Effects of shear on proteins in solution

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Section: Review

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Abstract

The effects of "shear" on proteins in solution are described and discussed. Research on this topic covers many decades, beginning with investigations of possible denaturation of enzymes during processing, whilst more recent concerns are how the quality of therapeutic proteins might be affected by shear or shear related effects. The paradigm that emerges from most studies is that shear in the fluid mechanical sense is unlikely by itself to damage most proteins and that interfacial phenomena are critically important. In particular, moving gas-liquid interfaces can be very deleterious. Aggregation of therapeutic proteins on nanoparticles shed from solid surfaces is a recent concern because of potential consequences on patient safety. It is clear that labeling such damage as "shear" is a mistake as this inhibits clear investigations of, and thinking about, the true causes of damage to proteins in solution during processing.

Keywords

Aggregates; enzymes; human therapeutics; interfaces; proteins; shear;

Introduction

Biological materials such cells and proteins are delicate and this is often considered to cause problems in bioprocessing. One such problem is "shear"; a term loosely used to mean "mechanical forces" or "hydrodynamic forces" and even more loosely, interfacial phenomena. Such shear effects are important in processing of proteins in solution e.g. in the manufacture of enzymes (Ghadge et al. 2005) and in the fermentation, purification, formulation, fill and finish operations for protein drug products (Rathore and Rajan 2008).

Damage to proteins, whether by fluid mechanical shear or related phenomena, might be caused by changes to their secondary and/or tertiary structures through unfolding, or by disruption of the quaternary structure of a multi-subunit protein, or by promotion of protein aggregation to give soluble or insoluble aggregates. This damage may be manifested as loss of enzyme activity or reduced therapeutic efficacy and increased immunogenicity of protein drug products.

Because most damage to proteins during processing will increase with higher fluid flow rates, for example in passage through a pump or ultrafiltration unit, it has not always been clear that any effects are truly attributable to shear in the fluid mechanical sense, which is caused by velocity gradients in moving liquids. In particular, loss or damage might be due to interfacial effects that also increase with flow rate. Without a clear understanding of proteins and their behavior during processing, the generic term "shear" might be invoked to explain loss or damage at gas-liquid interfaces or caused by material and process incompatibilities. Such casual thinking might lead to inappropriate palliative measures and late discovery of such effects could lead to significant losses of time and money in the manufacture of protein drugs, as well as potential risks to patients.

Following a brief discussion of early research in this field and some general background, recent literature concerning gas-liquid, solid-liquid and oil-water interfaces will be reviewed here. Studies concerning manufacture, formulation, filling, storage and delivery will be considered. Recent concerns about "shear" in microfluidic devices and during microencapsulation will be mentioned. Based on a significant body of literature, it will be suggested that fluid mechanical shear, interfaces and other adventitious effects are collective properties of any processing system. They should be considered together.

Background

The common measure of fluid mechanical shear is the shear rate, or sometimes the shear stress. It is difficult to identify typical industrial shear rates and exposure times, because they vary tremendously within processing equipment and there may be significant, usually unidentifiable, "hot spots". As described below, it is usually easier to define shear rates in laboratory equipment in which most studies are undertaken.

Early work on shear damage to proteins began with a study by Charm and Wong (1970). Activity loss of catalase and carboxypeptidase solutions in a narrow gap coaxial cylinder viscometer, and in flow through a cylindrical tube, was attributed to breakage of tertiary structure by shearing. Narrow gap coaxial cylinder viscometers (Figure 1a) are commonly used for laboratory shearing studies, along with parallel plate and cone

Figure 1 here

and plate viscometers (Figure 1b). Flow through capillary tubing has also been popular, simulating flow through pipework and other processing equipment.

Charm and Lai (1971) found that shearing inactivated catalase during circulation of a solution through ultrafiltration devices but rennet did not appear to be damaged. This was attributed to recovery of tertiary structure and activity on standing. It was claimed that the important parameter was $\dot{\gamma}\theta$ i.e. the shear rate, $\dot{\gamma}$, multiplied by the time of exposure, θ. Values of this parameter up to 10^6 were tested and were enough to cause significant loss of activity. Tirrell and Middleman (1975) considered that exposure of an enzyme to a hydrodynamic flow field might distort an enzyme molecule causing a temporary loss of activity. They studied urea hydrolysis by urease *under* shear and reported both temporary and permanent inactivation. Charm and Wong (1981) summarized work on shear inactivation of a number of enzymes (and interferon, heparin and fibrinogen) in capillary flow and claimed that turbulent flow caused greater losses than those expected from streamline flow under the same conditions. These studies suggested that proteins in solution are susceptible to shear (as described by the parameter $\dot{\gamma}\theta$) and that this should be considered in the design of enzyme processing equipment.

However, Thomas et al. (1979) showed that alcohol dehydrogenase at approx. 1 mg ml^{-1} was not significantly inactivated when sheared at 30˚C in a coaxial cylinder viscometer at 683 s^{-1} for up to 5 h ($\dot{\gamma}\theta \sim 10^7$). At higher shear rates and temperatures, there was still little loss of activity although some aggregation occurred. Thomas and Dunnill (1979) found a similar lack of shear damage using catalase and urease and also no significant losses of activity in a capillary even at shear rates up to 10^6 s^{-1}. It was concluded that shear alone does not readily damage proteins and that other effects such as air/liquid interfacial inactivation must be occurring in conjunction with the shear. Even in a small agitated reactor with a notional shear rate of 9000 s^{-1} there was only a small loss in activity over 15 h ($\dot{\gamma}\theta \sim 5 \times 10^8$), provided air was excluded. Virkar et al.(1981) extended concentric cylindrical viscometer studies to 26000 s^{-1} and no loss of alcohol dehydrogenase activity was observed in 1h ($\dot{\gamma}\theta \sim 10^8$). A variety of pumps was also studied (with air excluded) and again negligible damage was observed up to 1500 passes. It was concluded that damage to proteins in a single pass through a (properly primed) pump will be negligible and even in operations involving recycle, damage will be small for a realistic number of passes. Nevertheless, as Narendranathan and Dunnill (1982) noted, damage to proteins during industrial-scale ultrafiltration is observed and seems to correlate with pumping rate. Shear is naturally assumed to be the cause, but it is very common in such systems for air to be entrained in the pump. In this circumstance, gas-liquid denaturation may be an important damaging mechanism.

In support of this idea, acid phosphatase was denatured in laminar flows in the presence of a gas-liquid interface (Donaldson et al. 1980) and that the denaturation of β-lactoglobulin by shaking its solution could be reduced to a very low level by the addition of surfactants or large polymers such as polyethylene glycol (Reese and Robbins 1981). Subsequently, Lee and Choo (1989) showed that the rate of denaturation of a lipase in a stirred tank reactor decreased significantly in the presence of polypropylene glycol or when the reactor was completely filled. It was concluded that inactivation of lipase is a shear-induced interface effect. Similarly, Maa and Hsu (1997) looked at the synergistic effects of shear and gas-liquid interfaces on recombinant human DNAase and recombinant human growth hormone in a coaxial cylinder shearing device and a rotor/stator homogenizer (shear rate times exposure time up to 2×10^7). In either case there was no effect on the rhDNase solution, which remained clear, but irreversible aggregation of recombinant human growth hormone was observed, rising with air-liquid interfacial area and protein concentration. The extent of this aggregation did not depend consistently on the level of shear. One might conclude that no single device could predict the behaviour under “shear” of

these proteins. Maa and Hsu (1997) also found foaming could be very detrimental but could be prevented with antifoam.

The paradigm that emerged from these studies is that a moving gas-liquid interface is probably required to cause "shear" damage to most globular proteins in processing. Proteins might be denatured at the interface and then mixed back into the solution as the interface is continually renewed. Lencki et al.(1993) showed that shear might enhance chemical or thermal enzyme inactivation, possibly by promoting coagulation of denatured protein, reducing renaturation. There may also be other effects attributed to "shear"; for long exposure times, one might worry about heavy metal, plasticizer, lubricant or sealant contamination, or local hot spots near an impeller bearing, and it has been known for a long time that proteins can also be denatured at solid-liquid interfaces (Sandwick and Schray 1987). In experimental work it is very difficult to control for adventitious effects but fortunately the conditions and times of exposure in industrial equipment will generally not be as extreme as those employed experimentally. However, it is important that unnecessary air-liquid interfaces are avoided in processing, for example by ensuring pumps are probably primed. Unfortunately, solid-liquid interfaces are unavoidable and may be very important in some processing operations and when protein solutions are stored for long periods, as discussed later.

Since this early work, there have been many publications concerning the effects of shear on proteins in solution, and the debate has continued. A excellent summary can be found in Jaspe and Hagen (2006), who showed that there was no evidence that shear rates up to ca. 2×10^5 s^{-1} in a silicon capillary tube destabilized (ferric equine) cytochrome c. In another thorough study, this time on immunoglobulin-G1, Bee et al. (2009a) sheared high concentration solutions in a parallel plate rheometer at a shear rate of 20000 s^{-1} (with exposure times up to 300 s) and in a capillary rheometer at shear rates up to 250000 s^{-1} (with exposure times up to 30 ms). Shear alone did not cause aggregation although prolonged exposure resulted in some minor, reversible aggregation. It was concluded that air-bubble entrainment, adsorption to solid surfaces, contamination by particulates or pump cavitation stresses are much more important than shear. Although the same general conclusion has been reached by most workers, there remain dissenting voices. In particular, there has been a series of studies by J.B. Joshi and coworkers. For example, Ghadge et al.(2005) studied cellulase deactivation in closed stirred tanks with several impeller types and a range of power inputs. The deactivation was correlated with the "average turbulent normal stresses", which are discussed later. It is difficult to criticize the methodology of these workers, who

claim to have excluded an air-liquid interface and who used a reasonable protein concentration. Although the temperature was high (50˚C) and the agitation extended (6 h), a thermostable enzyme was used. Even if adventitious effects such as solid-liquid interfacial effects or the accidental introduction of air had occurred, which is likely, it would be surprising if these could have been correlated with "average turbulent normal stresses". However, there are reasons to think that proteins are not affected by turbulence alone, as discussed later. The identification by Elias and Joshi (1998) of some proteins that can be damaged by shear alone remains contentious, and at least in some cases was based on a misrepresentation of the literature (e.g. Maa and Hsu (1997) looked at the *synergistic* effects of shear and gas-liquid interfaces, not shear alone). The activity of lipase and lysozyme, which were identified as needing an air-liquid interface and relatively low enzyme concentrations for deactivation (Mohanty et al. 2001), was lost by first order processes in a partly filled stirred tank (Patil et al. 2000). The rate constant depended on the power input per unit volume and the hold-up, as might be expected for an interfacial effect. Similar results were obtained in a bubble column and in an inclined film (Ghadge et al. 2003), with the rate constant correlated with the volumetric mass transfer coefficient, k_La. It was claimed that polyethylene glycol could reduce inactivation in stirred tanks (Patil et al. 2000), and that it might be helpful in manufacture to reduce the fermenter impeller speed during the stationary phase.

It is worth considering that most "shear" experiments use low viscosity, water-like, solutions. However, some enzyme and human therapeutic protein formulations (e.g. high protein concentrations >50 mg ml^{-1}) may be very viscous, as is blood plasma, in which case shear *stresses* ought to be considered. van der Veen et al. (2004) studied inactivation of α-amylase in a highly viscous solution by simple shear in a customized cone and plate viscometer. The maximum shear stress was equivalent to a shear rate of $\sim 10^7$ s^{-1} in aqueous solution. It was discovered that enzyme inactivation correlated non-linearly with shear and time but only after a shear stress of 25 kPa was exceeded. It may be significant that this work was performed at temperatures up to 110ºC where temperature-related adventitious effects might be anticipated.

In passing, Harrington et al. (1991) showed for several immobilised enzymes, shear had no effect on the maximum reaction velocity nor on the Michaelis constant. This suggested that shear at levels found in industrial processes were unlikely to distort the structure of a globular protein significantly despite the earlier report of Tirrell and Middleman (1975).

Under the paradigm that that interfacial effects are probably required to cause "shear" damage to most enzymes (globular proteins) in processing, the claims of Charm and Wong (1981), Charm and Lai (1971), Charm and Wong (1970) and possibly Tirrell and Middleman (1975) must be considered incorrect. It is interesting that these workers found inactivation to be time-dependent and very low enzyme concentrations were used in at least some of the cited studies. Both are consistent with adventitious effects. If inactivation is indeed through interfacial effects, it might be expected that fractional loss of activity would drop with increasing protein concentration. This was observed by Thomas et al. (1979) and was confirmed for cellulases by Kim et al. (1982). It would seem advisable to maintain high protein concentrations during laboratory experiments (and processing) to minimize the fractional damage.

It is clear that understanding the effects of "shear" on proteins in solution is important, not only because processing of enzymes continues but because of the large increase in the production of monoclonal antibodies and other human therapeutic proteins, particularly in the last decade, and more recently production of virus-like particles (VLPs). Formulation, filling and finishing operations are of great importance (Rathore and Rajan 2008), and there are potential shear effects in drug delivery e.g. in syringes. The high protein concentrations required in some drug formulations e.g. for high subcutaneous dosing can result in major challenges in manufacturing and it is interesting that many formulations of human therapeutics are labeled, "Do not shake". It is clear that particulate matter, such as protein aggregates, is undesirable in therapeutics because of the resulting increased immunogenicity (Sauerborn et al.2008; Schellekens and Jiskoot 2006; Schellekens 2002; Fradkin et al. 2009; Fradkin et al.2009; Rosenberg 2006). Even with enzymes, there are increasing reports of their use in oil-aqueous systems where different interfacial effects might be implicated in loss of activity. Organic solvents might be used to improve solubility of hydrophobic substrates, but may lead to deleterious effects on enzymes. The key papers are reviewed below, starting from the view that interfaces are of vital importance in "shear" damage. Other reviews are available and may be worth consulting (Patro et al. 2009; Rathore and Rajan 2008) and there are short but excellent recent summaries by Jaspe and Hagen (2006) and Bee et al. (2009a). Although protein adsorption and denaturation on solid surfaces is important in the biocompatibility of biomedical devices, such concerns are excluded from the discussion, except where protein losses on solid surfaces might be implicated in "shear" effects on proteins.

General Aspects

Most published work on "shear" of proteins in solution is experimental but a few papers refer to the thermodynamics and kinetics of protein unfolding and some to underpinning fluid flow theory. Following unfolding at an interface, proteins may aggregate, which may be particularly undesirable in therapeutics. These general aspects are briefly reviewed here.

Cromwell et al. (2006) reviewed protein aggregation in bioprocessing, noting that aggregates may arise from several mechanisms and be classified in numerous ways. Firstly, reference is often made to "soluble" aggregates, which the authors define as neither visible nor removable by a 0.22 μm filter, unlike insoluble aggregates. Both types are of concern in the production of therapeutic proteins. A second distinction is between covalent and non-covalent aggregates, where the former arise from covalent bonding between two or more monomers. This type of aggregation is likely to be stable, whereas non-covalent interactions between monomers may be easily reversible. Finally, Cromwell et al. (2006) discuss whether denaturation is a prerequisite for aggregation, as is commonly believed, and point out that small perturbations in protein structure may expose hydrophobic surfaces, causing self-interactions and consequential aggregation..

Iyer and Ananthanarayan (2008) have reviewed the literature concerning enzyme stability. Inactivation (*in vitro*) is a two step process in which enzymes unfold reversibly before being irreversibly inactivated. Thermodynamic or conformational stability confers resistance to unfolding or partial unfolding of protein that remains in equilibrium with the native form. Sanchez-Ruiz (2010) reviewed protein kinetic stability, which determines longer-term, irreversible inactivation. In considering the former, Walstra (2001) suggested that the stability of globular proteins can be expressed as the free energy change between the folded and the unfolded state. For a small globular protein, the maximum value of this parameter is 20 to 65 kJ mol^{-1} or about 5×10^{-20} J per molecule. Walstra (2001) pointed out that larger proteins may have regions that can unfold independently. In any case, with such a low free energy change, suggesting marginal stability of the molecules, it is not unreasonable to consider that shear or interfacial forces might unfold a protein. According to Walstra (2001), a shear stress of 1 Pa would provide a deformation energy of about 10^{-25} J per molecule. This would imply a shear stress of about 5×10^{5} Pa would be required to denature a small globular protein. In water, this would require a shear rate of 5×10^{8} s^{-1}, far higher than any shear rate applied in experiments using shear alone. Similarly, Jaspe and Hagen (2006) estimated that a shear rate in water of at least 10^{7} s^{-1} would be required to

unfold cytochrome c with a free energy of unfolding of ca. 42 kJ mol^{-1}. On the other hand, Walstra (2001) used surface tension estimated that air-liquid interface adsorption of a 40 kDa protein at a surface concentration of 2 mg m^{-2} can impart energies of about 400 kJ mol^{-1} to the adsorbed protein, i.e. of the order of 10^{-18} J per molecule. This should mean that proteins can be unfolded at interfaces, as observed. Similar estimations by Bee et al.(2009a) suggest that the forces at air-water interfaces are about 140 pN, which was compared to the 20-150 pN required to unfold proteins using atomic force microscopy.

Although the resistance of a protein to unfolding, i.e. its thermodynamic or conformational stability, is typically described by the free energy of unfolding, Wierenga et al.(2006) concluded that the key parameter was actually the activation energy for unfolding. The fast unfolding of β-lactoglobulin, the slower rate for cytochrome c, and the slowest rate for ovalbumin was consistent with this concept.

The non-ionic surfactants, Tween 20 and 80, at the appropriate molar ratios can increase the free energy of unfolding by about 1 kcal mol^{-1} i.e. ca. 4 kJ mol^{-1} (Chou et al. 2005). This is significant compared to the 20 to 65 kJ mol^{-1} free energy of unfolding estimated by Walstra (2001) for a small globular protein. This may be a reason why surfactants like Tween confer some protection against interfacial denaturation, as discussed later.

It is relatively easy to estimate the forces on a molecule in simple shear, in for example a coaxial cylindrical viscometer. However, fluid flow in processing is often turbulent and it is sometimes claimed that turbulence can result in additional forces on molecules above those caused by the mean flow. For example, Joshi et al.(2001) and Ghadge et al.(2005) claimed that deactivation of some enzymes in processing equipment could be correlated with the "volume average turbulent normal stress". However, even these authors recognized that enzyme molecules are much smaller than the Kolmogorov length scale of turbulence, even at high local energy dissipation rates. At this scale, the energy carried by turbulent eddies is being dispersed as heat; indeed smaller eddies have little kinetic energy and are unlikely to cause shear damage to a protein. According to Ghadge et al.(2005), the maximum energy dissipation rate was about 150 kW m^{-3} at 1 kW m^{-3} power input per unit volume to a stirred tank. This gives a Kolmogorov microscale of ca. 10 µm, assuming isotropy. This implies that turbulence is meaningless at the scale of an enzyme. Even more bizarrely, Krstic et al. (2007) claimed that the effect of shear forces is expressed by the rotation of molecules in turbulence, where they absorb enough energy to break chemical bonds. There is simply no evidence for this claim.

Gas-liquid interfaces

There have now been many studies specifically investigating how proteins behave at gas-liquid interfaces. Four studies have concerned lysozyme. In the first two (Caussette et al. 1998, 1999), bubbling N_2 through a lysozyme solution strongly enhanced inactivation that otherwise depended on pH and temperature. Inactivation in a stirred reactor was a first order process that depended on the agitator power and the area of the various interfaces, including the gas-liquid interface (Colombie et al. 2001). Postel et al. (2003) suggested that the hydrophobic nature of the air-water interface caused partial deactivation of lysozyme on adsorption.

Concerning proteins other than enzymes, Sluzky et al. (1992) studied the kinetics of the aggregation of insulin in the presence of air-liquid interfaces. As mentioned above, such interfaces have a hydrophobic character, and it was suggested that partially denatured monomers interact with each other to minimize the surface energy by shielding exposed hydrophobic moieties from the aqueous environment. Insulin solutions could be stabilized by adding surface active agents such as sugar-based non-ionic detergents (which are primarily surfactants). In shaken vials, the formation of insoluble aggregates of recombinant human growth hormone could also be prevented by the addition of the non-ionic surfactant and common excipient Tween 20, at an appropriate molar ratio to the protein (Bam et al. 1998). Tween also seemed to protect a recombinant fusion protein (of human growth hormone and human albumin) from shaking in microcentrifuge tubes with an air surface present (Chou et al. 2005). From such studies it has been concluded that Tween and similar surfactants can protect proteins from air-liquid interfaces by binding to the protein surface (Chou et al. 2005; Bam et al. 1998). It might also compete with soluble protein aggregates for interfaces, preventing the formation there of insoluble aggregates (Kreilgaard et al. 1998). Tween is a common excipient used in biopharmaceutical formulations and used for this purpose.

Immunoglobulins are an important class of therapeutic proteins. Mahler et al. (2005) claimed that agitation in the presence of a (hydrophobic) gas-liquid interface caused aggregation of immunoglobulin-G1 (IgG1). In this case, Tween 80 seemed to stabilize small aggregates and prevent further aggregation. Besides antibodies such as IgG1, antibody fragments might also be susceptible to damage at air-liquid interfaces. Harrison et al.(1998) showed that a recombinant scFV antibody fragment suffered a first order loss of activity in a partially filled, agitated vessel. However, there may be a protective effect of antifoams in agitated fermentation broths.

Some insight into protein denaturation at gas-liquid interfaces might be obtained from studies on foaming. Clarkson et al. (1999a, b) suggested that more rigid proteins, such as lysozyme and catalase, display a lower surface activity and degree of damage than flexible proteins such as bovine serum albumin and pepsin, solutions of which can create stable foams. However, Pereira et al. (2003) suggest that bovine serum albumin is actually a "hard" protein, showing little molecular compressibility and little structural change at adsorption, whereas (charged) β-casein is a soft protein. Further work is needed to relate protein molecular compressibility, unfolding energy for unfolding, surface activity and ease of denaturation.

The conclusion of these studies is that many if not all proteins can be damaged at air-liquid interfaces, which should be avoided if possible, but also that some non-ionic surfactants may be effective at preventing damage. High protein concentrations should also be beneficial in reducing losses, at least relatively.

Solid-liquid interfaces

A general review of the mechanisms of protein adsorption on a solid surface is to be found in Nakanishi et al. (2001) and a broad study of the adsorption of eighteen proteins on a titanium oxide can be found in Imamura et al. (2008).

Sluzky et al. (1992) studied the kinetics of the aggregation of insulin in the presence of solid-liquid interfaces. For hydrophobic solid surfaces such as Teflon, the monomer was denatured at the surface followed by formation of microaggregates. This did not occur with hydrophilic surfaces e.g. glass. Insulin solutions could be stabilized against aggregation by adding surface active agents such as sugar-based non-ionic detergents.

Siedlecki et al. (1996) studied changes in the three-dimensional structure of human von Willebrand Factor (vWF) adsorbed onto a hydrophobic surface that consisted of a self-assembled monolayer of octadecyltrichlorosilane deposited on coverslips and sheared with an atomic force microscope or by flow (up to 4.2 Pa). vWF is a large, multimeric, globular, plasma glycoprotein. Initially, adsorbed protein was in its native conformation but a shear stress of about 3 Pa caused protein unfolding. This possibly corresponded to a shear rate of ca. 3200 s^{-1} in plasma, or ca. 1000 s^{-1} in whole blood, although of course this does not mean that shear alone at such levels would be effective in causing damage.

Colombie et al. (2001) suggested that polytetrafluoroethylene (PTFE) was much more effective at causing aggregation of lysozyme than glass, which confirms the view that hydrophobicity is the governing factor in these phenomena. Similarly, fibronectin maintained its native conformation on adsorption to a hydrophobic bare silica whilst suffering strong unfolding on hydrophobic polystyrene, particularly at low bulk concentrations (Baujard-Lamotte et al. 2008).

Biddlecombe et al. (2007) invented a shear device with a solid-liquid interface and no air-liquid interfaces. Shear rates up to 3.4×10^{4} s^{-1} could be achieved. This was used to show that a combination of shear and a solid-liquid interface could cause significant levels of immunoglobulin-G4 (IgG4) aggregation and precipitation. More recent work by Biddlecombe et al. (2009) demonstrated that the loss of monomeric IgG4 loss was correlated to surface roughness in the same shear device. The authors' hypothesis was that the rougher surface increases the surface area for protein unfolding to occur and shear contributes more to active transport to the surfaces.

As described more fully later, proteins can interact with subvisible solid particles shed from surfaces of processing equipment. Bee et al. (2009b) showed how a humanized IgG1 antistreptavidin interacts with such micro- and nano-particles. Adsorption to stainless steel microparticles was irreversible and led eventually to the formation of soluble protein aggregates. Exposure to air-solution interfaces also caused aggregation but independently of the effects of the microparticles.

Oil-water interfaces

It seems reasonable that protein damage can also occur at aqueous-organic interfaces. Several studies have been undertaken on enzymes in oil-water systems, particularly for enzymes that have organic substrates. Ross et al. 2000a, b) investigated the inactivation of several enzymes by 12 organic solvents in a bubble column. Although inactivation correlated with the area of solvent exposed and depended on aqueous-organic interfacial tension, as expected for an interfacial mechanism, no clear correlation with any enzyme property was observed. Baldascini and Janssen (2005) used a stirred cell to show that interfacial inactivation of a epoxide hydrolase in octane/water increased as mixing intensity increased. They proposed that this was due to an increase in the rate of desorption of inactivated enzyme molecules from the surface. On the other hand, some lipases are susceptible to interfacial

activation by structural rearrangements at the oil (substrate)-water interface (Otero et al. 2005). For these enzymes, interfaces would seem to be advantageous.

Jabbal-Gill et al. (1999) showed that a range of vaccine antigens were also damaged by exposure to organic solvents in the preparation of polymeric lamellar substrate particles (PLSP) as adjuvants. It is also likely that human therapeutic proteins might be damaged during microencapsulation, which often involves the use of organic solvents. Kwon et al. (2001) observed that during microencapsulation of recombinant human insulin, the water-organic interface caused aggregation. A 10-fold molar excess of SDS allowed recovery of conformational changes caused by the organic solvent methylene chloride and it was suggested this was due to the protective effect of the surfactant binding to the protein.

Industrial processing, formulation, filling and delivery

Filtration, mixing, pumping, filling, lyophilization, transport and delivery are common industrial processes that significantly expose enzymes and therapeutic proteins to fluid dynamic shear and interfaces. Failure to investigate and address these risks early in the development of a process for manufacture of a therapeutic protein can be costly and potentially compromising to quality. Rathore and Rajan (2008) provide a holistic overview of potential risks of importance during such product development. The following highlights specific examples of common problems where the root causes are under investigation.

In manufacturing processes, protein solutions are often concentrated and purified by ultrafiltration (UF), in which the solution is pumped through hollow fiber or other membranes through which some solution components and not others can permeate. Tangential flow filtration is often used to achieve very high concentrations of a monoclonal antibody for subcutaneous delivery. The result could be concentrations as high as 125 mg mL^{-1} at the membrane surface (Shire 2005). Losses in such cases are sometimes blamed on shear, but unfolding and aggregation at interfaces is more likely (Shire 2004, 2005). It is commonly believed that enzymes can be inactivated during ultrafiltration (and also in membrane reactors) by "shear", either in the pump or in the unit operation itself. For example, Krstic et al. (2007) claimed significant losses of endopectinase activity in laboratory-scale, cross-flow ultrafiltration were due to shear. However, the length of operation was very long, the protein concentration was relatively low, there was significant loss of activity even without flow ("shear"), and it is not at all clear that air was excluded. The authors, like many others, were determined to link

the activity losses to shear. This is dangerous without a much more careful study of potential adventitious effects. On the other hand, Bodalo et al. (2004) claimed enzyme inactivation in such systems is caused mainly by adsorption on the membrane surface, and Prazeres and Cabral (1994) suggested that enzyme leakage through the membrane, loss of enzyme activators and other effects such as local heating and air entrainment may also be significant. Paolucci-Jeanjean et al. (2001) also considered leakage and adsorption as the main reasons for activity loss, with the latter dominating. These workers recommend that the tangential velocity in membrane reactors is not decreased to reduce shear, as this unexpectedly resulted in higher enzyme inactivation. Finally, Paolucci-Jeanjean et al. (2001) and Portugal et al. (2008) purified β-lactoglobulin by ultrafiltration and suggested the impact of membranes may be more relevant when it is the permeate of commercial interest, as a retained protein would not be affected by passage through the membrane.

Pumps, such as lobe, peristaltic and/or piston pumps (Figure 2) are frequently used during microfiltration (e.g.

Figure 2 here

ultrafiltration and diafiltration) and filling operations and reportedly cause aggregation of proteins if the process and excipients are not carefully controlled. As mentioned earlier, Narendranathan and Dunnill (1982) found that damage to proteins during UF seems to correlate with pumping rate. Whilst aggregation is often observed during membrane filtrations, it is now accepted that this is attributable to micro-cavitation that occurs in the pumps and valve (van Reis and Zydney 2007). Albumin was observed to aggregate after repeated pumping through a lobe pump and subsequently, increased levels of aggregation were observed following long-term stability trials (Gomme et al. 2006 a, b). These authors claim that repeated exposure to shear in the lobe gap was likely the cause of the prolonged aggregation. The experimental apparatus was not devoid of air/solid interfaces and therefore, other possibilities may exist. In the same set of studies, aggregation was not seen for an IgG antibody, which suggests that not that (unsurprisingly) care needs to be taken in extrapolating the behavior of one protein in one set of circumstances to any other protein or system.

In a recent and related early development study, strong evidence was found of aggregation of virus-like particles adsorbed to an adjuvant during recirculation studies involving a peristaltic pump (Figure 3). In these studies,

Figure 3 here

increased back pressure in scaled down equipment demonstrated a proportional increase in aggregation, as measured by light scattering. While this result was initially attributed to pump “shear” increasing as a result of

higher back pressures, additional studies showed that if a different tubing material was used, the levels of aggregation were independent of back pressure. This result is interesting as peristaltic pumps are frequently advertised as a mechanism to minimize "shear" during processing. Thus, protein aggregation via a peristaltic pump is unlikely related to hydrodynamic shear mechanisms and requires further investigation.

Other potential sources of aggregation during and post-filling include contaminating particulates from the pump and equipment during processing e.g. during vial filling. Stainless-steel piston pumps are known to shed nanoparticles during operation that can lead to adsorption and subsequent aggregation of an IgG monoclonal antibody (Tyagi et al. 2009; Bee et al. 2009b) and indeed this may be a consequence of the micro-cavitation reported by van Reis and Zydney (2007). A key outcome of these studies was that particulates thought to provide substrates for aggregation in protein drug products were below the current particle size thresholds for which there is routine testing (e.g. in accordance with USP <788>). They may therefore go undetected. Fortunately, aggregation was very fast and in practice any increases in particle counts would be detected during screening and characterization tests. Furthermore, not all protein therapeutics will be susceptible to particulate induced aggregation and it may be possible to inhibit agitation-induced aggregation by careful choice of excipient (Serno et al. 2010). Nonetheless, it seems wise that aggregation should be evaluated routinely as part of early formulation development and that new and improved assay methods be developed to determine the quantity of nanoparticles in products. In this context, it should be noted that high throughput detection of IgG aggregation using extrinsic fluorescence has been reported (He et al. 2010).

As part of aseptic processing, protein solutions are filtered. It has been shown recently that IgG1 and the excipient polysorbate 80 can adsorb to sterilizing filters, and it was recommended that this adsorption should be considered in manufacturing and formulation (Mahler et al. 2010). Besides the effects of steel nanoparticulates mentioned earlier, other solid interfacial interactions during storage and delivery can result in losses, for example of monoclonal antibodies to surfaces of glass vials. This may require significant, compensating overage (Shire et al. 2004) which is costly and creates a potential risk of overdosing. Similarly, losses of recombinant factor VIII by adsorption onto PVC container surfaces during storage have been observed (McLeod et al. 2000). There may even be losses to container closures (Sharma 2007) and residual metals (e.g. tungsten in syringes; Bee et al. 2009c) are known to interact with protein products, causing aggregation. Syringes, stoppers and other surfaces are treated with silicone oil for lubrication or to inhibit protein binding but silicone oil can actually induce protein aggregation (Jones et al. 2005; Thirumangalathu et al. 2009). Silicone oil emulsions also induce

loss of soluble protein, probably through adsorption onto silicone-oil droplet surfaces (Ludwig et al. 2010). Finally, therapeutic proteins in solution are sometimes delivered to patients through catheters using portable pumps. Tzannis et al. (1996) showed severe activity losses by irreversible structural changes of interleukin 2 by adsorption to catheter tubing. It is clear that there are many ways in which proteins may be lost or damaged though interaction of proteins with solid surfaces.

Most therapeutic proteins are delivered in vials that will be accidentally or deliberately shaken between filling and delivery. Kiese et al. (2010) showed that shaking vials or heat could cause IgG1 aggregation but that the insoluble aggregates caused by the former could reversibly dissociate into soluble aggregates. This aggregation was probably due to the air-liquid interface in the vials. Brych et al. (2010) showed that eliminating the headspace prevented the aggregation of IgG A in shaken vials and this may be a better approach than a label saying "Do not shake". Of course, it may not always be practical to fill vials to capacity and therefore an alternate format (e.g. vacuum filled syringe) may be recommended.

Conclusion

This review has summarized the literature concerning "shear" damage to both enzymic and therapeutic proteins. It is clear that hydrodynamic shear alone is rarely or never the cause of damage or losses. In careful studies, interfacial effects are generally discovered to be the predominant mechanisms. Whilst such effects may be of commercial importance in enzyme processing, potentially adverse consequences on patient safety mean that it is critical that "shear" damage to therapeutic proteins is understood and controlled, as far as is practical To this end, fluid mechanical shear and interfacial and other adventitious effects should be considered collective properties of any processing system. The compatibility of a particular drug and formulation with the processing system should be evaluated early in the development cycle and studies should be designed carefully prior to clinical evaluation with the knowledge of potential interactions and processing conditions upon scale up. In this way the quality and safety of the commercial product might be ensured. Mahler et al. (2009) describe some current techniques for detecting and characterizing aggregation, and some "emerging technologies", whilst Bee et al. (2009b) suggest a protocol for assessing the sensitivity of therapeutic proteins to surfaces found in processing equipment and during storage. Table 1 shows a list of recommended screening studies that can be

Table 1 here

used to assess product sensitivity to shear forces and materials that are common in biopharmaceutical processes. Data acquired from the studies can be used to aid formulation design (e.g. addition of surfactants) and/or

equipment selection. Of course, these studies should be implemented by individuals who have knowledge of the potential equipment/conditions and who know (for example) to take precautions to eliminate air interfaces and other adventitious effects, where these variables are not being evaluated directly. Further evaluation of molecular properties via characterization testing may eventually provide crucial information to support such empirical approaches.

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Fig. 1: Viscometers commonly used for shear studies: (a) narrow gap coaxial cylinder viscometer (Contraves), (b) cone and plate and parallel plate viscometers (Malvern Instruments).

Fig. 2: Pumping systems for vaccines and therapeutic protein formulations: (a) peristaltic pump (Ismatec FTM300), (b) stainless steel piston pump (Bausch and Stroebel), (c) lobe pump (Unibloc 300/350).

Fig. 3: Small scale pump and tubing recirculation study for drug formulation of virus-like particles adsorbed to adjuvant. Aggregation was observed as measured by dynamic light scattering after a significant number of recirculation events (denoted as dimensionless time: Q – flow rate; t – time; V – formulation volume). Platinum-cured silicone (Tubing #1) and polypropylene based tubing with USP mineral oil (Tubing #2) were used in a peristaltic pump and ~25 ml of drug formulation was recirculated at 90 ml min^{-1} for 24 hours. Solid lines indicate low pump pressure and dotted lines indicate higher outlet pump pressure (~3.5 psi). All pressures are gauge.

Data from R Mahajan, M Walker, E Walker, PK Yegneswaran and D Geer, Merck, Sharp and Dohme Corp (2007): Process development approach to assess the effect of formulation and filling process on physical stability of biological liquid formulations, (http://aiche.confex.com/aiche/2007/techprogram/P94697.HTM).

Table 1 – Common laboratory scale screening methods to evaluate pre-market drug product formulations and their sensitivity to potential unit operations.

Lab Scale Screening Methods	Product Unit Operations	Common Scale-Dependent Parameters	Potential Solutions
Direct agitation (i.e. mixing studies)	Mixing, resuspension	◊ RPM (i.e. tip speed) ◊ Geometric similarity (e.g. fluid height to vessel diameter) ◊ Power input and unit	◊ Floating stir bars for small scale ◊ Large batch volumes and concentrating bulk ◊ Minimize mixing speed

		volume	
Indirect agitation (i.e. product shaking)	Shipping and handling	◊ Pressure ◊ Vibrational frequency ◊ Headspace / fluid volume	◊ Enhanced secondary packaging ◊ Lyophilization
Pump studies	Filtration and filling	◊ Flow rate, pressure ◊ Pump type ◊ Material composition and dimensions	◊ Screen materials (e.g. tubing material) ◊ Slower flow rates ◊ Time-pressure filling system
Mock drug delivery and dosing	Delivery to patient	◊ Syringe characteristics (e.g. silicone/tungsten, needle size) ◊ Headspace composition / volume ◊ Catheter / IV set materials	◊ Optimize needle size ◊ Specialized syringes (e.g. baked-on silicone, luer-lock, low tungsten) ◊ Screen materials
Material compatibility	All	◊ Volume / surface area ◊ Material composition (e.g. filter and vial glass/elastomer selection) ◊ Headspace composition ◊ Storage conditions	◊ Additional drug overage (not recommended) ◊ Surfactants ◊ Optimize materials via stability screening ◊ Lyophilization

Fig 1a:

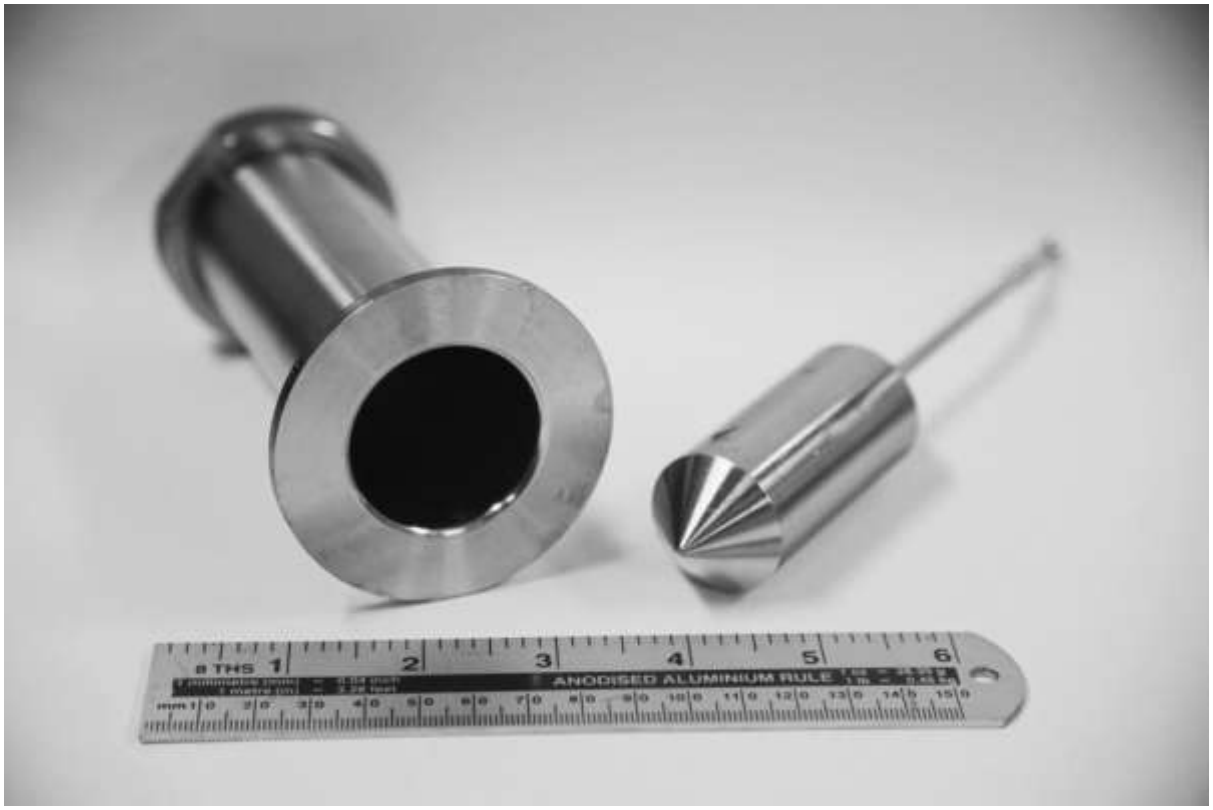

8 THS
ANODISED ALUMINIUM RULE

Fig 1b:


8 THS
ANODISED ALUMINIUM RULE

Fig 2a:

Fig 2b:

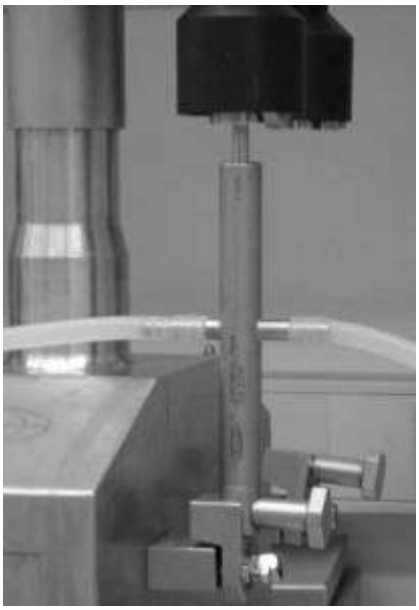


Fig 2c:

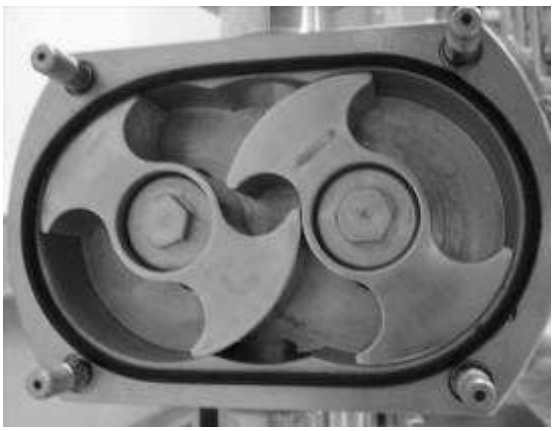
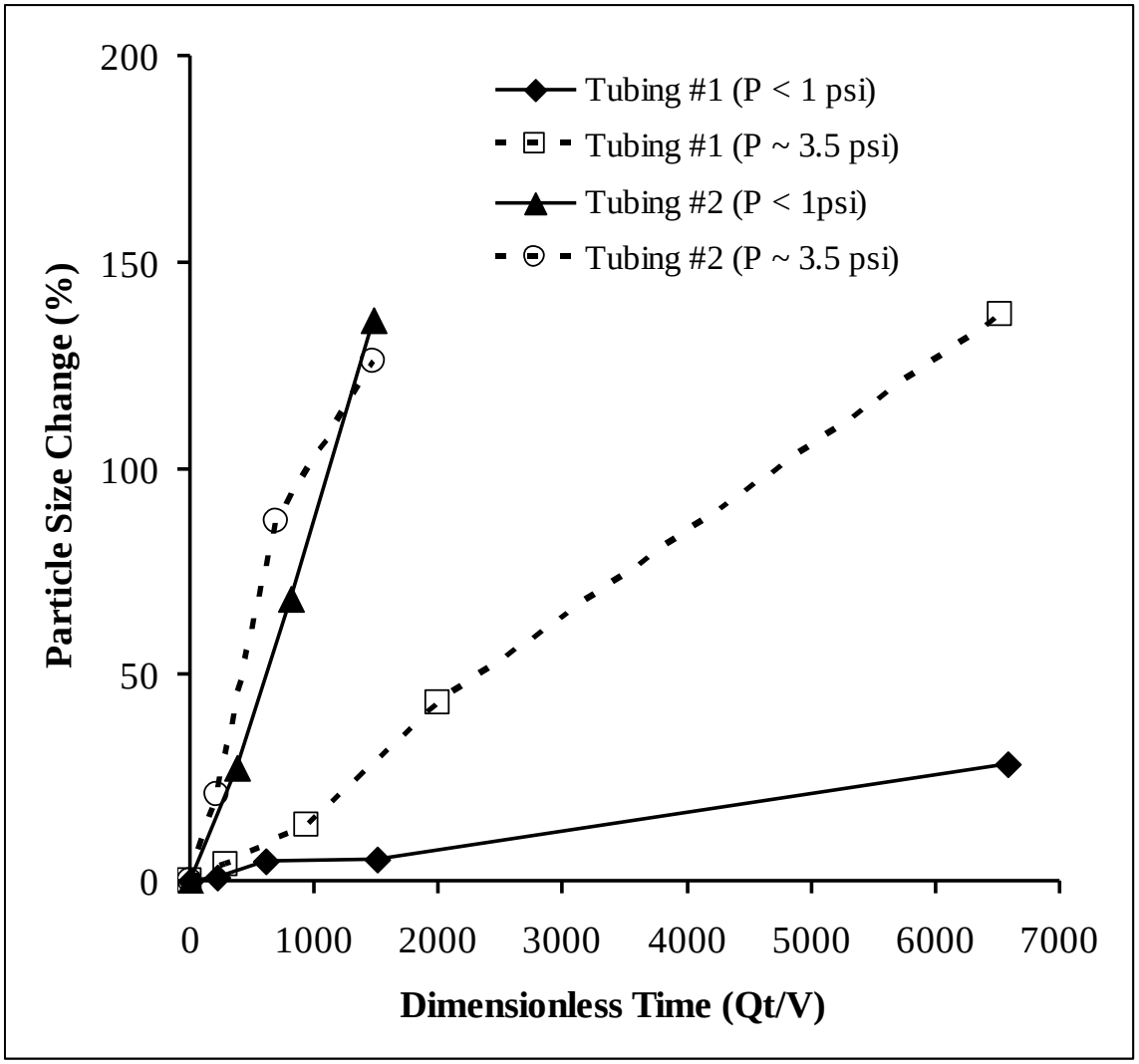


Fig 3:


Tubing #1 (P < 1 psi)
Tubing #1 (P ~ 3.5 psi)
Tubing #2 (P < 1psi)
Tubing #2 (P ~ 3.5 psi)
Particle Size Change (%)
200
150
100
50
0
0
1000
2000
3000
4000
5000
6000
7000
Dimensionless Time (Qt/V)