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Response of a Concentrated Monoclonal Antibody Formulation to High Shear

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Abstract

There is concern that shear could cause protein unfolding or aggregation during commercial biopharmaceutical production. In this work we exposed two concentrated immunoglobulin-G1 (IgG1) monoclonal antibody (mAb, at >100 mg/mL) formulations to shear rates of between 20,000 and 250,000 s^{-1} for between 5 minutes and 30 ms using a parallel-plate and capillary rheometer respectively. The maximum shear and force exposures were far in excess of those expected during normal processing operations (20,000 s^{-1} and 0.06 pN respectively). We used multiple characterization techniques to determine if there was any detectable aggregation. We found that shear alone did not cause aggregation, but that prolonged exposure to shear in the stainless steel parallel-plate rheometer caused a very minor reversible aggregation (<0.3%). Additionally, shear did not alter aggregate populations in formulations containing 17% preformed heat-induced aggregates of a mAb. We calculate that that the forces applied to a protein by production shear exposures (<0.06 pN) are small when compared with the 140 pN force expected at the air-water interface or the 20 to 150 pN forces required to mechanically unfold proteins described in the atomic force microscope (AFM) literature. Therefore, we suggest that in many cases air-bubble entrainment, adsorption to solid surfaces (with possible shear synergy), contamination by particulates, or pump cavitation stresses could be much more important causes of aggregation than shear exposure during production.

Keywords

protein aggregation; shear; monoclonal antibody; stability; pumping

INTRODUCTION

Therapeutic protein formulations can be exposed to shear stresses during their commercial production. Many studies have demonstrated that the magnitude and duration of shear

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exposure, in the absence of the air-water interface, during protein production does not cause protein aggregation (Harrison et al. 2003). In classic shear studies, short time exposures to high shear did not cause degradation of catalase, urease, or alcohol dehydrogenase (Thomas and Dunnill 1979; Thomas et al. 1979). More recently, an elegant study utilized the high sensitivity of cytochrome-c fluorescence to unfolding to demonstrate that even shear rates of 200,000 s^{-1} did not result in any detectable protein unfolding (Jaspe and Hagen 2006). However, the literature does contain some reports of shear-induced protein unfolding or aggregation. Deactivation of α-amylase was caused by shear in highly viscous starch solutions (van der Veen et al. 2004), though it should be noted that in solutions at the much lower viscosities typical of therapeutic protein formulations, the shear rate required to achieve an equivalent shear stress would require shear rates of over 10^7 s^{-1} (Jaspe and Hagen 2006). Shear has also been reported to induce amyloid fibril nuclei in β-lactoglobulin solutions prone to fibrillation (Akkermans et al. 2006; Hill et al. 2006).

Sometimes the effects of agitation on protein stability have been attributed to shear stresses even though stresses at the air-water interface were present. For instance, shear was cited as the cause of aggregation of β-lactoglobulin and human serum albumin aggregation after agitation in vials containing headspace that contributed air-water interfacial stresses (Oliva et al. 2003). Other studies have reported subtle shear-induced damage to proteins when the air-water interface was carefully eliminated. Exposure of recombinant human growth hormone (rhGH) to over 100,000 s^{-1} shear rate for 16 hr without exposure to an air-water interface did not result in any aggregation, but did result in changes to the thermal stability profile of the protein suggestive of minor conformational changes (Maa and Hsu 1996). In the same work, the potent effect of the air-water interface on rhGH was highlighted by the extensive aggregation caused by low-shear bubble exposure (Maa and Hsu 1997).

A critical point to note is that it is not possible to completely remove the influence of solid-liquid interfaces in shear experiments. Thus, surface adsorption and associated conformational perturbations of proteins could act synergistically with shear-induced desorption to result in greater rates of protein unfolding or aggregation. For example, during peristaltic pumping of recombinant interleukin-2, exposure to silicone rubber tubing surfaces (and not shear) caused a 97% loss in bioactivity (Tzannis et al. 1997). Stirring enhanced the inactivation of lysozyme caused by exposure to various interfaces (Colombie et al. 2001). And a synergistic shear/surface mechanism caused the aggregation of an immunoglobulin-G4 (IgG4) molecule exposed to high shear rates in a stainless steel chamber (Biddlecombe et al. 2007). We note that mAb IgG4 subtypes may be prone to an additional instability where the molecule can dissociate into ‘half-antibodies’(Bloom et al. 1997), although this problem may be corrected by engineering of the primary sequence of commercial or development products (Salfeld 2007). Similarly, sub-visible stainless steel particles shed from a piston pump in the laboratory setting caused the aggregation of an IgG (Tyagi et al. 2009), and particle formation during filling of an IgG was eliminated by replacement of a radial piston pump with a rolling diaphragm pump (Cromwell et al. 2006). These studies suggest that exposure of a protein to product contact surfaces or particulate contamination, and the possible synergistic effects of shear, could be much more damaging than the effect of shear alone. We speculate that difficult-to-identify, uncontrolled effects in long-duration shear experiments like leaching of contaminants from metal or polymers could cause protein aggregation, leading to incorrect identification of shear as a cause of aggregation.

There remains some concern within industry that larger molecules may be more sensitive to shear-induced damage. Shear can damage cells during fermentation mixing (Cherry and Papoutsakis 1986), and shear has been shown to stretch and degrade DNA molecules during processing (Lengsfeld and Anchordoquy 2002; Meacle et al. 2007; Shaqfeh 2005). Because many of the new therapeutic proteins currently entering the market or under development

are monoclonal antibodies (mAbs) with molecular weights near 150 kDa, they might be expected to be more susceptible to shear-induced damage than many smaller proteins. In fact, a model developed by Jaspe *et al*. (Jaspe and Hagen 2006) can be used to predict that shear rates as low as 10,000 s^{-1} could induce unfolding of a mAb.

It is useful to assess what the actual shear rate and exposure times might be for typical commercial operations and to discuss the other stresses that may be present. The shear rate (γ) is defined as the fluid velocity (v) gradient perpendicular to the direction of flow: $\gamma = dv_z/dx$ (Wilkes 2006). For a Newtonian fluid, the shear stress (τ) is the product of the shear rate (γ) and the viscosity (μ) and the force (F) applied is the product of the shear stress and the surface area that the stress acts upon: $\tau = \mu \cdot \gamma$ and $F = \tau \cdot A$ (Wilkes 2006). The shear rates expected during many physical processes have been tabulated (Steffe 1996; Williams 2007). Mixing processes typically expose protein formulations to relatively low shear rates of about 50 s^{-1} for up to several hours, and pipe flow exposes formulations to up to about 2000 s^{-1} for up to several seconds. In general, we estimate that most of the fluid inside a pump will experience similar order-of-magnitude shear rates as in the adjacent pipe flow, assuming that the internal dimensions are similar to the pipe dimensions. We calculate that self lubricated pumps (piston or rotary lobe) operated at a high relative piston velocity of 1000 mm/s and a low clearance of 0.05 mm, estimated from design specifications found in the literature (Neumaier 1997), would apply a shear rate of 20,000 s^{-1} to a small fraction (less than 1%) of the pumped solution. Slightly more complex but similar calculations for a specific lobe pump have been published that correspond to a gap shear rate of about 16,000 to 20,000 s^{-1} (Gomme et al. 2006).

Cavitation is an additional pumping stress that violently creates and destroys micro-bubbles inside pumps and valves. During rotary lobe pumping of human albumin, a pump with larger self-lubricated clearances caused more aggregation than a pump with smaller clearances (Gomme et al. 2006). This was attributed to greater cumulative shear due to the inefficiency of the pump with larger clearances (Gomme et al. 2006). An alternative explanation, although not explored in the original work, is that “gap cavitation” was occurring and causing aggregation (Gomme et al. 2006; Neumaier 1997).

All biopharmaceuticals are filtered during production. During crossflow filtration the protein may be exposed to shear rates of 1000 to 10,000 s^{-1} for a few seconds as it passes over the membrane (Davis 2001). Multiple passes through pumps and valves and the concomitant microcavitation and air bubble entrainment, rather than shear, have been cited as a cause of aggregation during filtration (Narendranathan and Dunnill 1982; van Reis and Zydney 2007). Adsorption to the membrane during filtration is also sometimes a cause of aggregation as demonstrated by the deactivation of aminoacylase by adsorption to an ultrafiltration membrane (Bodalo et al. 2004).

Finally, therapeutic proteins are exposed to shear at the end of production during the vial filling operation. We calculate that dispensing a protein solution into vials through a 20 gauge by 10 cm needle at a rate of 0.5 mL/s would expose the protein to a shear rate of 20,000 s^{-1} for about 50 ms (Wilkes 2006).

In this work we characterized the aggregation state of a monoclonal antibody formulation before and after exposure to high shear rates in different buffer solutions. We used a parallel plate rheometer to expose a mAb to shear rates near the maximum shear rate expected during processing for prolonged periods of time, and we used a capillary rheometer to apply much higher shear rates for shorter periods of time. Additionally, we investigated the effect of shear on protein aggregates by passing a previously aggregated mAb formulation through the capillary rheometer.

MATERIALS AND METHODS

Materials

Two monoclonal antibodies (mAb) were used in this work and were both donated by Amgen Inc. (Thousand Oaks, CA). The first was a humanized immunoglobulin-G1 (IgG1) antistreptavidin mAb ("mAb-AS") that is not a commercial or development product. This is a highly purified monomeric molecule that was used in most of the studies reported in this work. The IgG1 mAb-AS has a molecular weight of 145 kDa (including 3 kDa of glycosylation) and an isoelectric point (pI) of 8.7. mAb-AS was prepared at various concentrations in two different buffer systems: buffer A, 10 mM sodium acetate (pH 5.0); and buffer B, 10 mM sodium phosphate with 150 mM sodium chloride (pH 7.0).

The second IgG1 mAb ("mAb-X") is a proprietary Amgen Inc. monoclonal antibody. This antibody has properties (molecular weight, glycosylation, pI) similar to IgG1 mAbs in general. mAb-X was available at concentration of 31.4 mg/ml dissolved in 10 mM sodium acetate (pH 5.2) buffer with 9% w/v sucrose and 0.04% w/v polysorbate 20 ("buffer C").

All other chemicals used were of reagent grade or higher quality.

Application of Shear to Monomeric mAb-AS in the Capillary Rheometer

A Bohlin Instruments RH2000 Capillary Rheometer (Malvern Instruments Ltd, UK) was used to expose high-concentration (ca. 100 mg/mL) mAb-AS solutions to shear rates of up to 250,000 s^{-1}. This instrument uses a piston to drive fluid through a narrow capillary. A diagram of the measurement geometry and mathematical analysis of the relevant fluid mechanics can be found in the text by Wilkes (Wilkes 2006). A custom thread assembly was made that could accept a longer 1/16 inch OD capillary instead of the standard short capillary die, thus increasing the time that the mAb solution was exposed to high shear.

A 1/16 inch OD stainless steel capillary 304 mm long with a 0.318 mm ID was attached to the rheometer bore (9.5 mm ID) outlet. The tip that pushes the piston into the bore was made from PTFE. We attached a 20 mm piece of 1.59 mm ID silicone tubing to the end of the capillary to reduce the fluid velocity from about 10 m/s to 0.4 m/s at the outlet. This modification eliminated spraying of the mAb solution from the capillary (which would constitute a potentially large air-water interface stress) and allowed a gentle steady dropwise collection down the side of a polypropylene receiving tube.

The maximum wall shear rate can be calculated based on the capillary internal diameter and the flow rate using standard fluid mechanics relationships, (Wilkes 2006) and the average shear rate can be shown to be two-thirds of the maximum shear rate (Jaspe and Hagen 2006). We applied maximum (wall) shear rates of 250,000 s^{-1} and 150,000 s^{-1} for 30 and 51 ms respectively in our experiments. The exposure time is based upon the residence time of the fluid in the capillary. Calculations for our system confirm that we were operating in the laminar flow regime (Wilkes 2006).

Application of Shear to Aggregated mAb-X in the Capillary Rheometer

We applied shear to a formulation containing aggregates of mAb-X in buffer C to determine if shear could break aggregates apart or induce further aggregation. To produce aggregates, a 31.4 mg/mL mAb-X solution in buffer C was heated at 50°C for 3 days. There was no visible particle formation after the incubation. This heat stress generated ca. 17% aggregates in the dimer to decamer range; with more 90% of aggregates in the dimer to hexamer range. The highly aggregated suspension of mAb-X in buffer C was exposed to a shear rate of 150,000 s^{-1} in the capillary rheometer. The suspension of mAb-X was then diluted in buffer

C to 0.93 mg/mL, analyzed by SEC and AUC, and the results compared with those for an unsheared mAb-X suspension.

Application of Shear to mAb-AS in the Parallel-Plate Rheometer

A maximum shear rate of 20,000 s^{-1} was applied to high-concentration mAb-AS solutions (ca. 100 mg/ml) for either 10 or 300 s at controlled room temperature (23 °C) using an ARES Rheometer (TA Instruments, New Castle, DE) with a parallel-plate fixture. A diagram of the measurement geometry and mathematical analysis of the relevant fluid mechanics can be found in the text by Wilkes (Wilkes 2006). The sample is sandwiched between two plates, one of which is rotated relative to the other: the maximum shear rate is applied at the rim and the average shear rate is two-thirds of the maximum (Wilkes 2006). In three separate replicate experiments mAb-AS solution (104 mg/mL in buffer A) was sandwiched between two 20 mm parallel stainless steel plates with a gap of 0.035 mm. A single drop was carefully deposited to avoid inclusion of air-bubbles as the top plate was lowered, although we cannot be certain that no very small micro-bubbles were present during the measurement. The capillary rheometer may have an advantage in this regard since the applied pressure might minimize or redissolve any micro-bubbles in the fluid. About 10 μL of the sheared mAb-AS solution was collected from the plate and diluted into 2.00 mL of buffer A for a final mAb-AS concentration of about 0.5 mg/mL. A control protein sample without applied shear was prepared in which the protein solution was put into the assembly without starting the plate rotation. The protein solutions were then analyzed by SEC for monomer and aggregate levels.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed using two different systems. The mobile phase consisted of 100 mM sodium phosphate and 300 mM sodium chloride (pH 7.0). Control samples were run for comparison with samples for all studies.

SEC of mAb solutions sheared in the capillary rheometer was performed with a TSK-GEL Super SW3000 column (Tosoh Bioscience LLC, Montgomeryville, PA) using an Agilent HP 1050 HPLC system (Agilent Technologies Inc., Santa Clara CA) with a flow rate of 0.3 mL/min.

SEC of mAb-AS solutions sheared in the parallel plate rheometer were performed with a TSK-GEL G3000SW_{XL} column and SW guard column (Tosoh Bioscience LLC, Montgomeryville, PA) using a Beckman HPLC system (Beckman Coulter Inc., Fullerton, CA) with a flow rate of 0.6 mL/min. In addition to UV detection a Wyatt MiniDawn® (Wyatt Technology Corp., Santa Barbara, CA) was used for online light scattering detection.

Field Flow Fractionation

Asymmetric Field Flow Fractionation (AFFF) was performed using an Agilent autosampler and pump (Agilent Technologies Inc., Santa Clara CA) connected to a Wyatt Eclipse™ AFFF separations module (Wyatt Technology Corp., Santa Barbara, CA), with a Wyatt Dawn® Heleos™ Multi-Angle Light Scattering (MALS) detector and an Agilent UV detector set to 280 nm. The separations were performed using a 10 kDa molecular weight cutoff regenerated cellulose membrane in the 25 cm separation channel with a 350 μm spacer, and 1.6 cm breadth. The mobile phase contained 100 mM sodium phosphate and 250 mM sodium chloride (pH 6.8). The samples introduced into the AFFF were first diluted to about 1 mg/mL in the original buffer. 35 μg of mAb-AS in buffer A or 20 μg of mAb-AS in buffer B were injected into the separation channel with a focus flow of 1 mL/min. The separation was performed with a channel flow of 1.0 mL/min and a constant crossflow of 2.0 mL/min for 10 min. The crossflow was then decreased from 2.0 mL/min to 0.07 mL/min

over 10 min, held at 0.07 mL/min for 5 min, and finally the system was flushed without a crossflow.

The molecular weight profile of the eluting mAb-AS species was determined by fitting the MALS and UV data to the Zimm Equation function in the Wyatt Astra® software (Zimm 1948). A refractive index increment (dn/dc) of 0.185 mL/g was assumed in the data analysis. The results were reported as the apparent molecular weight of protein species in each peak or shoulder detected.

Analytical Ultracentrifugation

Analytical ultracentrifugation in the sedimentation velocity mode (AUC-SV) was used to determine aggregates levels in samples sheared in the capillary rheometer. An Optima XL-I analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) with absorbance optics set to 280 nm was used to collect AUC-SV data. The samples to be measured were diluted in the appropriate buffer to about 1 mg/mL immediately before AUC-SV measurements. Three replicate samples were placed in each of three 12-mm charcoal-filled Epon double sector cells and the cells were mounted in a four-hole An60 Ti rotor. The instrument settings were as follows: scanning range, 5.95-7.2 cm; angular velocity, 45,000 rpm; temperature, 20 °C.

UV scans were collected without delay until monomer boundary reached bottom (at least 70 scans were collected). AUC-SV data were analyzed using the Sedfit 9.4 software program. Sedimentation coefficient distributions, *c(s)*, were determined for each replicate sample using the procedures previously described by Schuck *et al*. (Schuck 2000; Schuck et al. 2002). The values of the densities and viscosities of the buffers (buffers A, B, and C) were entered as estimated using the Sednterp program (Haynes et al. 2006). The partial molar volume of mAb-AS and mAb-X were set to 0.73 mL/g. The frictional ratio and the meniscus position were allowed to float during fitting. The data were analyzed with a resolution of 200 increments between 2 and 20 Svedbergs. All other parameters were set to the default values. The data for the three individual cells were each analyzed at confidence level of 0.6 and the area percentages of the high molecular weight species (HMWS) peaks were reported as the average of the three replicate measurements. The sedimentation coefficients were reported as the values normalized to the standard conditions ($s_{20,w}$) and the frictional coefficients of the observed species determined according to the tutorial review by Lebowitz *et al*. (Lebowitz et al. 2002).

Dynamic Light Scattering

Dynamic light scattering (DLS) analysis was performed on samples of mAb-AS sheared in the capillary rheometer that had been diluted in the appropriate buffer to about 10 mg/mL. A Zetasizer® Nano-Z or ZS (Malvern Instruments Ltd, UK) was used to perform the DLS measurements. Control buffer samples without protein that had been passed through the capillary rheometer were found to contain some larger particles (ca. 0.5 μm and above). Thus, because the instrument was shedding some larger particles, samples were first centrifuged at 12,000 ×g for 5 min before reading on the DLS. Three measurements were reported as the z-average hydrodynamic diameter.

RESULTS AND DISCUSSION

Results of Exposure of Monomeric mAb-AS to High Shear in the Capillary Rheometer

No aggregation of mAb-AS was detectable after 30-51 ms exposures to shear rates of up to 250,000 s^{-1} (Table 1). These experiments were performed at protein concentrations of over 100 mg/mL in two different buffer systems. The mAb recovery and aggregates level determined by SEC, the aggregates level found using AUC, and the z-average diameter

measured by DLS of sheared samples all indicate that there was no aggregation caused by application of shear in the capillary rheometer (Table 1). The sedimentation coefficients measured by AUC give frictional coefficients (f/f_0) of about 1.6 for monomer and 1.8 for the dimer which are comparable to a previous AUC analysis published for a monoclonal antibody solution containing monomer and dimer (Gabrielson et al. 2007). The shear exposure applied during these experiments can be converted into shear stress, force, cumulative shear stress and cumulative force exposures (Table 2). Comparison of the anticipated shear exposure during a filling operation with our experiments shows that we exceeded the estimates of shear exposure during filling operations by all measures (Table 2).

Results of Application of Shear to Aggregated mAb-X in the Capillary Rheometer

Exposure of the highly aggregated (ca. 17% soluble aggregates) mAb-X solution to 150,000 s^{-1} shear rate did not result in any significant change in the levels of soluble aggregates assayed by SEC ($p = 0.1$) and AUC ($p = 0.2$) (Table 3). Significance was determined using the t-test function to compare data before and after shear exposure in Microsoft Excel® using $p < 0.05$ as the criteria for statistical significance. Therefore, shear did not increase mAb-X aggregation or break previously formed mAb-X aggregates apart.

Results of Exposure of mAb-AS to High Shear in the Parallel-Plate Rheometer

A small peak presumed to reflect protein aggregates (ca. 0.3%) was observed in the triplicate SEC chromatograms of mAb-AS after exposure in a parallel-plate rheometer to a 20,000 s^{-1} shear rate in buffer A for either 10 or 300 s. Light scattering analysis of the peak gave a mass equivalent to 40 to 60 monomer units. No other aggregation or monomer loss was observed in the sheared samples. The peak was not detected upon reanalysis of the sheared samples after storage at 4-8 °C for 1 week. The peak was not observed in control samples that were exposed to the stainless steel parallel plate assembly without shear. The peak was also observed in samples exposed to shear in the parallel plate rheometer for only 10 s and therefore with 30-times less cumulative shear stress and force exposure. We calculate that the surface area to volume in the parallel plate rheometer was five times that of the capillary rheometer and so it is also possible that a synergistic effect of mAb adsorption to the stainless steel combined with exposure to shear caused the small amount of aggregation in the parallel plate assembly. The time of exposure was also much greater in the parallel-plate rheometer which could be important in processes involving adsorption and desorption. In other recent work we found that this mAb can be aggregated by stainless steel under low-shear conditions (Bee et al. 2009, in-press). This is also consistent with previous reports that concluded that stainless steel can cause the aggregation of other mAbs (Biddlecombe et al. 2007; Tyagi et al. 2009).

Comparison of Measured Protein Unfolding Forces with Calculated Shear and Interfacial Forces

To estimate the impact of shear on protein stability we can compare the measured atomic force microscope (AFM) force required to unfold a protein with order-of-magnitude estimates of the forces that may be applied by exposure to shear (Table 2). AFM experiments have been used to unfold a variety of protein molecules with the general result that unfolding requires about 20 and 150 pN for α-helical proteins and β-sheet proteins respectively (Brockwell 2007). The force to pull apart amyloid-β aggregates was about 45 pN (Lyubchenko et al. 2006), which may be similar to the forces needed for domain-domain separations in larger proteins. In our introduction we noted that shear of only 10,000 s^{-1} can be predicted, using the bead model of Jaspe and hagen (Jaspe and Hagen 2006) to be enough to unfold a mAb: the failure of the bead model to accurately predict the shear threshold for unfolding of mAb-AS could be explained by the fact that denaturant and mechanical unfolding are not necessarily correlated (Brockwell 2007;Jaspe and Hagen 2006).

We can calculate an order-of-magnitude estimate of force applied to a mAb protein molecule in a shear field using the equations given in our introduction. Corrections for slip at the molecular level were not applied. We applied a factor of three to account for the fact that the local maximum shear rate applied to a small area on a spherical particle is three times the fluid shear rate (Cherry and Papoutsakis 1986). The average shear rate applied to a particle is half the fluid shear rate since half the force is applied to rotation of the particle (Cherry and Papoutsakis 1986). To estimate values for average shear rates we also applied a factor of two-thirds to account for the average shear rate in a capillary or parallel plate. The maximum force is applied to a small part of the protein molecule to those molecules near the wall and is nine times the average force exposure. The viscosity can vary from a few mPa·s to over 100 mPa·s for highly concentrated mAb solutions (Shire et al. 2004). For a 10.5 nm mAb protein molecule, the maximum shear force is about 0.06 pN assuming a viscosity of 3 mPa·s and a 20,000 s^{-1} shear rate. This corresponds to our estimate of the shear exposure during final filling (Table 2). AFM-based estimates of the force required to unfold β-sheet proteins range from 150 to 220 pN depending on the pulling speed used (Best et al. 2003). Therefore, for a mAb in a 20,000 s^{-1} shear flow, the applied force is three-orders of magnitude lower than the experimental AFM forces required to unfold β-sheets in a protein or to pull β-sheets motifs apart. In experiments where we applied shear in the capillary rheometer we calculate that the maximum force was about 0.8 pN and therefore two-orders of magnitude lower than the forces in AFM-based unfolding studies. The shear rate necessary to achieve a maximum force of 150 pN on a protein (average force = 17pN) in a 3 mPa·s fluid is 5×10^{7} s^{-1} (Table 2). In AFM experiments tension is applied to a tethered protein, whereas in shear flow the equal elongational and rotational shear forces applied to a protein are predicted to cause a dynamic instability rather than complete unfolding above a critical shear rate (Alexander-Katz and Netz 2008; Jaspe and Hagen 2006). These direct comparisons of shear forces with the forces required to mechanically unfold proteins by AFM should be used with caution: we are not aware of any current evidence to suggest that the stresses applied by shear would induce the same kind of unfolding as that caused by direct mechanical unfolding. In fact, the degree to which aggregation-prone molecules are unfolded is generally unknown, although only moderate tertiary structure changes may be required in some cases. These order-of-magnitude comparisons still have utility since they serve as benchmarks of the fundamental physical forces that have been unequivocally found to unfold proteins. If we postulate that a protein becomes aggregation-prone at only 5% unfolding and assume that a linear correlation with AFM (or air-water interface) unfolding forces is valid, then this would still correlate to 7.5 pN maximum force (average force = 0.8 pN) and a shear rate on the order of 2 million per second (still 100 times greater than expected during processing).

We can also evaluate the cumulative force×time exposure. Again, taking the AFM unfolding force of 150 pN applied to a β-sheet protein at 100 nm/s for a 20 nm extension we estimate that the average cumulative force×time exposure in AFM unfolding is $15{,}000\times10^{-15}$ N·s (Table 2). This value is four orders of magnitude larger than our estimated cumulative shear force exposure during final filling (Table 2). Furthermore, computational simulations for collapsed polymers in shear flows predict threshold shear rates of greater than 10^{8} s^{-1} for the onset of instability for protein molecules (Alexander-Katz and Netz 2008). The fact that we observed the same small amount (ca. 0.3 %) of transient aggregate in mAb exposed to shear in the parallel plate for both 300 s and 10 s indicates that it is likely that this aggregation was due to a protein-metal surface interaction, or possibly incomplete elimination of small air bubbles in the parallel plate fixture.

In contrast, we calculate that protein molecules will be easily unfolded by the surface tension forces at the air-water interface. This seems reasonable since there are many reported instances of air-water interface induced aggregation in the literature. The force on a

protein across the interface is the product of the surface tension and the interface length. Taking the depth of the air-water interface (Adamson and Gast 1997) to be 2 nm and using an air-water surface tension of 0.07 N/m we estimate an applied force 140 pN. Clearly, this estimate is very close to the forces actually measured in AFM protein unfolding studies. The potency of the air-water interface to cause protein unfolding and aggregation could be enhanced by its regenerative nature, mass transport effects, and additional interface compression stresses that are associated with agitation and shear.

Cavitation is another potentially damaging stress that frequently occurs under conditions where high shear is present. Cavitation might be expected to damage proteins by the creation of small vapor bubbles to which proteins may adsorb. If solutions are degassed before cavitation occurs, violent collapse of these bubbles may occur, further damaging adsorbed proteins. Thus, we speculate that cavitation-induced protein damage may be exacerbated if solutions are degassed by filtration immediately before pumping (Neumaier 1997). Likewise, minimization of shear by increasing pump clearances could actually cause more protein damage by increasing cavitation during pumping (Gomme et al. 2006; Neumaier 1997). Acoustic cavitation from ultrasound devices has also been shown to cause protein aggregation through the generation of reactive oxygen species (ROS) that oxidize protein molecules (Riesz and Kondo 1992). Although we can only speculate that a cavitating pump could cause generation of ROS it may be worth evaluating the effects of different operating parameters on pump cavitation and the resulting impact on protein stability during pumping.

CONCLUSIONS

Our experimental data show that “pure” shear exposure during normal commercial unit operations should not cause antibody unfolding or aggregation. AFM-based estimates of the forces required to unfold proteins suggest that shear rates greater than 10^7 s^{-1} would be needed to generate the forces required to fully unfold a protein. The trace amount of reversible aggregates we observed in the stainless steel parallel-plate rheometer was most likely caused by a synergistic effect of adsorption of the mAb to stainless steel and shear. Forces exerted on proteins adsorbed at the air-water interface, however, are within the range needed to cause protein unfolding. Aggregation observed during the production of protein therapeutics is therefore not likely caused by shear per se, but rather by the air-bubble entrainment, exposure to solid surfaces, contamination by particulates, or pump cavitation that is often associated with shear.

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Table 1

Results of the characterization of mAb-AS solutions exposed to high shear in the capillary rheometer. The tolerances are the instrument standard deviations of three replicate sample readings.

Buffer	[mAb-AS] (mg/mL)	Maximum Shear Rate (s^{-1})	Monomer recovery by SEC (%)	Soluble oligomers (%)		MW of mAb species detected by AFFF-MALS (kDa)	DLS z-average diameter (nm)
				SEC	AUC		
A	108	0	100.0 ± 0.1	1.85 ± 0.08	Not done	139 (peak)	9.0 ± 0.1
A	108	150,000	102.3 ± 0.1	1.88 ± 0.06	0.9 ± 0.9	140 (peak)	10.0 ± 0.4
B	1.0	0	100 ± 1	1.74 ± 0.03	0.0 ± 0.0	Not done	11.0 ± 0.2
B	103	0	100.0 ± 0.5	2.04 ± 0.02	Not done	142 (peak) 284 (shoulder)	11.4 ± 0.1
B	103	150,000	100.1 ± 0.4	2.06 ± 0.04	0.0 ± 0.0	143 (peak) 277 (shoulder)	11.3 ± 0.1
B	103	250,000	99.6 ± 0.2	2.07 ± 0.04	0.7 ± 0.7	142 (peak) 288 (shoulder)	11.3 ± 0.1

Table 2

Comparison of the shear rate, exposure time, shear stress, maximum force, and cumulative shear stress applied in our experiments with an estimate of the shear forces applied to a protein solution during filling. A viscosity of 3 mPa·s was assumed in calculations. The average cumulative shear stress and forces were calculated with a factor of two-thirds and a half applied to the shear rate to account for the average shear rate in space and the elongational fraction of the applied force respectively. The maximum force was calculated with a factor of 3 applied to the maximum fluid shear rate to account for the locally higher maximum shear rate applied to a particle in shear flow. The measured forces from AFM unfolding studies and calculations of forces at the air-water interface are included for comparative purposes. See discussion for details of calculations.

	Maximum Shear Rate (s^{-1})	**Exposure Time (ms)**	**Average Shear Stress τ (Pa)**	**Maximum Applied Force (pN)**	**Average Cumulative Shear Stress: Shear Stress × time (Pa·s)**	**Average Cumulative Force: Force × time (N·s)**
Filling through a 20 gauge needle at 0.5 mL/s	20,000	50	20	0.06	1	0.3×10^{-15}
Parallel plate	20,000	300,000	20	0.06	6000	2000×10^{-15}
Parallel plate	20,000	10,000	20	0.06	200	70×10^{-15}
Capillary rheometer	150,000	50	150	0.5	8	3×10^{-15}
Capillary rheometer	250,000	30	250	0.8	8	3×10^{-15}
AFM unfolding[a]	NA	200[a]	NA	150[a]	NA	$15{,}000\times10^{-15}$
Air-water interface forces[b]	NA	NA	NA	140[b]	NA	NA
Shear rate to achieve 150 pN max. force	5×10^{7}	NA	NA	150	NA	NA

[a] Calculated using data from the AFM literature (Best et al. 2003).

[b] Calculated using data contained in “Physical chemistry of surfaces” (Adamson and Gast 1997).

Table 3

Results of the characterization of a highly aggregated mAb-X solution in buffer C after exposure to high shear in the capillary rheometer. The tolerances are the instrument standard deviations of three replicate sample readings.

Aggregated [mAb-X] (mg/mL)	Shear Rate (s^{-1})	Soluble oligomers (%)	
		SEC	AUC
34.1	0	17 ± 1	16 ± 1
34.1	150,000	19.1 ± 0.5	18 ± 2