ELSEVIER

International Journal of Pharmaceutics 127 (1996) 191–201

international journal of pharmaceutics

Protein nebulization II. Stabilization of G-CSF to air-jet nebulization and the role of protectants

Ralph W. Niven[a,*], Steven J. Prestrelski[1b], Michael J. Treuheit[a], Anna Y. Ip[a], Tsutomu Arakawa[b]

[a]*Department of Pharmaceutics and Drug Delivery, Amgen Inc., Thousand Oaks, CA 91320, USA*
[b]*Department of Protein Chemistry, Amgen Inc., Thousand Oaks, CA 91320, USA*

Received 20 April 1995; revised 16 June 1995; accepted 19 June 1995

Abstract

Air-jet nebulization induces aggregation and degradation of granulocyte-colony stimulating factor (G-CSF). The air-water interface as a site for destabilization of G-CSF is investigated and possible mechanisms of stabilization by polyethylene glycol (PEG) and Tween 80 are proposed. Equilibrium surface tension measurements show that G-CSF is highly surface-active, but dynamic surface tension measurements made using a maximum bubble pressure surface tensionometer show that G-CSF and surfactants have little surface activity in the interval between formation and destruction of aerosol generated within a nebulizer. Bubbling of air through solutions of 4 mg/ml G-CSF for up to 1 h resulted in $\approx$ 10% aggregation of total protein suggesting that destabilization at the air-water interface occurs. Polyethylene glycol (PEG) and Tween 80 are effective in reducing aggregation and degradation of G-CSF but their respective modes of protection differ. Protection is improved by increasing the PEG molecular weight and concentration indicating that PEG may influence stability through steric effects. In contrast, the protective effects of Tween 80 appear to relate to the micellar properties and not to the surface activity of the surfactant.

Keywords: Aggregation; Degradation; G-CSF; Nebulization; Protein; Pulmonary delivery; Stability; Surface tension

1. Introduction

Methods for pulmonary delivery of proteins include propellant metered-dose inhalers, dry-powder inhalers and nebulizers (Patton and Platz, 1992; O'Hagan and Illum, 1990). The nebulizer is a logical first choice for development of a protein for pulmonary delivery (Aitken et al., 1992; Vogelmeier et al., 1990; Hubbard et al., 1989; Shak et al., 1990) as most recombinant proteins are purified and stored as an aqueous concentrate as opposed to a bulk powder. Thus, the translation of a formulation for nebulizer use would, at first glance, appear to be a more readily accomplished task than the development of a powder or suspension formulation. Unfortunately, stability of aqueous solutions on the shelf may not translate to stability during nebulization and the

* Corresponding author. Current address: Megabios Corp., Burlingame, CA 94010, USA.
[1] Current address: Alza Corp., Palo Alto, CA 94303, USA.

SSDI 0378-5173(95)04209-S

protein may be denatured by several mechanisms including drying, shear and surface effects (Charm and Wong, 1970; Andrews, 1991).

It was shown that air-jet nebulization induces the loss of enzymatic activity of lactate dehydrogenase (LDH) and results in aggregation and degradation of recombinant human granulocyte-stimulating factor (G-CSF) (Niven et al., 1994). The addition of polyethylene glycol (PEG) had a marked stabilizing effect on these proteins but the mechanisms involved in the denaturation of these proteins have not been clarified. In this study, the nature of G-CSF aggregation is further examined and the 'protective' effects of Tween 80 and different molecular weights of PEG are evaluated. The results are discussed with reference to the air-water interface generating characteristics of the nebulizer.

2. Materials and methods

2.1. Protein analysis

Recombinant human N-methionylated G-CSF bulk solution (Lot nos. T6803 and 4) was obtained from Amgen manufacturing at a concentration of 4 mg/ml in 1 mM HCl. The protein was in a highly purified monomeric form (99%) as determined from various analytical methods including reverse-phase high-performance liquid chromatography (HPLC) and size exclusion chromatography (SEC). Gel electrophoresis in the absence and presence of sodium dodecylsulfate (SDS and native PAGE) was carried out as described previously (Niven et al., 1994). SEC was carried out using a Waters (Milford, MA) HPLC system, a Biosep SEC 2000 column (250 × 7.5 mm, 7 μm particle size) and 0.1 M sodium phosphate, pH 6.9, as a mobile phase. Samples were placed in a refrigerated autosampler (4°C) (WISP 717+, Waters, Milford, MA) before injection. The flow rate was set at 1.0 ml/min and absorbance of eluent was monitored at 280 nm.

2.2. Nebulization

Nebulization was performed using the same procedures outlined previously (Niven et al., 1994). Briefly, G-CSF solutions were aerosolized using a 316 stainlesss steel, Collison 3-jet nebulizer. A starting volume of 10 ml of protein solution in 1 mM HCl was placed in the nebulizer reservoir, the inlet air pressure was fixed at 40 psig (10.0 l/min) and the nebulizer was operated for 10 min unless otherwise stated. The temperature of solutions during nebulization was monitored via a temperature probe (8402-20, Cole-Parmer Inst. Co., Niles, IL). In these studies, the output was not examined and samples were removed from the nebulizer reservoir at set times for further analysis.

2.2.1. Reversibility of aggregation and degradation

To study the possibility of reversibility, nebulization was halted after 10 min and samples of the remaining reservoir fluid were incubated and analyzed for aggregation and degradation. A 100-μl sample was immediately subjected to SEC analysis as a control. Samples of the remaining fluid were split into three groups and stored at −70, 4 and room temperature (22°C). An additional group was maintained at room temperature, but an aliquot of 50% w/v PEG1000 solution was added to the samples to produce a final concentration of 1% w/v PEG. Samples were removed from the storage conditions at intervals over the next 3 days and examined by SEC to monitor any changes in the fraction of aggregate in the nebulized solution.

2.2.2. Effect of G-CSF concentration

The concentration dependence of aggregate formation was studied by varying the initial concentration of the protein from 0.1 to 4 mg/ml in 1 mM HCl.

2.2.3. Effect of air-pressure

The effects of varying the applied air-pressure from 10 through 60 psig was evaluated to determine if the extent of aggregation would be influenced by the atomization conditions. That is,

increasing the air pressure increases the rate at which fluid is cycled within the nebulizer and should increase the available energy to atomize the fluid upon each passage through the nebulizer jets.

2.2.4. Effect of solution temperature

Since evaporative cooling occurs during nebulization, it is possible that the nature and extent of aggregation could be influenced by the changing solution temperature. To assess this possibility, G-CSF at 4 mg/ml was nebulized in a water-jacketed reservoir (Ip and Niven, 1994) held at 4, 10 and 22°C.

2.2.5. Effect of additives

Certain excipients when added to protein solutions can exert a protective effect on the proteins during nebulization as observed previously with PEG1000 (Niven et al., 1994). Further experiments were performed using additional PEG of varying size (PEG8000, PEG1000 and PEG400, Carbowax, Union Carbide), polyoxyethylene sorbitan monooleate (Tween 80, Mol. Wt 1310) and two amphiphilic surfactants, Zwittergent 3-08 (Mol. Wt 280) and 3-16 (Mol. Wt 392), (Calbiochem, La Jolla, CA). The concentration of rhG-CSF in the starting solutions was always 4 mg/ml in a 1 mM HCl buffer.

2.3. Surface area generation

To determine if the air-water interface was involved in the aggregation of G-CSF two types of experiment were performed.

(1) Air at 0.5 psig was bubbled through 10 ml of 4 mg/ml G-CSF solution via a 0.45-μm HPLC degassing frit for a period of 1 h. Samples were removed during this period and analyzed by SEC. Thus significant air-water interface was generated in a different manner to the air-water interface generated during nebulization.

(2) To avoid the production of any air-water interface during 'nebulization', the liquid feed inlets of the nebulizer were connected to an external reservoir from which fluid was pumped through the atomizer jets at a rate ($\approx$ 300 ml/min) normally generated by the expanding air-pressure (Niven and Brain, 1994). Additionally, the nebulizer jets were submerged in a solution of G-CSF that was continuously pumped back to the external reservoir. The net result was that fluid forced through the nebulizer jets did not produce any air-water interface. A total of 200 ml of solution was used and samples were removed during a 1-h experiment and analyzed by SEC.

2.4. Surface tension

2.4.1. Equilibrium surface tension

The surface tension of aqueous solutions was measured using the ring method of Denuoy (Leukenheimer and Wantke, 1981) or a Wilhemy plate coupled to a Krüss K10T surface tensiometer (Krüss Co., Hamburg, Germany). All measurements were made at 20°C. Samples were allowed to equilibrate for at least 1 h prior to measurement. Values were adjusted for density as described by Harkins and Jordan (Harkins and Jordan, 1930). The surface tension of pure water was measured before and after all measurements. If changes in the surface tension of pure water were noted after completing a measurement with a protein solution, this would indicate that protein had deposited on the Wilhemy plate and was therefore influencing results. In the case of G-CSF this did not occur.

2.4.2. Dynamic surface tension

To provide additional data on the influence of 'protectants' during the formation of aerosol within the nebulizer, the dynamic surface tension of G-CSF, aggregated G-CSF, Tween 80, and PEG solutions was measured using a Krüss BP2 bubble pressure surface tensionometer. The surface tension of the additives was measured with and without G-CSF in the time range of 250 ms to 2 s at 22°C.

3. Results

3.1. Nebulization

3.1.1. Reversibility of aggregation and degradation

When G-CSF is subjected to air-jet nebuliza

Fig. 1. Native gel of isolated aggregate and monomeric peaks. Lanes 1 and 2 correspond to the aggregate and monomeric peaks, respectively, obtained after 4–10 min nebulization of 1 and 4 mg/ml G-CSF at 40 psig as indicated in the figure.

tion, two types of damage are observed; aggregation and degradation (Niven et al., 1994). The aggregate, which is predominantly a self-associated dimer, was detected by a peak eluting before the native monomer using SEC, and degradation was observed after native gel electrophoresis as a band migrating faster than the native protein. The aggregation observed by SEC should cause slow-mobility bands (possibly with smearing) on the native gel. The absence of such bands suggests that the aggregation is reversible in the conditions used to run the native gel (Niven et al., 1994). There is also the suggestion that the dissociated form of the aggregate may correspond with the degraded form. This was tested by collecting the aggregate fraction eluting from SEC and subjecting it to native gel analysis. The aggregated sample consists only of degraded protein and the monomer remaining after nebulization has not been degraded (Fig. 1). Further analysis by peptide mapping and mass spectrometry (details not given) showed no resolvable differences between the degraded protein and the starting material, lending support to the contention that the degraded G-CSF was deamidated. Reversibility of aggregation was also tested by SEC. Incubation at room temperature has no effect on the amount of degradation (data not shown). Conversely, room temperature incubation exhibits dramatic effects on the amount of aggregation. At −70 and 4°C, there is negligible loss of aggregate over the length of the study period of 110 h (Fig. 2). However, samples maintained at room temperature over this time exhibit a progressive loss of aggregate and a corresponding increase in absolute quantity of monomer eluting from the column. These results illustrate the instability of the nebulized aggregate and confirm that the aggregation process is re-

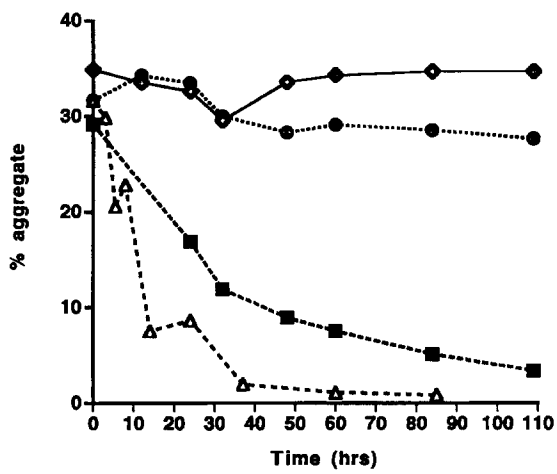

Fig. 2. The change in the aggregate fraction of nebulized samples after incubation at various temperatures. The aggregate reversed to monomer at room temperature and this process was accelerated in the presence of the PEG1000. (◆) −70°C; (●) 4°C; (■), 22°C; (△) 22°C + 1% w/v PEG1000 ($n = 1$).

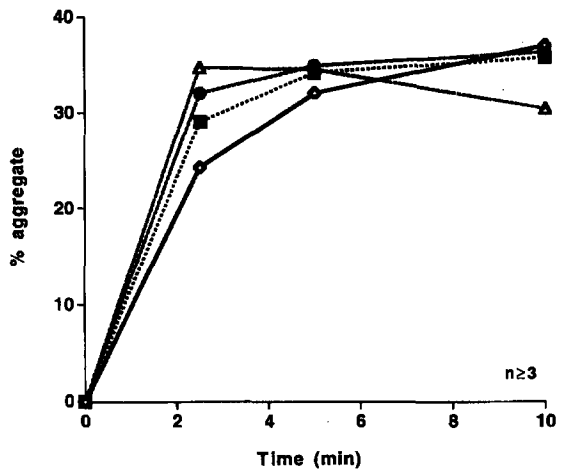

Fig. 3. Concentration dependence of G-CSF aggregation during nebulization. The ordinate represents the fraction of G-CSF which has aggregated after a period of nebulization. (△), 4 mg/ml; (●), 1 mg/ml, (■), 0.4 mg/ml, (◆), 0.1 mg/ml ($n > 3$). Error bars are not shown for clarity.

versible. This is supported by the SDS-PAGE results which show no evidence of covalently-linked aggregates. This observation is also technically important, as it shows that the nebulized samples must be stored at or below 4°C before SEC analysis. Reversibility was not observed in phosphate-buffered saline (PBS). The aggregate generated by nebulization also remains unchanged when the nebulized sample is diluted into PBS and incubated in PBS at room temperature.

3.1.2. Effect of G-CSF concentration

Fig. 3 shows the effect of G-CSF concentration on the formation of aggregate during nebulization as monitored by SEC. There is little effect of concentration in the range studied, although, at the lowest concentration of G-CSF, a smaller fraction of aggregate may be formed during nebulization. G-CSF was also air-jet nebulized at the same G-CSF concentrations for chemical degradation analysis using native gel electrophoresis. In this case, only the 10- min nebulized samples were analyzed as summarized in Table 1. These results also show slight differences in the amount of degraded product.

3.1.3. Effect of air-pressure

An increase in the air pressure to generate aerosol might be expected to increase the extent of G-CSF aggregation as more energy is being introduced to the system during nebulization. The net result is a reduced droplet size and an increased aspiration rate (a greater cycling rate) (Niven and Brain, 1994). However, the air-pressure does not increase the generation of G-CSF dimer during nebulization. After 20 min, the percentage of aggregate has reached a plateau at each tested air pressure giving values of: 29.2 ± 9.3 at 10 psig; 32.3 ± 1.0 at 20 psig; 36.5 ± 3.0 at 40 psig; and 33.1 ± 6.3 at 60 psig (mean ± S.D.; $n \geq 3$).

3.1.4. Effect of solution temperature

Based upon the results found for the influence of storage temperature on the stability of aggregate, it was thought that the changing temperature of solutions during nebulization might influence the aggregation profiles. This was not the case and the aggregation vs. time curves of solutions nebulized at 4, 10 and 22°C were similar to curves obtained without temperature control.

3.1.5. Effects of additives

The effect of PEG molecular weight on G-CSF stabilization during aerosolization was examined using molecular weights of 400, 1000 and 8000 at concentrations of 0.001 and 1.0% w/v. The results are shown in Fig. 4. At 0.001% w/v the stabilization effects of PEG are marginal, and there is no apparent relationship between the PEG molecular weight and protein stability (Fig. 4A). In contrast, a strong molecular weight dependence is observed at a PEG concentration of 1% w/v (Fig. 4B).

Table 1
G-CSF degradation after 10 min nebulization

G-CSF concentration (mg/ml)	Degradation (%) (standard error)[a]
0.4	32 (± 2)
1.0	36 (± 1)
4.0	40 (± 1)

[a]The extent of degradation as estimated from densinometric scans of Coomassie Blue-stained native gels ($n = 3$)

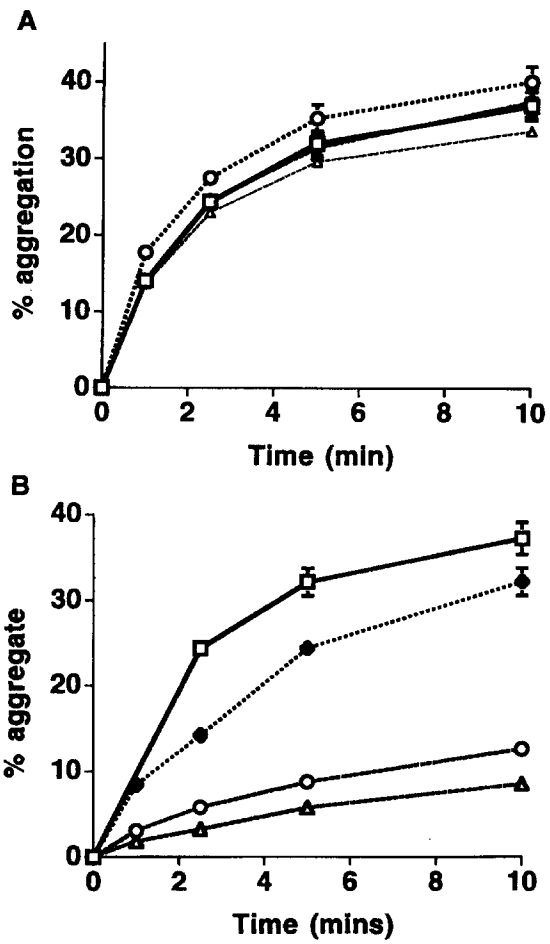

Fig. 4. Effect of PEG concentration and molecular weight on stabilization of G-CSF during aerosolization. (A) 0.001 % w/v, (B) 1.0 %w/v. (□), G-CSF control; PEG400; (◆), PEG1000; (○), PEG8000. ($n = 3 \pm$ S.D.).

Tween 80 stabilizes G-CSF against aggregation and degradation in a concentration-dependent manner (Fig. 5). Essentially no effect is observed below 0.001% w/v and complete stabilization was observed above 0.05% w/v. This sharp drop in the equilibrium surface tension values of Tween 80 over a narrow range of concentrations is consistent with changes in solution properties that occur upon reaching the critical micelle concentration (CMC) and a value of 0.008 mM was found which is similar to literature values for Tween 80 (Wan and Lee, 1974). The Zwittergent molecules were chosen because their respective CMC values differ substantially from each other and Tween 80 (CMC Zwittergent 3-08, ≈ 330 mM; Zwittergent 3-16, ≈ 0.05 mM (Neugebauer, 1992)). Nebulization of G-CSF for 10 min in the presence of each surfactant at concentrations above and below their CMC values again show that protection is significantly enhanced only at values above the CMC value of the surfactant (Fig. 6).

3.2. Surface area generation

During the bubbling experiment almost 10% aggregation of the total available protein occurs. Solutions of G-CSF containing 0.01% w/v Tween 80 are unaffected by air-bubbling. When solution is pumped through the nebulizer jets at 300 ml/min, while submerged in the protein solution, no aggregates are produced. These results indicate that aggregation is associated with the production of air-water interface.

3.3. Surface tension

Table 2 summarizes the results of the equilibrium and dynamic surface tension measurements made on G-CSF solutions and of several additives. A 4-mg/ml solution of G-CSF has an equilibrium surface tension value of 47.8 mN/m and a 0.1-mg/ml solution a value of 50.4 mN/m,

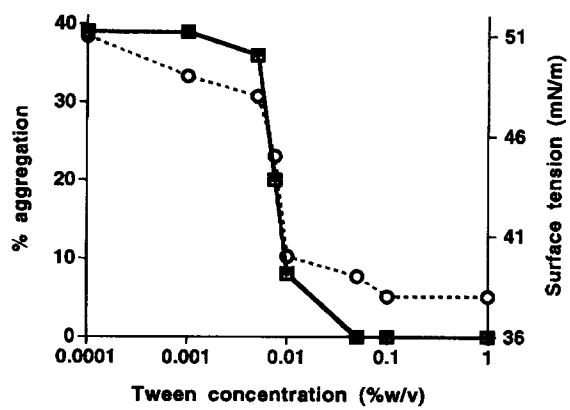

Fig. 5. The degree of aggregation of G-CSF after 10 min nebulization (10 ml initial volume; 40 psig) in the presence of various concentrations of Tween 80 (■). This curve is shown overlaid with the surface tension curve for the corresponding Tween 80 solutions (○) ($n = 1$).

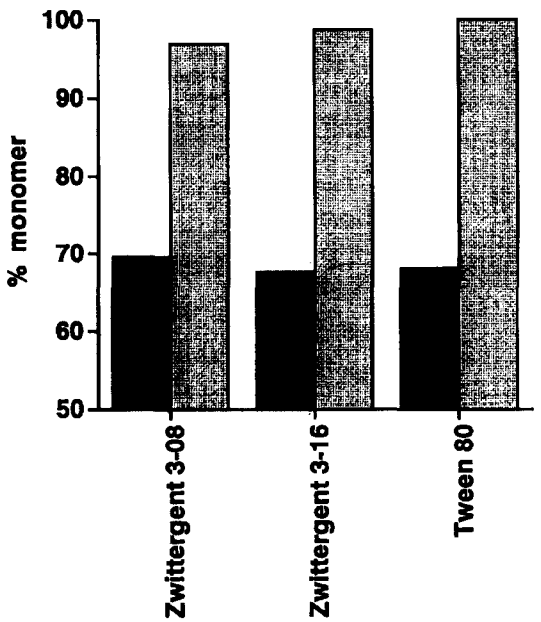

Fig. 6. The aggregation of G-CSF after 10 min nebulization in the presence of different surfactants above (■) and below (■) their respective CMC values (Zwittergent 3-08, 0.01% w/v, 20% w/v; Zwittergent 3-16, 1×10^{-5}% w/v, 0.01% w/v; Tween 80 1×10^{-4}% w/v and 0.05% w/v ($n = 2$)).

indicating that this protein has significant surface activity (pure water at 20°C has a surface tension of 72.8 mN/m). This suggests that G-CSF concentrates preferentially at the air-water interface and, even at low concentration, the surface concentration is close to saturation once equilibrium is reached. In comparison, the dynamic surface tension measurements made on G-CSF linearly extrapolated to $t = 0$ indicate that the number of G-CSF molecules exposed to newly formed surface do not lower the interfacial tension. The apparent surface tension of a 4-mg/ml G-CSF solution in 1 mM HCl is 74.8 compared to 71.5 mN/m for the buffer alone. A similar observation is made with Tween 80 at or below concentrations of 0.01% w/v where the surface tension at $t = 0$ is always close to that of pure water. However, the PEG solutions at 1% w/v have extrapolated $t = 0$ values that are close to their equilibrium values indicating that the PEG molecules do not exhibit significant surface activity.

4. Discussion

The results suggest that one site where aggregation and degradation of G-CSF occurs is at the air-water interface produced during the atomization process. The fact that aggregation occurs upon bubbling air through G-CSF solutions, and that other research has shown proteins to be denatured at air-water interfaces (Andrews, 1991; Thurow and Geisen, 1984; Donaldson et al., 1980), supports the contention. If it is assumed that surface denaturation also predominates over any other form of denaturation, the next question to ask is how does it occur? The most straightforward explanation is that protein molecules presented to the air-water interface undergo some degree of unfolding or conformational change that favors both aggregation and degradation. To support this hypothesis requires an understanding of the atomization process, and a brief discussion of nebulization follows. The velocity of air emerging from a venturi in the Collison nebulizer is close to the speed of sound. The expanding gas jet results in a pressure drop causing the aspiration of liquid from a reservoir which then interacts with the air stream (Mercer et al., 1968). At this region of interaction, the gas literally shreds the fluid surface. The liquid is rapidly accelerated and stretched into ligaments that break up into primary droplets. This primary spray will repeatedly breakup depending upon the size and level of instability of the droplets that are produced. The aspiration rate of fluid in the 3-jet Collison nebulizer at 40 psig is over 300 ml/min (Niven and Brain, 1994) ($>$ 100 ml/min/jet). The diameter of the orifice of the exit jets is $\approx$ 1 mm and the distance between the exit jet and the container walls is 1.4 cm. If we assume no radial expansion of a 1-mm diameter liquid jet emerging from the nebulizer and, for simplicity, no creation of droplets, a highly conservative estimate of the rate of air-liquid interface production can be made based upon the generation of a cyclindrical jet of fluid. The surface area of a cylinder is $2\pi rh$, where r, is the radius and h is the length of the cylinder. The volume of the cylinder is $\pi r^2 h$. The surface to volume ratio is therefore $2/r$. Hence the surface generated through each jet per minute will be 2

Table 2
Equilibrium and dynamic surface tension data

Conc. (% w/v)		Dynamic[a] (mN/m) (−) G-CSF	(+) G-CSF	Equilibrium (−) G-CSF
Water	-	-	72.8	72.0 (20°C)
G-CSF	0.4	74.6	-	47.8
	0.1	73.8	-	48.3
	0.04	73.3	-	49.2
	0.01	73.0	-	50.4
Tween 80	0.001	73.7	73.2	49
	0.01	73.7	73.4	40
	0.1	[b]	[b]	38
	1.0	[b]	[b]	38
PEG 400	1.0	71.9	67.4	65
PEG 1000	1.0	68.8	65.4	65
PEG 8000	1.0	67.6	63.1	64

[a]Data extrapolated back to $t = 0$ using linear regression.
[b]Values not determined due to significant curvature in plots; possibly due to presence of micelles in solution

$\times$ volume flow rate/r = (2 $\times$ 100/0.05) = 4000 cm^2/min/jet or 200 cm^2/s for all nebulizer jets. The true surface area that is generated will obviously be much larger (a) due to droplet production and (b) due to bulk transfer of solution from the interior to the surface of droplets, but the example serves to illustrate that even this 'low' rate of surface expansion vastly exceeds the diffusion coefficients of surfactants which are typically in the order of 10^{-6} cm^2/s. Consequently, one can assume that in the time of existence of the de novo surface before it is returned to the nebulizer reservoir (1.4 cm will be traversed in $<$ 10 ms) the molecular composition of the surface will reflect that of the bulk fluid. The surface tension that exists during this period will therefore be approximately constant and only dependent upon the combined influence of the protein and protectant at their 'initial' surface concentrations. If we now consider the surface concentrations in terms of molar quantities we know that 10 ml of water translates to 0.55 mol and 4 mg/ml G-CSF = 2.1 $\times$ 10^{-4} mol. This is equivalent to $\approx$ 2600 molecules of water for every protein molecule. It has been estimated that proteins can 'structure' or bind water to the extent of 0.5 g/g (Richards, 1977). In the case of G-CSF, this turns out to be about 500 water molecules per G-CSF. This means that, on average, the G-CSF molecules cannot be expected to be in direct apposition to each other but may only be separated by five to six molecules of water $\approx$ 10 Å. It is therefore quite plausible that adjacent proteins can interact during atomization if they become sufficiently close together so that forces of attraction can effect aggregation. The scenario becomes further complicated when the extent of aggregation is considered. The aggregation is self-limiting and a plateau is reached at 35–40% of the total protein. One explanation is that the the presence of dimer may inhibit its own formation by inhibiting the interaction of the monomer molecules. Since dimer is the predominant aggregated form produced, it would appear that the conditions for monomer to bind to dimer are not favorable. As the levels of dimer increase the opportunity for monomer to monomer interaction will therefore diminish. However, this idea suggests that aggregation is concentration dependent and would decrease as the G-CSF concentration is decreased. Yet, the concentration of G-CSF in the range of 0.1–4 mg/ml does not substantially affect the fraction of aggregate that is formed. Another explanation is that a fraction of the starting G-CSF is predisposed to aggregation. But this seems unlikely given the high purity of the starting material received from manufacturing. The results also indicate that dimer formation is not in equi-

librium with the monomer as no significant change in the extent of dimer formation occurs by changing the nebulization air pressure. If an equilibrium existed then a change in the the plateau levels would be expected as more energy is applied to the system. Clearly, a full explanation for the self-limiting nature of aggregate formation remains to be resolved.

The nebulization-induced inactivation and degradation of proteins can be ameliorated by various additives. However, the results suggest that the mechanisms of protection differ between the PEG and the surfactants.

Dynamic surface tension measurements together with equilibrium surface tension measurements provide some insight into how the Tween and PEG influence protein stability (Table 2). Linear extrapolation of the surface age curves of the PEG to $t = 0$ gives values that are not disimilar to their respective equilibrium values. This suggests that over time the net transfer of PEG molecules from the interior to the surface is quite small, but that the PEGs do intrinsically lower the interfacial tension at $t = 0$. This 'initial' lowering of the surface tension may be sufficient to reduce or prevent some aggregation at the interface and is supported by the fact that as the dynamic surface tension of the PEGs is reduced, so is the aggregation of G-CSF. A more likely explanation is that PEG sterically hinders monomer to monomer interactions at the surface. This contention is supported by the results showing that G-CSF aggregation is reduced by an increase in PEG molecular weight and concentration. An increase of the hydrodynamic size of the PEG molecules and their numbers at the surface would be expected to increase the probability that they can interfere with G-CSF monomer interactions. It is also unlikely that the PEG molecules interact directly with the protein. Titration differential scanning calorimetry data (data not shown) does not demonstrate any thermodynamic interaction between the G-CSF and the PEG. This is supported by evidence from the literature demonstrating PEG has a large exclusion volume and does not bind to proteins directly (Arakawa and Timasheff, 1985).

The stabilizing action of the surfactants seems quite different from that of the PEG. Extrapolation of surface age plots for Tween 80 at concentrations of 0.01% w/v or less gives $t = 0$ values that are not dissimilar to the surface tension of water (Table 2). Higher concentrations of the Tween 80 unfortunately do not generate linear plots of surface tension vs. time and no extrapolation could be made. However, there is a correlation between G-CSF aggregation and the *equilibrium* surface tension of Tween 80 (Fig. 5). The large drop in surface tension that occurs between 0.001 and 0.1% w/v suggests that the CMC of the Tween 80 is exceeded. Some further evidence indicating that the CMC is involved is shown in Fig. 6 where several surfactants of widely differing CMC values are only able to protect G-CSF at concentrations above their respective CMC values. In summary, the stability conferred by the surfactants may relate to their micellar properties and not to their surface activity as might first be expected.

Although the above synopsis is logical, it relies on a number of assumptions. The main one being the argument that destabilization only occurs at the interface. Instability could also occur at the wall-liquid interface (Edwards and Huber, 1991), although no adhesion of G-CSF to the glass container walls over time has been observed. Similarly, during droplet creation shear forces may be greater than those generated by simply forcing fluid through the nebulizer jets while keeping the nebulizer head submerged in liquid. It would be helpful if the total surface area produced in the nebulizer over time could be accurately measured. This would enable a calculation to be made for the approximate fraction of protein molecules in solution that would be exposed to surface over time. If a similar calculation could be performed from the 'air-bubbling' experiments it should be possible to estimate what fraction of the total observed aggregation/degradation of G-CSF during nebulization is due to surface denaturation. However, such an estimate is difficult to make. The 'gross' aerosol produced in the nebulizer is highly polydisperse (i.e. the unbaffled aerosol). Attempts to determine the median size of this aerosol using the Malvern Mastersizer X were

unsuccessful. The emitted aerosol passing through the laser beam was too large to measure. The experiments did, however, confirm that the size of droplets generated within the nebulizer cover the dynamic range of the instrument: that is, from <1 to greater than 600 μm. If a surface film thickness of 10 Å is assumed in the presence of protein, then using the cylindrical jet example described above, an estimate can be made of the overall fraction of protein molecules in solution that will be exposed to surface over a fixed time. For an initial volume of 10 ml and 20 min operation of the nebulizer a total surface area of 200 $cm^2 \times 60 \times 20 = 240\,000\ cm^2$ will be generated; 10 Å = 10^{-7} cm thus the total volume exposure can be calculated at $\approx$ 24 μl. This represents $< 0.25\%$ of all available protein molecules in solution. Nevertheless, the estimate is undoubtably a gross underestimate of the true surface area that is generated. If all fluid were atomized to 10-μm droplets the surface exposure would rise to at least 35%. These values also assume that individual droplets are static and in reality during the droplet's lifetime internal turbulence will present bulk volume to the surface and therefore increase the overall surface exposure of the molecules.

5. Summary

The aggregation and degradation of G-CSF induced by nebulization can be reduced by the addition of various additives including different molecular weights of PEG and by surfactants such as Tween 80. The mechanism of denaturation of the G-CSF is not fully understood but there is good evidence to suggest that the air-water interface is a major site of denaturation. The mechanism(s) of protection of PEG and Tween 80 appear to differ. The protection of PEG may relate to its hydrodynamic size and how it influences the conformation of the protein molecule at the air-water interface. The action of Tween 80 may relate to its micellar properties and not to its surface tension-lowering capacity. The results also highlight the difficulties involved with investigating protein stability during nebulization.

References

Aitken, M.L., Burke, W., McDonald, G., Shak, S., Montgomery, A.B. and Smith, A., Recombinant human DNase inhalation in normal subjects and patients with cystic fibrosis. A phase I study. *J. Am. Med. Assoc.*, 167 (1992) 1947–1951.

Andrews, A.T., Partial denaturation and renaturation of β-lactoglobulin at air-water interfaces. *Biochem. Soc. Trans.*, 272S (1991) 19.

Arakawa, T. and Timasheff, S.N., Mechanism of polyethylene glycol interactions with proteins. *Biochemistry*, 24 (1985) 6756–6762.

Charm, S.E. and Wong. B.L., Enzyme inactivation with shearing. *Biotechnol. Bioeng.*, 12 (1970) 1103–1109.

Donaldson, T.L., Boonstra, E.F. and Hammond J.M., Kinetics of protein denaturation at gas-liquid interfaces. *J. Colloid. Interface Sci.*, 74 (1980) 441–450.

Edwards, R.A. and Huber, R.E., Surface denaturation of proteins: the thermal inactivation of β-galactosidase (*Escherichia coli*) on wall-liquid surfaces. *Biochem. Cell Biol.*, 70 (1991) 63–69.

Harkins, W.D. and Jordan, H.F., A method for determination of surface and interfacial tension from the maximum pull on a ring. *J. Am. Chem. Soc.*, 52 (1930) 1751–1772.

Hubbard, R.C., Casolaro, M.A., Mitchell, M., Sellers, S.E., Arabia, F., Matthay, M.A. and Crystal, R.G., Fate of aerosolized recombinant DNA-produced α-1-antitrypsin: use of the epithelial surface of the lower respiratory tract to administer proteins of therapeutic importance. *Proc. Natl. Acad. Sci. USA*, 86 (1989) 680–684.

Ip, A.Y. and Niven, R.W. Prediction and experimental determination of solute output from a Collision nebulizer. *J. Pharm. Sci.*, 83 (1994) 1047–1051.

Leukenheimer, K. and Wantke, K.D. Determination of the surface tension of surfactant solutions applying the method of Lecompte de Noüy (ring tensiometer). *Colloid Polymer Sci.*, 259 (1981) 354–356.

Mercer, T.T., Tillery, M.P. and Chow, H.Y., Operating characteristics of some compressed air-jet nebulizers. *Am. Ind. Hyg. Assoc. J.*, 29 (1968) 66–78.

Neugebauer, J., *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*. Calbiochem, La Jolla, 1992, pp. 1–62.

Niven, R.W. and Brain J.D., Some functional aspects of air-jet nebulization. *Int. J. Pharm.*, 104 (1994) 73–85.

Niven, R.W., Ip, A.Y., Mittelman, S.D., Farrar, C., Arakawa, T. and Prestrelski, S.J., Protein Nebulization I. Stability of lactate dehydrogenase and recombinant granulocyte colony stimulating factor to air-jet nebulization. *Int. J. Pharm.*, 109 (1994) 17–26.

O'Hagan, D.T. and Illum, L., Adsorption of peptides and proteins from the respiratory tract and the potential for development of locally administered vaccine. *Crit. Rev. Ther. Drug Carrier Syst.*, 7 (1990) 35–97.

Patton, J.S. and Platz, R.M., Routes of delivery: case studies. II. Pulmonary delivery of peptides and proteins for systemic action. *Adv. Drug Deliv. Rev.*, 8 (1992) 179–196.

Richards, F.M., Areas, volumes, packing and protein structure. *Ann. Rev. Biophys. Bioeng.*, 6 (1977) 151–176.

Shak, S., Capon, D.J., Helmiss, R., Marsters, S.A. and Baker, C.L., Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc. Natl. Acad. Sci. USA*, 87 (1990) 9188–1992.

Thurow, H and Geisen, K., Stabilization of dissolved proteins against denaturation at hydrophobic interfaces *Diabetologia*, 27 (1984) 212–218.

Vogelmeier, C., Buhl, R., Hoyt, R.F., Wilson, E., Fells, G.A., Hubbard, R.C., Schnebli, H., Thompson, R.C. and Crystal, R.G., Aerosolization of recombinant SLPI to augment antineutrophil elastase protection of pulmonary epithelium. *J. Appl. Physiol.*, 69 (1990) 1843–1848.

Wan, S.C. and Lee, P.F.S., (1974) CMC of polysorbates. *J. Pharm. Sci.*, 63 (1990) 136–137.