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Reduced Subvisible Particle Formation in Lyophilized Intravenous Immunoglobulin Formulations Containing Polysorbate 20

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ABSTRACT

The first goal of this study was to determine the effects of the surface fraction of protein in lyophilized formulations of intravenous immunoglobulin on protein stability during long-term storage. We attempted to modulate surface fraction by either including polysorbate 20 (PS20) in the formulation or performing pre-drying annealing during lyophilization, but neither approach reduced surface fraction. Our second goal was to study the effects of formulation and processing conditions on protein aggregation and subvisible particle formation. If formulations were reconstituted immediately after lyophilization, protein aggregation detected by size exclusion chromatography was insignificant. However, with the higher resolution of damage afforded by subvisible particle analysis, it was found that high levels of particles were produced in the formulation containing trehalose and that the presence of PS20 greatly reduced particle concentrations. Size exclusion chromatography analysis showed that in formulations without trehalose during storage for 16 weeks at 50°C, there was loss of monomer and a concomitant increase in aggregates. In formulations containing trehalose there were no significant increases in aggregation or subvisible particle levels. Finally, we observed that inclusion of PS20 in the water used to reconstitute lyophilized formulations without PS20 reduced the formation of protein particles; documenting that protection by the surfactant occurred during reconstitution as well as during lyophilization.

Introduction

Traditionally, both aqueous and solid dosage forms have been employed for therapeutic protein formulations. Although development of lyophilized formulations tends to be more time-consuming and expensive than the development of liquid formulations, lyophilized formulations are often chosen for proteins whose instability in aqueous formulations restricts their shelf life.[1,2] Out of over 140 therapeutic protein drugs in the market in 2012, 64 were marketed as lyophilized products.[3]

Instabilities may still arise in lyophilized protein formulations. Protein degradation may derive from various stresses that protein molecules are exposed to during lyophilization.[4-10] During freezing, proteins may be subject to cold denaturation,[11] ice-water interfaces,[12] high salt concentrations,[4] and freezing-induced pH shifts.[13] Drying can cause unfolding of protein molecules as water is removed from their surfaces.[4,14] During long-term storage, in spite of the low translational mobility of protein molecules in glassy solids, local movement or fast dynamics (i.e., molecules independently or collectively jostling against one another on the picosecond timescale) may still result in chemical and physical degradation.[15] If unfolded or conformationally perturbed protein molecules are formed during lyophilization or storage in the dried state, aggregation may occur upon reconstitution.[16]

In addition, protein molecules could adsorb to, or be kinetically trapped at, the ice-water interface.[9,12,17-19] After sublimation of ice crystals during primary drying, these protein molecules would be found at the remaining glassy solid-air interface.[9,20,21] In 2 recent studies, the extent of damage (oxidation, deamidation, and aggregation) to 2 therapeutic proteins observed after long-term storage correlated directly with the amount of protein exposed on the glassy solid-air interface.[20,21] Annealing the frozen formulations prior to drying reduced the specific surface areas (SSAs) of the glassy solid and the fraction of protein exposed at the solid-air interface, which in turn significantly reduced degradation of the protein.[21]

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The first goal of this study was to test the hypothesis that conditions that result in lower amounts of a model antibody therapeutic on the surface of glassy solids would in turn reduce aggregation and particle formation rates during storage. Strategies can be employed on both lyophilization cycle and formulation components to vary the amount of protein on the surface. For example, a pre-drying annealing step during lyophilization can promote the growth of large ice crystals at the expense of the smaller ones and subsequently reduce the total ice-water interfacial surface area.[14,22] Non-ionic surfactants such as polysorbate 20 (PS20) could potentially compete with protein molecules for ice-water interface adsorption[9] and thus reduce the amount of protein per unit surface area.

A second goal of the study was to characterize the subvisible particle levels in reconstituted lyophilized formulations. Subvisible particle characterization is particularly valuable in detecting trace amount of protein aggregates.[23-25] Particle counting methods provide a much more sensitive means by which to detect and quantify protein aggregation than traditional methods such as size exclusion chromatography (SEC). In 2 recent examples, particle formation was observed after monoclonal antibodies were subjected to freeze-thawing[23] or pumping,[24] but no loss of monomer or formation of soluble aggregates could be detected with SEC. Limited studies have been performed on particle levels in reconstituted lyophilized protein formulations. A single published study found that the addition of sucrose and sorbitol significantly reduced the amount of subvisible particles observed in reconstituted lyophilized immunoglobulin G (IgG)1 formulations.[26]

In this study, aggregation and particle formation of intravenous immunoglobulin (IVIG) was compared in formulations with buffer alone, PS20, trehalose, or trehalose plus PS20. The specific surface areas (SSAs), the surface protein fraction, and the secondary structure of the lyophilized formulations were measured after lyophilization. To evaluate the effect of ice-water interfacial surface area, a post-freezing, pre-drying annealing step was included in the lyophilization cycle, and results were compared to those obtained when a cycle without an annealing step was used. After reconstitution, protein aggregation was monitored by SEC and subvisible particle analysis with the FlowCAM® instrument and the resonant mass measurement (RMM) instrument. Protein aggregation was also measured after reconstitution of dried formulations that had been stored at 50°C. Finally, the potential protective effect of PS20 during reconstitution was studied by analyzing aggregation after reconstituting of lyophilized IVIG formulations with water or with PS20-containing solutions.

Materials and Methods

Materials

IVIG (GAMMAGARD® LIQUID; Baxter Healthcare Corporation) was purchased from the University of Colorado at Boulder's Wardenburg Pharmacy and used before the expiration date. IVIG contains IgG 1, 2, 3, and 4 and is formulated with 0.25 mm glycine at pH 4.6-5.1.[27] High-purity α,α-trehalose dihydrate was purchased from Pfanstiehl (Waukegan, IL). All other chemicals were purchased from Fisher Scientific (Hampton, NH) and were of reagent grade or higher quality. Lyophilization glass vials (5 mL, catalog # 68000318) and butyl rubber stoppers (catalog # 19560042) were purchased from West Pharmaceutical Services (Linville, PA).

Methods

Sample Preparation

A total of 100 mg/mL IVIG in the original formulation buffer (250 mM glycine, pH 4.6-5.1) was dialyzed at 4°C against 2 mM sodium citrate solution at pH 4.6, with 3 changes in the external solution, at ~1000 times exchange volume each time. The dialyzed IVIG was then prepared at 1 mg/mL concentration in 4 different formulations: 2 mM sodium citrate; 2 mM sodium citrate and 0.03% PS20 (wt/vol); 2 mM sodium citrate and 5% (wt/vol) trehalose; and 2 mM sodium citrate, 5% (w/v) trehalose, and 0.03% PS20 (wt/vol). All formulations had a pH value of 4.6. To remove protein particles prior to experiments, the samples were centrifuged at 12,000 × *g* for 3 h with an sw28 rotor in a Beckman Optima LE-80k ultracentrifuge and then filtered through a 220 nm polyethersulfone low protein-binding syringe filter (Millipore, Billerica, MA). All formulations were prepared in at least 3 replicate vials for each sampling time point.

Lyophilization of IVIG Formulations

Samples (1 mL) were pipetted into 5 mL glass vials, and then the vials were loaded onto the shelves of an FTS LyoStar 3 lyophilizer at room temperature, following previously described cycles.[21] During the standard lyophilization cycle, the shelf temperature was initially set at 10°C, and samples were allowed for equilibration for 1 h. Then shelf temperature was reduced to −5°C at 1°C/min and held for 20 min. A second ramp of shelf temperature to −45°C at 1.3°C/min was then started. After holding the samples at −45°C for 400 min, primary drying was performed by ramping the shelf temperature to −20°C at 2.5°C/min, setting chamber pressure at 70 mTorr and holding samples under this condition for 1400 min. Then the shelf temperature was elevated to 33°C at 0.3°C/min to initiate secondary drying, and samples were maintained at 33°C and 70 mTorr for 4 h. After freeze-drying, all vials were back filled with dry nitrogen and sealed with stoppers in the chamber.

To evaluate the effect of pre-drying annealing, an annealing step was added before the primary drying of the standard lyophilization cycle. After holding the samples at −45°C for 400 min, the shelf temperature was increased to −5°C over 30 min and maintained at −5°C for 6 h. Then the shelf temperature was cooled down to −45°C at 1.3°C/min and held at this temperature for 6 h. Then the primary drying was initiated as described in the standard lyophilization cycle.

Long-Term Storage

After lyophilization, one set of samples was analyzed immediately. Other samples were stored at 50°C incubator and removed for analysis at weeks 2, 4, 8, and 16.

Reconstitution of Lyophilized Formulation

MilliQ water was used to rehydrate the lyophilized cake. Aliquots of 950 μL and 1 mL MilliQ water were pipetted into vials with or without trehalose, respectively. Then samples were allowed to sit on the lab bench for at least 30 min before analysis.

To evaluate the effect of PS20 during reconstitution, the 5% trehalose formulation was reconstituted with 950 μL MilliQ water containing 0.03% PS20.

Infrared Spectroscopic Analysis of IVIG Secondary Structure

In a nitrogen dry box, 500 mg KBr powder was mixed thoroughly with ~13 mg lyophilized cake (containing ~0.25 mg protein), ground with a pestle and mortar and then transferred to a stainless steel die (13 mm internal diameter). The die was pressed with hydraulic press (Carver Model "C", Wabash, IN) to form a pellet. The pellet was then analyzed with a Proto-3DS infrared spectrometer (Biotools, FL). For comparison, the spectrum of IVIG (20 mg/mL) in aqueous solution was also analyzed. Under single-beam transmission mode, 200 interferograms from 4000 to 1000/cm were collected and averaged. The spectra were processed with GRAMS® software for water vapor subtraction, second-derivative