Inverse Relationship of Protein Concentration and Aggregation

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Purpose. To determine the effect of protein concentration on aggregation induced through quiescent shelf-life incubation or shipping-related agitation.

Methods. All aggregation was measured by size-exclusion high-performance liquid chromatography. Aggregation was induced by time-dependent incubation under stationary conditions or by agitation caused by shaking, vortexing, or vibration using simulated shipping conditions.

Results. Protein aggregation is commonly a second- or higher-order process that is expected to increase with higher protein concentration. As expected, for three proteins (PEG-GCSF, PEG-MGDF, and OPG-Fc) that were examined, the aggregation increased with higher protein concentration if incubated in a quiescent shelf-life setting. However, aggregation decreased with higher protein concentration if induced by an air/water interface as a result of agitation. This unexpected result may be explained by the rate-limiting effect on aggregation of the air/water interface and the critical nature of the air/water interface to protein ratio that is greatest with decreased protein concentration. The non-ionic detergent polysorbate 20 enhanced the aggregation observed in the quiescently incubated sample but abrogated the aggregation induced by the air/water interface.

Conclusions. The effect of protein concentration was opposite for aggregation that resulted from quiescent shelf-life treatment compared to induction by agitation. For motionless shelf-life incubation, increased concentration of protein resulted in more aggregation. However, exposure to agitation resulted in more aggregation with decreased protein concentration. These results highlight an unexpected complexity of protein aggregation reactions.

KEY WORDS: aggregation; protein; agitation; shelf-life; polysorbate; stability.

INTRODUCTION

Aggregation is an agglomeration of proteins that frequently is irreversible when introduced into physiologic fluids, leading to inactivation or increased immunogenicity. Aggregation is a common problem with protein pharmaceuticals (1–3) and may compromise process isolation yields, limit shelf-life, cause failure in manufacturing, and prevent applications to new advances in delivery. Exposure of proteins to shear, agitation, and multiple surfaces is unavoidable and may induce aggregation. A wide range of potencies and doses demand concentrations that differ by five orders of magnitude (1 ug/mL to >100 mg/mL). The pharmaceutical scientist is challenged to stabilize a protein against this wide array of detrimental environments.

The implication of surface-induced protein aggregation was reported over 50 years ago (4–6). Research in protein aggregation over the last 20 years has shown that conformational dynamics plays a key role (7,8). The native state is least prone to undesirable aggregation whereas partial unfolded states are highly susceptible. The exposure of otherwise buried hydrophobic regions in the partial unfolded state is a reactive surface that triggers intermolecular protein attractions. Because air is more hydrophobic than water, the interface between the air and liquid is a denaturing surface in which aggregation originates (9). Situations that increase the area of this boundary, such as shipping-induced agitation or high-pressure filling lines, are particularly damaging. Strategies that favor the native conformation are helpful at preventing aggregation. Unfortunately, some of the strategies for stabilizing the native state of proteins are not compatible with parenteral applications.

Some practical approaches have empirically improved the aggregation problem for certain situations but have exacerbated aggregation for other situations. For example, the inclusion of detergents may prevent an agitation-induced aggregation but accelerate a shelf life induced aggregation (10). Often, a compromise or balance between the stabilizing and destabilizing conditions must be struck. A great need exists for the fundamental understanding of aggregation pathways and how different strategies impact the steps of these reactions.

Protein concentration is an important variable for ameliorating aggregation. The initial conformation related reaction leading to aggregation is expected to be first-order but the subsequent aggregation of nonnative states is expected to be a second- or higher-order process because the frequency of collisions varies with concentration (11). Therefore, aggregation is expected to accelerate with increased protein concentration (12). We have examined three different proteins, PEG-MGDF, PEG-GCSF, and OPG-Fc and observed that aggregation induced by agitation is inversely concentration dependent. This report documents these observations and provides a rationale for this unexpected result.

EXPERIMENTAL PROTOCOL

Materials

PEG-MGDF refers to pegylated megakaryocyte growth and development factor and has a molecular weight of the protein portion of 17,432 Da. PEG-GCSF refers to pegylated granulocyte colony stimulating factor and has a molecular weight of the protein component of 18,798 Da. Each contains a single polyethylene glycol polymer of ~20,000 Da attached at the N-terminus. OPG-Fc refers to osteoprotegerin protein fused at its C-terminus with the sequence from the Fc portion from an immunoglobulin with a combined molecular weight of ~107,000 Da. These proteins were produced at Amgen with MGDF and GCSF from heterologous expression in *Escherichia coli* and OPG-Fc fusion from heterologous expression in Chinese hampster ovary cells. Pegylation was at the α-amino group by alkylation (13). Polysorbate 20 was obtained from Mallinckrodt. Protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 0.86, 0.35, and 1.5 for PEG-GCSF, PEG-MGDF, and OPGFc, respectively, for a 1-cm pathlength and a 0.1% solution.

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All other reagents were of analytical grade and deionized double-distilled water, Milli-Q-grade, was used exclusively.

Aggregation Assay

All aggregation measurements were obtained using size-exclusion high-performance liquid chromatography (HPLC). Separations were performed using a Hewlett–Packard 1050ti HPLC system controlled by ChemStation software and a 7.5 mm × 30 cm stainless-steel TSKGEL G3000SWXL column (TosoHaas). For PEG-GCSF an isocratic elution was run at 0.5 mL/min with 100 mM sodium phosphate pH 6.4, and 10% ethanol (v/v) as the mobile phase. PEG-MGDF was run similarly but at a flow rate of 0.7 mL/min, pH 6.9, plus 0.5 M NaCl. OPG-Fc was run identically to PEG-MGDF except for the 10% ethanol in the mobile phase was omitted.

Stability Experiments

Samples were prepared by exchanging solvent conditions from the last step in purification to the formulation through diafiltration or dialysis. Samples of different protein concentration were obtained by dilution or concentration using an ultrafiltration device with the appropriate formulation buffer. Samples were sterile filtered with a 0.2-micron Gelman Acrodisc filter, filled with 1 mL of sample into 3-mL size glass vials, closed with silicon treated bromobutyl rubber stoppers, and crimp capped.

Stability samples under quiescent conditions were incubated at the appropriate temperature in an incubation oven without any movement of the vial during the duration of the stability experiment. Samples were stored in an upright configuration.

Stability samples exposed to agitation were subjected to one of the following procedures; shaking, vortexing, or vibration using simulated shipping conditions. For all agitation studies, sample vials were tightly packaged into boxes with individual dividers for each vial and the box was attached to the device for agitation. Shaking was accomplished by a Hoefer Scientific "Red Rotor" orbital shaker for approximately 400 rpm for 36–40 h at 23°C. Vortexing was accomplished by subjecting samples to high speed orbital mixing on a vortex mixer for 30 min at 23°C. Vibration using 4000 miles of simulated air transportation was accomplished in a testing lab using a specially designed vibration table. The vibration table utilized random vertical vibration with a programmed frequency range as described for Method A with a closed loop-automatic equalization by the American Society for Testing and Materials (14).

RESULTS

Direct Dependence of Protein Concentration and Aggregation of Quiescently Incubated Samples

The effect of PEG-GCSF concentration on aggregation was explored at 1, 5, and 10 mg/mL concentrations. Samples were incubated quiescently for varying times and varying temperatures. SEC-HPLC results for samples incubated at 45°C were typical of the observations and are illustrated in Figure 1. All of the material eluting earlier than the main peak was considered aggregated product. Figure 1 demonstrates that after 2 weeks of incubation at 45°C, the amount of aggregate peak increased with higher protein concentrations. Other samples incubated at different temperatures and for various times showed a similar result of increased aggregation with higher protein concentration (results not shown).

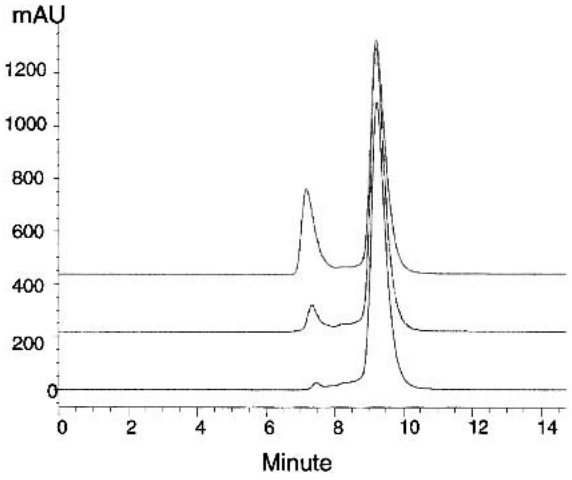

Fig. 1. Quiescent shelf-life (2 weeks) stability studies at 45°C of PEG-GCSF at varying concentrations according to SEC-HPLC. Samples were diluted to the same concentration prior to analysis. Material eluting prior to the main peak represents aggregated protein. The area of all peaks for each chromatogram was similar. The aggregation peak increased with increased concentration of protein; the top chromatogram was for 10 mg/mL, the middle chromatogram was for 5 mg/mL, and the bottom chromatogram was for 1 mg/mL. The formulation was that used for the unpegylated commercial GCSF product (NEUPOGEN®), 10 mM acetate, 5% sorbitol, pH 4.

Quiescent incubation of PEG-MGDF showed a similar result of enhanced aggregation with increased concentration. Figure 2 shows that aggregation grew over a 12-week period at 37°C and worsened as the concentration increased from 0.2 mg/mL to 2.0 mg/mL.

In a like manner, OPG-Fc demonstrated that upon quiescent incubation the aggregation was more pronounced at higher concentrations. Figure 3 demonstrates that over a 20-week incubation at 29°C, the most aggregation was observed for the 50 mg/mL sample, to a lesser extent for the 30 mg/mL sample, and the 10 mg/mL sample did not aggregate.

Inverse Dependence of Protein Concentration and Aggregation of Agitated Samples

Samples were subjected to an increased air/water interface by agitation with one of the following procedures: shaking, vortexing, or simulated shipping. Figure 4 shows the SEC-HPLC chromatograms for PEG-GCSF at different concentrations (1, 5, and 10 mg/mL) subjected to a vortex mixer. The portion of early eluting peaks demonstrated an inverse relationship with concentration, more aggregation was observed with lower concentrations. A similar relationship of increased aggregation with decreased concentration was observed for agitation associated with transportation in shipping (results not shown).

PEG-MGDF also showed (Fig. 5) that agitation by shaking increased the aggregation as the concentration decreased from 500 to 10 μg/mL. Figure 5 illustrates that samples of the

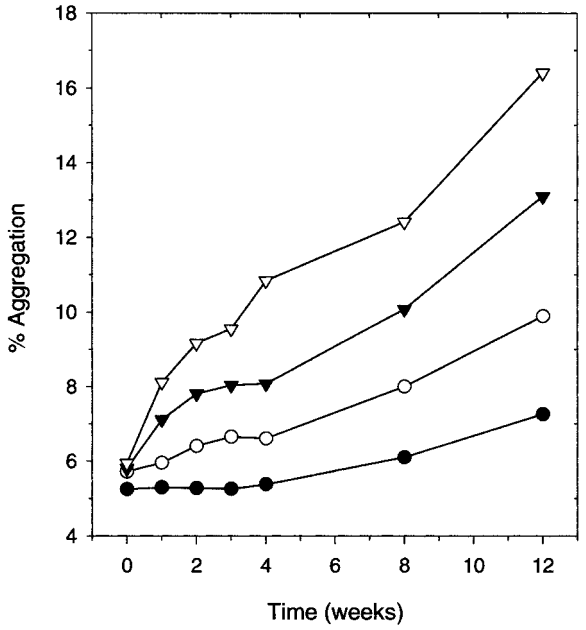

Fig. 2. Quiescent shelf-life stability studies at 37°C of PEG-MGDF at varying concentrations. The percent aggregation was determined as any peaks that eluted prior to the main peak on SEC-HPLC. The symbols represent the results for (▽) 2.0 mg/mL; (▼) 1.0 mg/mL; (○) 0.5 mg/mL; and (●) 0.2 mg/mL. The formulation was 10 mM acetate, 5% sorbitol, pH 5.

same concentrations not subjected to agitation showed no increase in aggregation.

OPG-Fc showed similar results due to agitation. Figure 6 demonstrated that agitation by vibration using simulated transportation resulted in OPG-Fc aggregation that increased

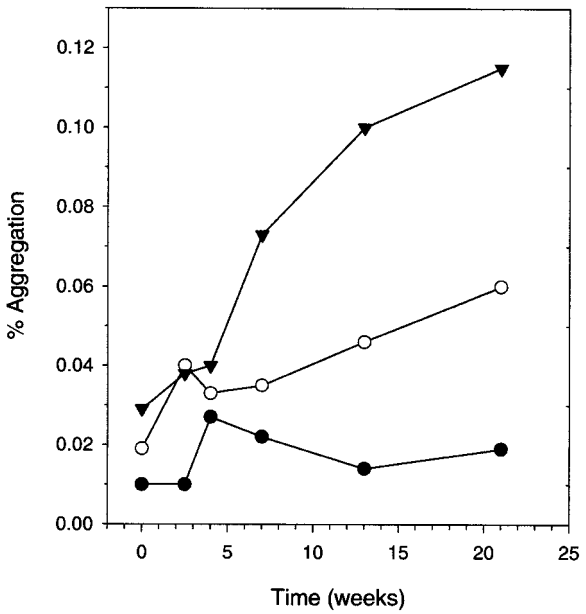

Fig. 3. Quiescent shelf-life stability studies at 29°C of OPG-Fc at varying concentrations. The percent aggregation was determined as any peaks that eluted prior to the main peak on SEC-HPLC. The symbols represent the results for (▼) 50 mg/mL; (○) 30 mg/mL; and (●) 10 mg/mL. The formulation was 10 mM acetate, 5% sorbitol, pH 5.

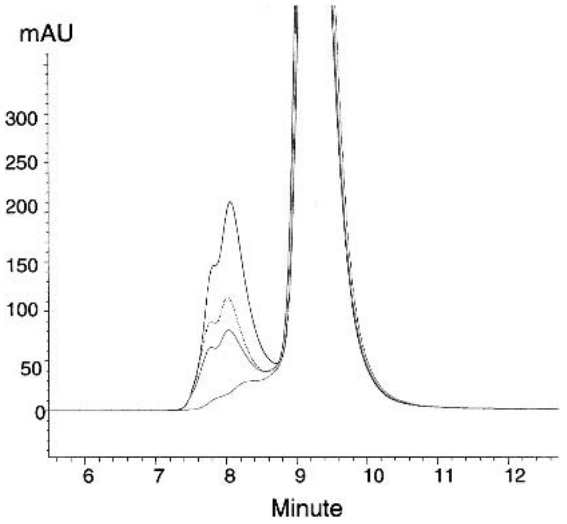

Fig. 4. Agitation-induced stability studies at 23°C of PEG-GCSF at varying concentrations according to SEC-HPLC. Samples were diluted to the same concentration prior to analysis. Material eluting before the main peak represents aggregated protein. The area corresponding to the sum of all peaks for each chromatogram was similar. The aggregation peak decreased with increased protein concentration; the chromatogram with the largest aggregate peak (top) was the 1 mg/mL sample, followed by the 5 mg/mL sample, then the 10 mg/mL sample, and the chromatogram with the least aggregate (bottom) was a 1 mg/mL sample not subjected to agitation. Agitation was induced by a vortex mixer. The formulation was the same as Figure 1.

as the concentration decreased from 30 to 1 mg/mL. Control samples (stationary) did not aggregate.

Opposite Effects of Polysorbate 20 on Quiescent and Agitated Induced Aggregation

The effect of polysorbate 20 concentration on the aggregation observed by quiescent incubation at 29°C and agitation induced by vibration using simulated transportation is illustrated in Table I. Analysis by SEC-HPLC showed that aggregation increased with higher concentrations of polysorbate 20 for the quiescent samples. Whereas, increased concentrations of polysorbate abrogated the aggregation induced by agitation associated with transportation in shipping.

DISCUSSION

Explanation for the Inverse Relationship of Protein Concentration and Aggregation

The unexpected result from this work is that agitation-induced aggregation was accelerated at lower protein concentrations. Agitation is commonly thought to cause aggregation of proteins by increasing the air/water interface area (15–19). The air/water interface forms a denaturing boundary between the hydrophobic air and hydrophilic water. Folded proteins are amphipathic with the hydrophobic groups buried into the interior of the protein and the hydrophilic groups exposed to the aqueous medium. Protein denaturation at air/water interfaces results from partial unfolding that exposes hydrophobic protein surfaces to the air. Aggregation then results from

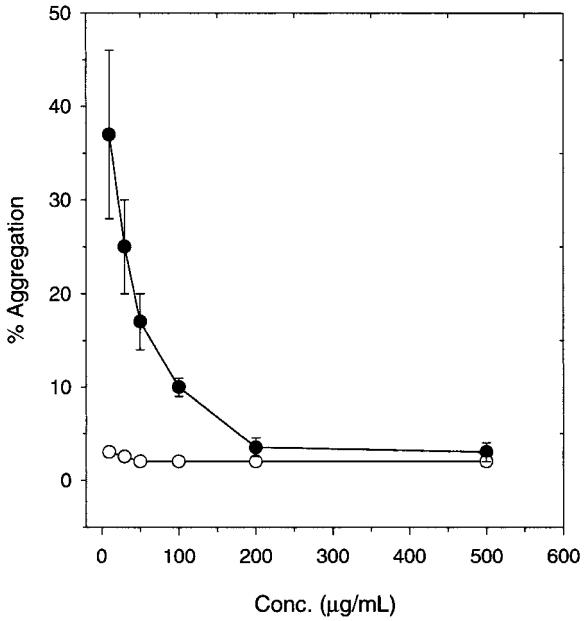

Fig. 5. Agitation-induced stability studies at 23°C of PEG-MGDF at varying concentrations. The percent aggregation was determined as any peaks that eluted prior to the main peak on SEC-HPLC. The symbols represent the results for (●) agitated samples, and (○) for stationary samples. Agitation was induced by shaking on an orbital shaker. The error bars represent the standard deviation for repeated measurements of 10 separate samples. The formulation was the same as Figure 2.

self-association through the exposed intermolecular hydrophobic interactions with other similar protein molecules. The rate of agitation-induced aggregation is expected to be the direct product of the concentration of the protein and the

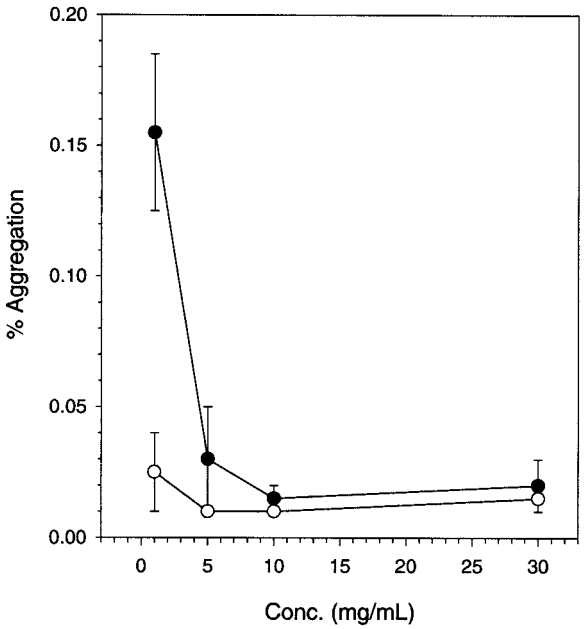

Fig. 6. Agitation-induced stability studies at 23°C of OPG-Fc at varying concentrations. The percent aggregation was determined as any peaks that eluted before the main peak on SEC-HPLC. The symbols represent the results for (●) agitated samples and (○) for stationary samples. Agitation was induced by vibration on a simulated shipping table. The error bars are ± variation of repeated measurements of 3 separate samples. The formulation was the same as Figure 3.

Table I. Effect of Polysorbate Concentration on Aggregation of PEG-GCSF (1 mg/mL)

% polysorbate concentration	Quiescent % aggregation after 59 weeks @ 29°C	With agitation[a] % aggregation (SD[b])
0	1.2	5.7 (1.4)
0.001	1.8	3.5 (0.7)
0.0025	2.5	2.4 (0.1)
0.004	2.7	2.2 (0.1)
0.007	3.5	2.2 (0.1)
0.01	5.9	2.2 (0.1)

[a] Agitation was accomplished by subjecting samples to 4000 miles of simulated air transport.
[b] Determined from four separate samples.

surface area of the air/water interface. However, our results for three different proteins showed (Figs. 4–6) the opposite effect with respect to protein concentration. We suggest, for agitation-induced aggregation reactions, the air/water interface is the rate-limiting reagent and the ratio of the air/water interface to protein is critical. For situations where the percentage of aggregation is measured, a high ratio of air/water interface to protein will result in accelerated aggregation reactions. Therefore, if the agitation rate is held constant and the protein concentration is decreased the ratio of air/water interface is increased which results in higher amounts of aggregation.

For the aggregation observed for three different proteins under quiescent conditions (Figs. 1–3) the rate of aggregation was directly dependent on the protein concentration. We suggest, that under quiescent conditions of storage the rate limiting reaction for aggregation is the productive collisional frequency of two protein molecules in solution. Such a reaction would be expected to be accelerated by protein concentration.

Explanation for the Polysorbate Effect

Polysorbate is a nonionic detergent that is known to concentrate at the boundary of the air/water interfaces. Polysorbate decreases surface tension measurements for aqueous solutions due to the enrichment of polysorbate at the air/water interface (20–22). Electron spectroscopy for chemical analysis (ESCA) was used to probe the elemental composition of spray dried powders of proteins in the presence and absence of polysorbate and showed that proteins preferentially concentrated at the particle surface but were displaced by polysorbate for contact with the surface (23). Using specular neutron reflection coupled with H/D labeling, protein and detergent showed a significant enhancement at the air/water surface interface of aqueous solutions (24). These studies suggest that polysorbate should ameliorate the air/water interface induced aggregation of proteins. Table I shows that for PEG-GCSF, polysorbate eliminated the aggregation induced by agitation associated with shipping. These results are adequately explained by the preferential surface activity of polysorbate resulting in a lowering of the surface tension and denaturing potential of the air/water interface. Consistent with these results, nonpegylated GCSF has been shown to be surface active, susceptible to aggregation induced by an air/water interface, and quenched by polysorbate (21).

Under quiescent shelf life studies, polysorbate has an opposite or detrimental effect on aggregation. Similar detrimen-

tal effects upon storage at elevated temperatures resulting from polysorbate have been reported (25). Table I showed that higher polysorbate concentration increased the amount of aggregate after incubation for 59 weeks at 29°C. For quiescent shelf-life studies the rate of aggregation is proportional to the productive collisional frequency of protein molecules rather than contact with the air/water interface as in the agitation-induced aggregation. Polysorbate must increase the productivity of collisional frequencies that lead to aggregation. We have no specific information regarding the interaction of polysorbate with PEG-GCSF. However, if the presence of polysorbate lowered the free energy of folding and caused an increase in the population of partially unfolded species, then increased aggregation would be expected. A related protein from the same family of four-helix bundle structures, human and porcine growth hormone, has been shown to aggregate and precipitate due to exposure to an air/water interface (19,26–29). The general mechanism for growth hormone aggregation is the hydrophobic interaction between partially unfolded states (29). Polysorbate is capable of binding to the intermediate state and preventing aggregation (30,31). Polysorbate was not effective at preventing the putative intermolecular interactions between partially unfolded PEG-GCSF molecules in quiescent shelf-life studies (Table I). The detrimental effect of enhancing aggregation was greater than the protective effect of preventing aggregation.

Previous work on the effects of agitation and interfacial interactions with insulin has been reported (32). The anomalous behavior of increased aggregation due to agitation-induced precipitation with decreased insulin concentration was observed. However, a different explanation for the inverse relationship of protein concentration and aggregation was provided (32). In aqueous solution, insulin exists in an equilibrium of monomer and multimers (principally hexamers in the presence of zinc). The hexamer's conformational stability makes it unlikely to denature at hydrophobic surfaces while the monomer is more susceptible to denaturation. Thus, decreased insulin concentrations increased the content of monomer in solution and resulted in an increased precipitation due to agitation. Consistent with this interpretation, removal of zinc and adding 60% ethanol eliminated insulin's self-association and the agitation-induced aggregation rates increased with increased concentration. Polysorbate or generic reduction of surface tension was not effective at reducing the agitation-induced aggregation of insulin. Other smaller surfactant molecules were effective, but the mechanism was not elucidated (33). The three proteins reported in our work are monomers and do not undergo the self-association reactions of insulin and a different phenomenon is responsible for the inverse relationship of protein concentration and aggregation.

The agitation-induced aggregation work described here uses simulated shipping, shaking, or a vortex mixer to enhance the air/water interface. Other environments that increase the exposure to denaturing interfaces or surfaces for pharmaceutically important proteins are freezing, thawing, lyophilization and spray drying, high pressure fill lines, filtration, and a myriad of other manufacturing circumstances. Those processes that increase the exposure of proteins to air/water interfaces might be expected to show the inverse relationship of concentration and aggregation as well as amelioration by polysorbate.

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