugation (13,000 rpm, 1 min) of the tubes. The filtrate was removed and the microspheres were resuspended with 300 μL of the release buffer. Venting of the tubes (stopper) with a 26 gauge needle was required to allow addition of the release buffer. The quality of the released protein was assessed by native SEC-HPLC and biological activity assays.

RESULTS AND DISCUSSION

Stability of Aqueous Protein Solutions in Organic Solvents

The recovery of native protein after microencapsulation is essential to the successful development of a controlled release protein formulation. Typically, the physical instability of proteins requires the use of stabilizing excipients to prevent denaturation and aggregation. The choice of excipients depends on the solvent environment and external stresses applied to the protein. For microencapsulation, homogenization or sonication of proteins in organic solvents may destabilize the native protein conformation. We therefore investigated the role of various excipients in stabilizing proteins under these denaturing conditions.

An initial rapid screen of aqueous rhGH formulations (10 mg/mL rhGH in 5 mM potassium phosphate, pH 8) was performed by emulsifying the solution in either methylene chloride or ethyl acetate (simulating the first emulsion step but without the polymer). Methylene chloride has been more commonly used for microencapsulation since PLGA is generally more soluble in methylene chloride than ethyl acetate. The emulsified solution was then diluted with excess buffer (second emulsion without polymer) and the quality and quantity of the rhGH was measured by native SEC-HPLC and absorbance. Emulsification of 10 mg/mL rhGH in methylene chloride resulted in a signifi-

cant loss of soluble monomeric protein (Table I). The addition of surfactants (Tween 20 and Tween 80) provided a slight increase in the yield of monomeric rhGH, while the use of a less hydrophobic surfactant, PEG, resulted in nearly complete recovery after methylene chloride treatment. The formulations containing 10 mg/mL mannitol or mannitol in combination with CMC (2.5 mg/mL of each) also gave a high yield of monomeric protein. Lower concentrations of mannitol or CMC alone were less effective in stabilizing rhGH in methylene chloride. These studies indicated that PEG, mannitol, and mannitol/CMC formulations stabilize rhGH during encapsulation.

In contrast to the methylene chloride results, rhGH formulations emulsified in ethyl acetate yielded very high recovery of soluble monomeric rhGH. PEG and mannitol were slightly more effective than buffer alone in stabilizing rhGH, but there was not an observable dependence on excipient concentration. Trehalose provided the best stability at the lower concentrations of 1 and 2 mg/mL (97% recovery, Table I). Combinations of PEG/trehalose gave slightly less recovery than trehalose alone at the same concentration. The yield for both the PEG/mannitol (10 mg/mL of each) and Tween 20/mannitol (1 mg/mL; 10 mg/mL) formulations were also significantly better than the buffer alone. Although ethyl acetate had less of a denaturing effect on rhGH, excipients (PEG, mannitol, and trehalose) were still required to improve the recovery of monomeric rhGH.

To achieve the goal of high protein concentrations, excipient- and buffer-free lyophilized rhGH was added to 10 mM sodium phosphate buffer, pH 8 with or without excipients. This pH condition was chosen as a compromise between maximizing solubility and minimizing degradation (higher pH: greater deamidation; lower pH: less solubility (pH 5–7) or greater denaturation (<pH 5)) (14). Without excipients in this buffer, rhGH reached a maximum solubility of 95 mg/mL. rhGH has been shown to bind Tween and is stabilized against surface denaturation by this interaction[15]. Addition of 1.0 mg/mL Tween 20 or Tween 80 did not significantly increase the solubility of rhGH (Table I). PEG at 10 mg/mL and mannitol at 50 mg/mL both provided a slight increase in solubility. Dextran 70 at 50 mg/mL increased the solubility of rhGH to 142 mg/mL and trehalose at 50 mg/mL provided the greatest solubility at 275 mg/mL rhGH. In contrast, 20 mg/mL of gelatin, a component previously used in microencapsulation of peptides and proteins (16,17), decreased the solubility of rhGH.

The stability of rhGH at the maximum solubility in each formulation was assessed by emulsification in organic solvents followed by dissolution in a stabilizing buffer (10 mM sodium

Table I. Screening of Aqueous rhGH Formulations in Organic Solvents[a]

Formulation	% Recovery[b]		Maximum Solubility[c] (mg/mL)	[Excipient] (mg/mL)
	EtAc	$MeCl_2$		
10 mg/mL rhGH (Prescreening)				
Phosphate buffer	93.5	53.2		
Tween 80	ND[d]	64.5		1.0 or 10.0[e]
Tween 20	ND	78.2		1.0 or 10.0[e]
PEG (3350 MW)	94.7	95.1		2.0, 5.0, or 10.0[e]
Mannitol	94.7	68.1–89.9		2.0, 5.0, or 10.0[f]
Trehalose	97.7	ND		1.0, 2.0, or 10.0[e]
CMC	ND	75.1		0.5 or 2.0
CMC/mannitol	ND	93.9		2.5:2.5
PEG/trehalose	96.1	ND		1:1, 2:2, or 10:10[e]
PEG/mannitol	96.3	71.7		2:2 or 10:10[e]
Tween 20/mannitol	97.6	ND		1.0:10.0
Maximum Solubility				
Phosphate buffer	93.7	85.7	95.3	
Tween 80	ND	58.3	95.2	1.0
Tween 20	97.6	69.3	97.6	1.0
PEG (3350 MW)	94.7	97.4	105	10
Mannitol	100	100	106(223)[g]	50 (111)
Dextran 70	ND	44.0	142	50
Trehalose	100	100	275(400)[g]	50 (100)
Gelatin	ND	97.0	70.5	20
CMC	ND	35.4	(200)[g]	10
CMC/mannitol	ND	61.5[h]	(200)[g]	50; 50

[a] All formulations contained 5 mM sodium phosphate, pH 8, and were emulsified and recovered from the organic solvents as described in the Materials & Methods section.

[b] % Recovery = Recovery of soluble non-aggregated protein (1-[total mass − recovered mass]/[total mass])*100%. Absorbance & SEC-HPLC.

[c] Maximum solubility as determined by addition of unbuffered rhGH to the excipient formulations in 5 mM phosphate, pH 8.

[d] ND = not determined.

[e] Results did not vary significantly as a function of excipient concentration.

[f] Recovery from methylene chloride increased with mannitol concentration, but recovery from ethyl acetate was not affected by mannitol concentration.

[g] Higher solubilities were observed for reconstitution of rhGH formulations that were lyophilized in mannitol, trehalose, or CMC.

[h] The lyophilized CMC/mannitol formulation formed a very viscous solution after reconstitution, and it was very difficult to syringe. The overall yield may therefore be incorrect due to this difficulty.

phosphate, pH 8). Without excipients, rhGH formed aggregates after the emulsification procedure resulting in less than complete recovery of the monomeric protein (Table I). However, the recovery of soluble protein was significantly greater at this protein concentration (95 mg/mL) than that observed at the lower protein concentration (10 mg/mL) in the initial screening. This result revealed that rhGH may be acting as a 'self-protectant' at high protein concentrations because only a limited amount of rhGH can interact at the interface between the oil and water phases resulting in a smaller fraction of denatured rhGH at higher protein concentrations. Also, at the high protein concentration (95 mg/mL), Tween 20 or Tween 80 decreased the recovery of soluble monomeric rhGH, and may have stabilized a partially denatured form of rhGH (see below). A slight increase in recovery of soluble monomeric rhGH was observed for the Tween 20 solution in ethyl acetate, indicating a potential difference in the solvent-surfactant interactions for these organic solvents. PEG and gelatin also provided a marginal increase in recovery of soluble monomeric rhGH under these conditions. Although dextran 70 increased the solubility of rhGH, it decreased the recovery of rhGH as compared to buffer alone. Mannitol and trehalose however yielded complete recovery of soluble monomeric rhGH after emulsification in either organic solvent.

The best stabilizers, mannitol and trehalose, were further studied after lyophilization with rhGH. Protein at 10 mg/mL in 5 mM potassium phosphate, pH 8 was lyophilized with either 5 mg/mL mannitol or 2.5 mg/mL trehalose. In addition, the CMC (0.5 mg/mL) and CMC/mannitol (2.5 mg/mL of each) formulations from the initial screening were also lyophilized at the same protein concentration in phosphate buffer, pH 8. The resulting lyophilized protein powders were then added to 10 mM sodium phosphate, pH 8, to achieve a maximum solubility of rhGH of 223 mg/mL in mannitol (111 mg/mL mannitol, 111 mM phosphate), 400 mg/mL in trehalose (100 mg/mL trehalose, 200 mM phosphate), and 200 mg/mL in the CMC (10 mg/mL CMC, 100 mM phosphate) or CMC/mannitol (50 mg/mL of each, 100 mM phosphate). The trehalose and mannitol solutions were slightly opalescent due to the high protein concentrations, but neither solution contained precipitated protein. The solutions containing CMC were very viscous and formed gel-like material shortly after reconstitution. These solutions were also assessed by emulsification in both organic solvents and subsequent dilution into buffer. Soluble monomeric rhGH was completely recovered for the trehalose and mannitol formulations after emulsification in the organic solvents. The CMC containing solutions provided low yields of monomeric rhGH and were very difficult to handle due to the high viscosity. These results indicate that both mannitol and trehalose provide high solubility and stability of rhGH under the conditions used for microencapsulation.

To further assess these excipients for protein stabilization, rhIFN-γ, a noncovalent dimeric protein, was formulated in different excipient solutions and then emulsified in methylene chloride. An initial formulation was selected based upon the current commercial formulation of Actimmune™ rhIFN-γ consisting of 0.1 mg/mL protein in succinate buffer, pH 5 with mannitol and Tween 20. Unlike rhGH, rhIFN-γ requires stabilization with excipients for lyophilization. Therefore, a solution of excipient-free protein was concentrated by ultrafiltration (10,000 MWCO membrane, Amicon cell) in 10 mM succinate buffer, pH 5. The maximum protein concentration achieved by this method was 134 mg/mL. This solution was then tested for stability in methylene chloride with and without additional excipients. Without excipients, the recovery of soluble dimeric protein was 51.6% (Table II). The addition of 0.1 mg/mL Tween 20 reduced the yield, while the addition of both Tween 20 (0.1 mg/mL) and mannitol (62 mg/mL) provided the same yield as the buffer alone. As observed for rhGH, the nonionic surfactant, Tween 20, reduced the recovery of native protein. The solutions containing mannitol as the only excipient resulted in slightly better recovery of soluble dimer. In contrast, trehalose at 50 mg/mL provided complete recovery of soluble dimer.

Maintenance of Native Conformation

To further confirm the ability of trehalose and mannitol to prevent protein denaturation in organic solvents, the protein integrity was assessed by circular dichroism. As shown in Table I, rhGH was monomeric after sonication in methylene chloride only when trehalose or mannitol were present as stabilizers. The size exclusion chromatogram for the recovered protein also yielded a single peak, unlike the excipient-free protein which eluted as several larger species (Figure 1). The earlier eluting species for the excipient-free protein did not elute at the same time as the rhGH dimer. These results indicated the possibility of the formation of a stable denatured state after solvent treatment. To investigate this possibility further, the earlier eluting species were collected and analyzed by circular dichroism. The circular dichroism spectra of these species indicated that the intermediate species (eluting between the native dimer and monomer) contained less α-helical structure as shown by the change in the minimum at 222 nm and more ellipiticity from the tryptophan-asparagine hydrogen bond as noted by the increased ellipiticity between 290 and 310 nm (18) (Figure 1). Previous studies of rhGH in propanol indicated a change in tertiary structure and maintenance of secondary structure, resulting in a compact molten globule form (19). The protein eluting before the native dimer peak had the same conformation as the intermediate species indicating a possible dimer (intermediate dimer) of this partially denatured state, while the dimer of the native monomer (native dimer) had the same conformation as the native monomer. This inter-

Table II. Screening of Aqueous rhIFN-γ Formulations in Methylene Chloride[a]

Formulation	% Recovery[b]	[Excipient] (mg/mL)
Succinate buffer	51.6	
Tween 20	36.1	0.1
Tween 20/Mannitol	59.0	0.1: 62
CMC	78.2	10
Mannitol	62.9	50
Mannitol	58.3	5
Trehalose	100	50
Trehalose	100	5

[a] All rhIFN-γ solutions were at 134 mg/mL in 10 mM succinate buffer, pH 5.

[b] % Recovery = Recovery of soluble non-aggregated protein (1-[total mass − recovered mass]/[total mass])*100%. Absorbance & native SEC HPLC. Note: All samples were >99% monomer by SDS-SEC HPLC.

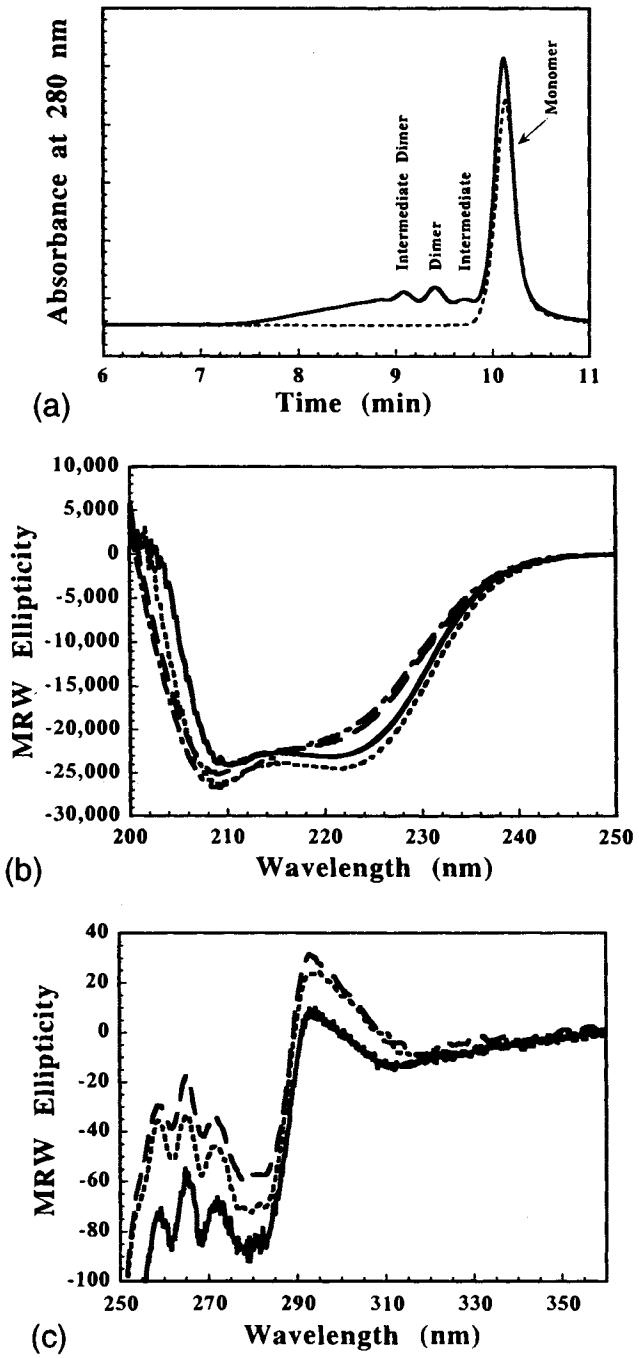

Fig. 1. (a) Native size exclusion chromatography (SEC HPLC) of rhGH after recovery from methylene chloride resulted in primarily monomeric rhGH (10.1 min) for protein formulated in trehalose or mannitol (solid line). However, without stabilizing excipients (dashed line), rhGH eluted as four species: monomer, intermediate (9.7 min), native dimer (9.4 min), and intermediate dimer (9.1 min). The purified species, monomer (—), intermediate (— —), native dimer (---), and intermediate dimer (— - —), were analyzed by far (b) and near (c) ultraviolet circular dichroism. The amount of purified intermediate dimer was too low for analysis by near UV circular dichroism.

mediate species was unfolded in 4.5 M guanidine hydrochloride (GuHCl), which was previously demonstrated to unfold native rhGH (20,21). The unfolded protein was then refolded by dilution to 0.5 M GuHCl resulting in the recovery of the native monomeric rhGH structure as measured by size exclusion and circular dichroism (data not shown). Therefore, denaturation of rhGH by organic solvents resulted in the formation of stable denatured state. Mannitol and trehalose were able to prevent the formation of this stable denatured state of rhGH, and provided high protein solubility.

In contrast to rhGH, the circular dichroism spectra of rhIFN-γ after emulsification in methylene chloride differed only slightly from the spectra for the native protein. The only major differences were observed in the near UV spectra (Figure 2). There was some loss of the phenylalanine contributions to the spectra (22) and this result could have been caused by the aggregation of the protein after emulsification in methylene chloride. Analysis of these samples in the cell-based bioactivity assay revealed that the succinate-buffered formulation maintained 49% biological activity. Addition of mannitol increased the recovery of biological activity to 69%, and trehalose provided complete recovery of biological activity. These results revealed that rhIFN-γ is prone to noncovalent aggregate formation and loss of biological activity after treatment with methylene chloride, but the native and biologically active state is maintained intact when formulated with trehalose.

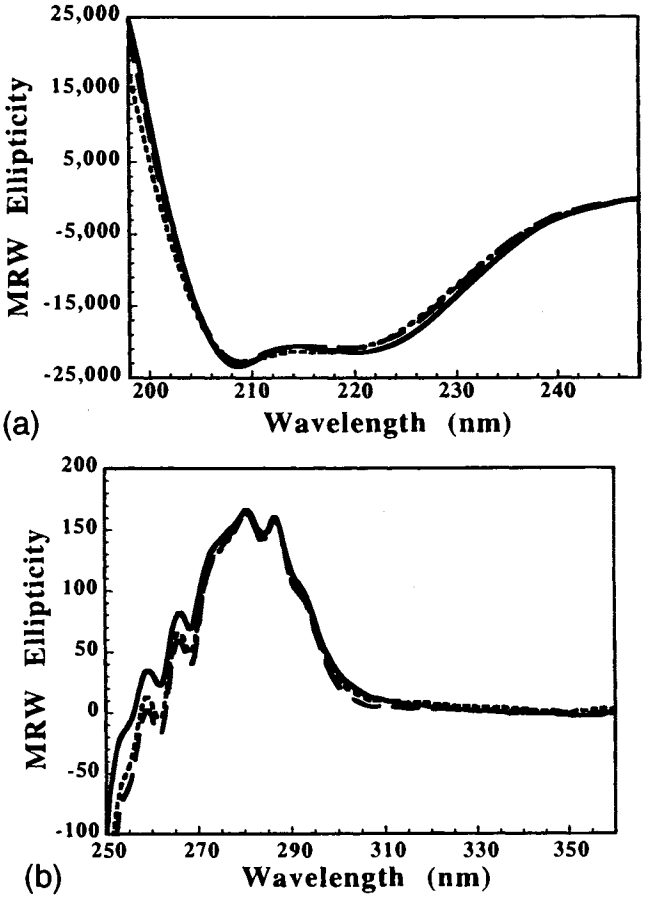

Fig. 2. rhIFN-γ formulated in 5 mM sodium succinate, pH 5 with (---) or without (——) trehalose was recovered from methylene chloride. The recovered protein and untreated sample (-) were analyzed by far (a) and near (b) ultraviolet circular dichroism.

Isoelectric Precipitation for Stabilization of Proteins in Organic Solvents

While trehalose and mannitol stabilized the aqueous protein formulations, other formulations may reduce or eliminate the need for stabilizing excipients. Previous studies have demonstrated that ionic surfactants can facilitate protein dissolution in organic solvents by hydrophobic ion pairing (5,6). This approach requires the use of an oppositely charged surfactant that binds to the protein through both hydrophobic and charge interactions, thereby reducing direct protein-organic solvent interactions and making the protein more neutral. The protein is required to have a positive charge for this technique since cationic surfactants may have toxic side effects (23). Unfortunately, nonionic surfactants were observed to destabilize rhGH in methylene chloride, and this protein has a low pI (5.2) that prohibits the use of negatively charged surfactants for hydrophobic ion pairing under non-denaturing conditions (rhGH is acid labile (18)). A similar charge neutralization effect can also be achieved by formulation of the protein at its isoelectric point. The neutral protein should more readily dissolve in the organic solvent, and may maintain its native conformation.

To test this hypothesis, excipient-free rhGH was added to 0.1 M acetate buffer (sodium or ammonium salt) with or without 100 mg/mL mannitol and the pH was adjusted to the pI (5.2) while mixing. The concentrated protein suspension was then assessed for stability in methylene chloride (Table III). The isoelectrically precipitated protein in either sodium or ammonium acetate yielded similar recovery of soluble monomeric rhGH to phosphate buffered rhGH at its maximum solubility (Table I) and was less concentrated than the phosphate buffered protein. The addition of 100 mg/mL mannitol to the acetate buffer greatly enhanced the recovery of soluble monomeric rhGH and the protein concentration for the ammonium acetate/mannitol formulation was comparable to the phosphate/mannitol formulation (Table I). Therefore, isoelectric precipitation of rhGH does provide some stabilization of rhGH, and may provide a method to achieve a high protein loading.

Table III. Screening of Isoelectric Precipitated rhGH Formulations in Methylene Chloride[a]

Formulation	% Recovery[b]	[rhGH][c] (mg/mL)
0.1 M NaAcetate	81.0	66.2
0.1 M NH_4Acetate	87.4	53.2
0.1 M NaAcetate 100 mg/mL mannitol	95.6	92.8
0.1 M NH_4Acetate 100 mg/mL mannitol	93.5	261

[a] Excipient-free rhGH added to formulation and titrated to pH 5.2 (pI). Supernatant was removed after centrifugation, and the resulting slurry (100 μL) was homogenized in methylene chloride (1 mL).

[b] % Recovery = Recovery of soluble non-aggregated protein (1-[total mass − recovered mass]/[total mass])*100%. Absorbance & native SEC HPLC.

[c] The protein concentration in the isoelectric precipitate was determined by 100× dilution of the untreated slurry into 5 mM sodium phosphate, pH 8, followed by absorbance measurement at 278 nm. After dissolution in phosphate buffer, the untreated precipitates were 97% monomer by SEC HPLC.

Stability of Solid Protein Formulations in Organic Solvents

Often it is difficult to achieve the high protein concentrations described above, and one is required to consider the use of a solid protein powder for microencapsulation. The use of a dry protein powder also eliminates the water-oil interface in the first emulsion. However, in solid-in-oil-in-water systems, the protein will still encounter an aqueous environment in the presence of the organic solvent during the second emulsion, even though this residence time is relatively short (minutes to hours). Ideally, this short exposure to a water-oil interface will not denature or dissolve the solid protein. The removal of water from the protein phase may then increase protein stability in the organic solvents.

To test the stability of solid protein in organic solvents, rhGH was lyophilized in different formulations. The dry solid protein was then homogenized (25,000 rpm, 1 min) in methylene chloride and recovered with excess phosphate buffer, pH 8. The use of rhGH lyophilized in potassium phosphate buffer (pH 8) resulted in a recovery of 83.1% soluble monomer (Table IV). The protein lyophilized in phosphate buffer (pH 8) containing either 5 mg/mL mannitol or 2.5 mg/mL trehalose was more stable, and greater than 90% recovery of soluble monomer was achieved for these formulations.

Table IV. Stability of Solid rhGH Formulations in Organic Solvents

Formulation	% Recovery[a] EtAc	% Recovery[a] $MeCl_2$	[Excipient]:[Protein][b] (mass ratio)
Homogenized Solids[c]			
Phosphate buffer	ND[d]	83.1	n/a[e]
Mannitol	ND	94.9	1:1
Trehalose	ND	95.2	1:4
Milled Solids[f]			
Ammonium bicarb.	ND	30.1	n/a
Phosphate buffer	ND	83.8	n/a
Mannitol	ND	85.0	1:1
Spray Dried Solids[g]			
Trehalose/PEG	98.9	44.1	1:4; 1:5
Trehalose/Tween 20	96.7	50.9	1:4; 1:5
Spray Freeze-Dried Solids[g]			
Trehalose/PEG	97.1	54.2	1:4; 1:5
Trehalose/Tween 20	93.4	31.8	1:4; 1:5

[a] % Recovery = Recovery of soluble non-aggregated protein (1-[total mass − recovered mass]/[total mass])*100%. Absorbance & SEC-HPLC.

[b] Ratio of excipient and protein concentrations.

[c] rhGH at 10 mg/mL was lyophilized in 10 mM phosphate buffer, pH 8 with or without excipients, and then homogenized at 25,000 rpm for 1 min in methylene chloride.

[d] ND = not determined.

[e] n/a = not applicable, buffer formulations without excipients.

[f] Lyophilized rhGH was air-jet milled and then emulsified in methylene chloride.

[g] rhGH at 10 mg/mL in 5 mM potassium phosphate buffer containing 2.5 mg/mL trehalose and 2 mg/mL of PEG or Tween 20 was spray dried or spray freeze-dried as described in the Materials and Methods section. The powders were then emulsified in either ethyl acetate or methylene chloride.

For solid-in-oil-water microencapsulation processes, the particles in the solid protein phase must be significantly smaller (<5 μm) than the final microspheres (~50 μm) to assure efficient encapsulation of the protein and minimize the initial release of protein. Homogenization of the lyophilized protein powders in the organic solvent usually resulted in protein particle on the order of 10 μm or greater. To generate small solid particles of protein, milling and spray drying methods were employed. The milling process generates high shear forces and heat that may denature or destabilize the protein. After milling and subsequent treatment in methylene chloride, the recovery of soluble monomeric rhGH was significantly decreased for the mannitol formulated protein (Table IV). The recovery of soluble protein was unchanged for rhGH formulated in phosphate buffer with and without milling. The phosphate buffer stabilized rhGH under these conditions as compared to the poor recovery from milled excipient- and buffer-free rhGH (30.1%). As discussed previously, the spray drying of rhGH required the use of a surfactant to protect the protein from denaturation at the air-water interface during the drying process (24). Therefore, the best organic solvent stabilizer, trehalose, was combined with Tween 20 or PEG for spray drying. Both the trehalose/Tween 20 and trehalose/PEG spray dried formulations yielded nearly complete recovery of soluble monomeric rhGH after emulsification in ethyl acetate. The methylene chloride emulsification of these same formulations, however, resulted in a very poor recovery, and these results further confirm the previous observation that surfactants facilitate the denaturation of rhGH in methylene chloride. Since spray drying requires the use of elevated temperatures that may denature proteins (24), the same protein formulations were spray freeze-dried. The yield of soluble monomeric rhGH for the spray freeze-dried solids was comparable to the recovery for the spray dried solids. These studies further demonstrated the requirement for a stabilizing excipient (trehalose or mannitol), and the detrimental effect of surfactants on rhGH emulsification in methylene chloride.

Another important property of solid protein formulations for microencapsulation is their propensity to partition between the aqueous and organic phases. In particular, for a solid-in-oil-in-water process, the protein must stay well dispersed in the oil phase (e.g. methylene chloride) to become homogeneously and completely encapsulated within the microspheres. To test this property of the rhGH lyophilized formulations, we homogenized the solid protein in methylene chloride and then added an equal volume of distilled water to the containers. The containers were inverted twice to allow contacting of the two phases. Each container was then centrifuged for 10 to 15 minutes at 3500 rpm to facilitate phase separation (more dense methylene chloride phase settles to the bottom). The excipient-free solid rhGH and the phosphate buffered rhGH partition to the interface between the water and methylene chloride. If the partitioning were based upon the solid density or buoyancy, one would expect the solid to be driven to the bottom of the container due to the gravitational force. However, rhGH can act as a surfactant and it is likely that the surfactant properties or rhGH are contributing to this phenomenon. In contrast, the rhGH formulations containing mannitol or trehalose both remain homogeneously suspended in the methylene chloride or settle to the bottom of the container. This partitioning is analogous to the processing step during the addition of the solid-oil suspension (protein in methylene chloride with polymer) to the PVA/water solution to form a solid-in-oil-in-water emulsion. During microsphere formation in this process step, the protein can either migrate with the water-organic solvent interface yielding protein at the surface of the microspheres or remain dispersed in the microspheres resulting in a homogeneous encapsulation of the protein in the microspheres. These results suggest that microencapsulation of excipient-free or phosphate buffered solid rhGH will result in a low protein loading and a high initial burst due to the amount of protein on the surface of the microspheres, where the water-oil interface is located during encapsulation. Thus, mannitol and trehalose not only stabilize the protein, but they also allow the protein to remain suspended in the organic solvent during processing.

The method used to produce the solid protein can also impact its distribution between the oil and water phases. In particular, spray-drying rhGH often resulted in the formation of hollow spheres which entrapped air. These protein particles were very buoyant due to the entrapped air and tended to migrate to the oil-water interface. The spray freeze-dried protein particles, in contrast, were fine 'snow flake' structures that remained homogeneously dispersed in the organic solvents. The milled protein powder also remained in the organic solvent or settled to the bottom as the result of their higher density caused by compaction of the solid protein. The spray freeze-dried and milled protein powders that remained well dispersed in methylene chloride contained either mannitol or trehalose as the stabilizer.

Microencapsulation of Proteins in PLGA Microspheres

The methods utilized above to screen formulations were next compared to the actual encapsulation of the proteins in PLGA microspheres. The formulated protein (solid or liquid) was homogenized in methylene chloride containing PLGA to form the first emulsion (aqueous protein) or a suspension (solid protein). This solution was then mixed with 6% PVA in water resulting in a water-in-oil-in-water emulsion (aqueous protein phase) or a solid-in-oil-in-water solution (solid protein phase). The resulting microspheres were hardened through the addition of excess water, filtered to remove fine particles (20 μm mesh), and then dried by purging air through an Amicon stirred cell containing a 20 μm mesh. The dried microspheres were then tested for protein loading and protein release at physiological conditions (see Materials and Methods section).

The encapsulation of rhGH in PLGA microspheres was performed with aqueous protein solutions containing mannitol, mannitol/CMC, CMC, gelatin, or trehalose. The maximum solubility of rhGH in each formulation was used in the first emulsion (Table V). Since the protein can undergo physical and chemical changes during extended storage at physiological conditions (37°C) and protein extraction from the microspheres often induces artifacts due to the additional processing, we focused on the stability of the rhGH released initially (24 hrs) from the microspheres. This released protein was generally found to be indicative of the quality of the protein released at later time-points and the remaining protein entrapped within the PLGA microspheres since this protein is exposed to the organic solvent throughout the encapsulation process (unpublished results).

Table V. Microencapsulation and Release of rhGH from PLGA Microspheres

Formulation	% Monomer[a]	[rhGH][b] (mg/mL)	[Excipient]:[rhGH] (mass ratio)
Aqueous rhGH[c]			
Mannitol	95	200	1:1
Mannitol/CMC	76	<100	1:4 for each
CMC	66	<100	1:20
Gelatin	67	<100	1:20
Trehalose	98	400	1:4
Solid rhGH[d]			
Trehalose	96		1:4
Mannitol	100		1:1
Ammonium Bicarb[e]	47		n/a[f]
Isoelectric Precipitate of rhGH			
0.1 M NH_4Acetate[g] 100 mg/mL mannitol	83		1:2
0.1 M NH_4Acetate[h] 100 mg/mL mannitol	80		1:2

[a] % Monomer is the area percentage of the peak representing monomeric rhGH as measured by SEC HPLC.

[b] The maximum rhGH concentration that could be achieved in each formulation was used in the first emulsion. The formulations containing CMC and gelatin were very viscous and could not be accurately dispensed to allow dilution for protein concentration measurements.

[c] The lyophilized formulations, which was dried at 10 mg/mL rhGH in 5 mM potassium phosphate, pH 8 with each excipient, were reconstituted with 5 mM sodium phosphate, pH 8, and used in the first emulsion.

[d] Solid rhGH was lyophilized at 10 mg/mL rhGH in 5 mM potassium phosphate, pH 8 with each excipient. The lyophilized powder was homogenized with methylene chloride to form the solid-in-oil suspension.

[e] Excipient-free rhGH (lyophilized in ammonium bicarbonate) was air-jet milled and homogenized with methylene chloride to form a solid-in-oil suspension.

[f] n/a = not applicable; This formulation was excipient and buffer-free.

[g] rhGH isoelectrically precipitated in 0.1 M 0.1 M NH_4Acetate and 100 mg/mL mannitol was lyophilized as a concentrated suspension. The solid protein was then homogenized in the methylene chloride/polymer solution.

[h] Lyophilized rhGH from the isoelectric precipitation was air-jet milled and then encapsulated in PLGA by homogenization in the methylene chloride/polymer solution.

Again, both the mannitol and trehalose formulations were the best excipients for stabilizing rhGH. The initial protein released for microspheres made with these formulations was >95% monomeric and was fully bioactive in the *in vitro* cell-based assay. The addition of CMC or gelatin to increase the viscosity of the protein solution in the first emulsion resulted in a greatly reduced protein solubility and stability.

Similar results were also observed for the encapsulation of solid rhGH in the PLGA microspheres. Protein released from the microspheres made with excipient-free rhGH was aggregated and yielded <50% monomeric rhGH. The microspheres prepared with either the trehalose or mannitol formulated solid rhGH released monomeric rhGH that was fully bioactive. The rhGH isoelectrically precipitated with 100 mg/mL mannitol and ammonium acetate was also lyophilized and encapsulated in the PLGA microspheres. Either the lyophilized solid or the milled lyophilized solid protein was homogenized in the methylene chloride/polymer solution. The protein initially released from these microspheres was approximately 80% monomer, and these microspheres exhibited a very large initial burst (>60% of total protein) indicating that this formulation may partition to the water-oil interface during the formation of the microspheres. The results of these encapsulation studies paralleled the screening results, validating the utility of preformulation screening to select stable protein formulations for microencapsulation.

Further verification of the screening results were obtained for encapsulation of rhIFN-γ in PLGA microspheres. An aqueous solution of rhIFN-γ (80 mg/mL) in 5 mM succinate buffer, pH 5 with 100 mg/mL trehalose was used in the first emulsion. The final microspheres were incubated at physiological conditions. The protein released from these microspheres was >95% monomer by SDS-size exclusion chromatography, >90% dimer by native size exclusion chromatography, and completely bioactive (1.6×10^7 IU/mg). Thus, trehalose prevented denaturation of rhIFN-γ under the conditions used for microencapsulation.

CONCLUSIONS

Excipients were required to stabilize rhGH and rhIFN-γ during microencapsulation. The stabilizing excipients were not identical to those used to stabilize these proteins against other stresses (e.g. agitation). For example, Tween 20 stabilizes both proteins against agitation (25,26), but its use in microencapsulation resulted in a significant loss of soluble protein and did not enhance protein solubility. Protein stabilization was however achieved with sugars, mannitol and trehalose, both of which may interact with the aqueous protein. Previous studies with sugars and proteins demonstrated that sugars cause preferential hydration of proteins, resulting in stabilization of the compact native state (27). The formation of a hydration layer around the compact protein may reduce the protein - organic solvent interactions preventing protein denaturation.

A similar analysis may be applied to the isoelectric precipitates. The protein in its neutral state flocculates to form large noncovalent 'native-like' aggregates and mannitol, which caused preferential hydration of the precipitate, further stabilized these aggregates during emulsification in organic solvents.

Even the dry protein powders of rhGH, which yield native rhGH upon reconstitution, must contain a stabilizing excipient to prevent denaturation during microencapsulation. Stability of the dry protein in organic solvents also was increased through the addition of the polyhydric alcohol, mannitol, or the sugar, trehalose. Previous work by Carpenter and others indicated that sugars such as trehalose may stabilize proteins in the dry state through hydrogen bonding (i.e. water substitution (28–30)). If the sugar covers the protein surface in the dry state, it may prevent direct protein-solvent interactions during microencapsulation. This hypothesis may also explain why surfactants have a destabilizing effect for both the aqueous and solid protein formulation. The surfactants may increase protein-solvent contacts through direct hydrophobic interactions with both components. Previous work with rhGH

indicated stoichiometric binding with Tween 20 (15) and this binding may have contributed to the destabilizing effect of Tween 20. When used with a more polar solvent, methylene chloride, protein denaturation occurred even in the presence of a stabilizing sugar, trehalose.

The results of these formulation screening studies suggested a model for protein stabilization as shown in Figure 3. The stabilizing excipient (cosolvent) may cause preferential hydration of the protein resulting in a hydration layer over the surface of the protein. Within each aqueous droplet (Figure 3a), the protein would then be shielded from the organic solvent by both the hydrating excipient and the water layer. In the case of the dry protein, the stabilizing excipient may act as a water substitute and occupy the surface of the protein preventing direct protein-organic solvent interactions (Figure 3b). Thus, the protein would not be subjected to the harsh environment of the organic solvent during the microencapsulation process.

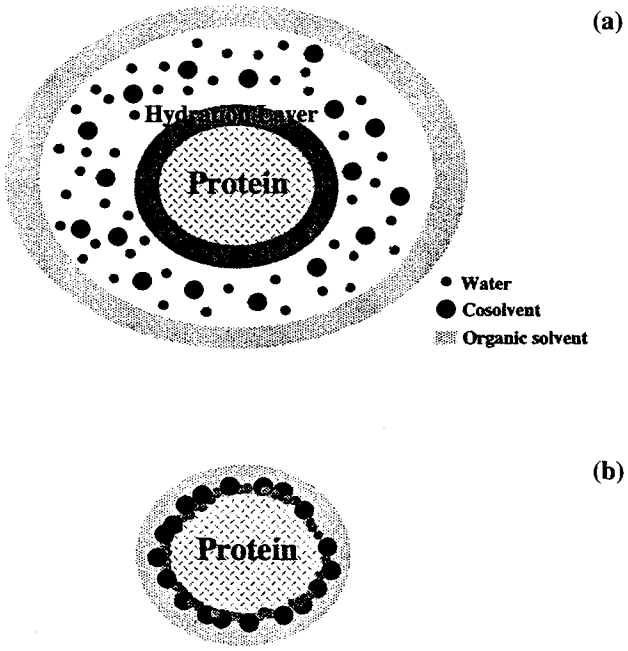

Fig. 3. Representation of the effects of excipients (cosolvents) on stabilization of proteins in organic solvents. Aqueous protein droplets (a) or solid protein particles (b) suspended in the organic solvent. As described previously, proteins are often stabilized by cosolvents (e.g., buffers, excipients, etc.) through a mechanism referred to as preferential hydration (31). Cosolvents that cause preferential hydration (e.g., sugars) alter the thermodynamics of the solution such that the protein attempts to minimize its solvent accessible surface area (minimize its chemical potential) resulting in stabilization of the compact native state (27). On the other hand, cosolvents that bind to a protein (e.g., sodium dodecyl sulfate) facilitate the formation of an extended denatured protein structure. In the presence of organic solvents, the stabilizing excipients cause preferential hydration of the protein, thereby minimizing the protein-organic solvent interactions and maintaining the protein in the compact native state. For the solid protein in the organic solvents, the excipients act as water substitutes at the protein surface preventing direct protein-organic solvent interactions that can denature the protein.

This proposed model for protein stabilization in organic solvents was also supported by the microencapsulation of these proteins (Table V). The stabilizing excipients, trehalose and mannitol, prevented denaturation of rhGH and rhIFN-γ during microencapsulation. Other excipients (e.g. dextran 70) that cause preferential hydration, but do not hydrogen bond with the protein, were not effective stabilizers. The method of formulation screening yielded similar results to the microencapsulation. Thus, this method should be employed in future microencapsulation studies.

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