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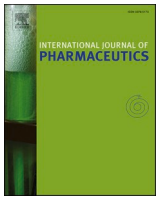

Review

Shear stress as a driver of degradation for protein-based therapeutics: More accomplice than culprit

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ABSTRACT

Protein degradation is a major concern for protein-based therapeutics. It may alter the biological activity of the product and raise the potential for undesirable effects on the patients. Among the numerous drivers of protein degradation, shear stress has been the focus around which much work has revolved since the 1970s. In the pharmaceutical realm, the product is often processed through several unit operations, which include mixing, pumping, filtration, filling, and atomization. Nonetheless, the drug might be exposed to significant shear stresses, which might cooperatively contribute to product degradation, together with interfacial stress. This review presents fundamentals of shear stress about protein structure, followed by an overview of the drivers of product degradation. The impact of shear stress on protein stability in different unit operations is then presented, and recommendations for limiting the adverse effects on the biopharmaceutical formulations are outlined. Finally, several devices used to explore the effects of shear stress are discussed.

1. Introduction

Process validation is required in the biopharmaceutical industry to verify product conformity and safety (Rathore and Sofer, 2005). Understanding and controlling each stage of the process is thus mandatory (Conner et al., 2014; Shire, 2009), with particular attention to those aspects that can lead to out-of-specification products. A key aspect of the biopharmaceutical industry is the preservation of biotherapeutic stability, which guarantees drug safety and efficacy. The stability of a pharmaceutical product is defined in the World Health Organization (WHO) guidelines on stability testing of pharmaceutical products (WHO, 1996) as "the ability of a pharmaceutical product to retain its chemical, physical, microbiological and biopharmaceutical properties within specified limits throughout its shelf-life". A product may be sensitive to numerous stability stressors, such as temperature, pH or ionic strength changes, oxidation, light exposure, interfaces, and shear (ICH, 1996). In particular, shear has received considerable attention due to its frequent occurrence in manufacturing lines. Exposure to shear stress conditions was postulated to be responsible for aggregation and particle formation (Wang et al., 2010), which can have side effects on humans or lead to drug instability issues (Brange and Havelund, 1983; James et al., 1981; Lehr et al., 2002; Zweifach, 1955). In addition, it could cause a decrease in drug potency due to a lower dose in solution or induction of immunogenicity (Ratanji et al., 2014; Rosenberg, 2006; Schellekens, 2005), with consequences ranging from patient discomfort to permanent damage and potential death (Hoots, 2006; Reipert et al., 2007). Since the immune system is thought to more easily recognise aggregates than the native parent protein (Den Engelsman et al., 2011), the product may be unacceptable if there is even a small amount of aggregates (Carpenter et al., 1999). For such reasons, products must be tested according to the US and European Pharmacopoeias's procedures for subvisible ("Particulate contamination: Sub-visible particles," 2016; USP 788, 2006) and visible particles ("Particulate contamination: visible particles," 2008; USP 29-NF 24, 2006), to make sure that the product is suitable for administration to patients.

Along with shear stress, interfacial stress is another potential contributor to product instability, whose control is not straightforward. Aggregation of therapeutic proteins can result from stresses encountered at vapour-liquid, solid–liquid, and liquid–liquid interfaces (Babinchak and Surewicz, 2020; Duerkop et al., 2018a; Perevozchikova et al., 2015). A recent theory holds that the loss in product stability in a bioprocessing line is due to the combined effect of interface and shear stress, and it is unlikely that shear stress alone causes protein aggregation (Nesta et al., 2017). Nevertheless, it is practically difficult to deconvolute the effects of shear and interfacial stress on product stability. Much work has been invested in figuring out how to evaluate the impact of shear stress on protein stability. In this sense, separating shear

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Nomenclature

D_i	inner nozzle diameter, m
K	unfolding kinetic constant, -
N	rotational speed, s^{-1}
Q	volumetric flowrate, m^3/s
r	distance from the centre of the pore, m
R	universal gas constant, J mol^{-1} K^{-1}
R_i	inner cilinder radius, m
R_o	outer cilinder radius, m
SH	shear history, -
t_{exp}	exposure time to shear rate, s
T	temperature, K
u_{liq}	liquid velocity at the point of atomization, m/s
u_{av}	average velocity at the mixing point, m/s

Greek letters

γ	shear rate, s^{-1}
$\bar{\gamma}$	average shear rate, s^{-1}
δ_{ij}	Kronecker delta, -
ΔG^0	unfolding Gibbs free energy difference, J mol^{-1}
θ	cone angle, deg
τ	viscous stress, Pa
ω	angular velocity, rad/s

Subscripts

lam	laminar
turb	turbulent

Abbreviations

BSA	Bovine Serum Albumin
CD	Circular Dichroism
CFD	Computational Fluid Dynamics
DLS	Dynamic Light Scattering
DNS	Dinitrosalicylic acid
DSC	Differential Scanning Calorimetry
DSF	Differential Scanning Fluorimetry
HPLC	High-Performance Liquid Chromatography
HSA	Human Serum Albumin
MAB	Monoclonal antibody
MF	Microfiltration
MFI	Micro-flow imaging
NS	Native State
NMR	Nuclear Magnetic Resonance
NTA	Nanoparticle Tracking Analysis
PIV	Particle Image Velocimetry
PLL	Poly-L-lysine
SANS	Small-Angle Neutron Scattering
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SE-HPLC	Size-exclusion High-Performance Liquid Chromatography
SEC	Size Exclusion Chromatography
SEC-MALS	Size Exclusion Chromatography-Multiangle Light Scattering
SVP	Subvisible Particle
TEM	Transmission Electron Microscopy
TFF	Tangential Flow Filtration
UF	Ultrafiltration
US	Unfolded State
USP	United States Pharmacopoeia
UV-vis	vis Ultra-Violet visible
WHO	World Health Organisation

forces from other denaturation factors, mainly solid–liquid and air–liquid interfaces, poses unique challenges since it is difficult to quantify shear stress under interface-free conditions. The values of the shear stress threshold appear not only dependent on the determination method, i.e., the flow condition in the adopted device, but also on the evaluated product (Sieck et al., 2013). For instance, Brückl et al. found that shear stress was unlikely to unfold rhGH, IgG1 up to shear rates of at least 10^4 s^{-1} under interface-free conditions (Brückl et al., 2016b). Nonetheless, in another work (McBride et al., 2015), even low shear rates were able to promote aggregate formation for human insulin. If finding the shear stress threshold experimentally is not straightforward, molecular dynamics (MD) approaches could potentially come to the rescue to study protein behaviour upon shear. A recent study focusing on the rotational dynamics of a small protein, i.e., ubiquitin, under different shear flows highlighted that MD can help understanding the effects of mechanical stress induced by shear flow on proteins (Papež et al., 2023).

Nevertheless, the actual contribution of interfacial and shear stresses on protein degradation is still under debate, as it is difficult to deconvolute these two factors experimentally or *in silico*. Particularly given that these two sources of stress are frequently encountered in several critical stages of the biopharmaceutical manufacturing process.

This article summarises the impact of multiple industrial operations involving high levels of shear, i.e., mixing, pumping, filtration, filling, and atomization, on the final product quality. In particular, relevant studies dealing with shear-induced protein aggregation and particle formation are presented. Recommended mitigations for limiting undesired effects on the product are also discussed, and an overview of the main techniques used to assess viscous-stress-induced instability is provided.

2. Background

2.1. Shear stress

Various forces can be exerted on a fluid element. Among these, it is worth mentioning viscous stresses, which are defined as a force per unit area acting on a fluid element and are commonly expressed in Pa. These stresses can be classified as normal stresses, if applied perpendicularly to the surface or shear stresses, if applied tangentially to the surface and generated by velocity gradients in the fluid.

For clarity, Fig. 1 shows the viscous stresses that can act on an element isolated from a generic fluid.

The viscous stress is a tensor comprising three normal components and six shear components. The fluid velocity gradient is defined as the shear rate (γ), which has units of inverse time (s^{-1}) (Bekard et al., 2011a).

The forces that the fluid in motion exerts on the surface of an immersed body (i.e., a protein) are the integral over the surface of the viscous shear and the normal stresses.

In most cases, the rheological behaviour of formulations containing therapeutic proteins can be assimilated to that of Newtonian fluids, except for highly concentrated drug products, which may exhibit shear thinning behaviour (Rathore et al., 2012b). For such non-Newtonian fluids, a non-monotone relation exists between the shear stress and the shear rate (Malkus et al., 1990).

2.2. Protein structure

Proteins are complex structures made of polymer chains, whose building blocks are named amino acids. It is essential to distinguish configuration and conformation when dealing with protein structure.

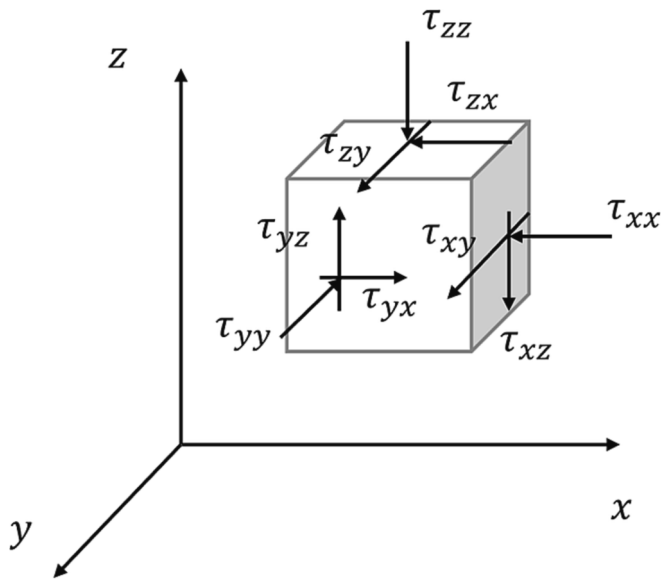

Fig. 1. Viscous forces acting on a fluid element.

The former is the absolute arrangement of atoms in space around a given atom, while the latter refers to the tri-dimensional arrangement of groups of atoms that can change without breaking any covalent bond. Given this premise, proteins can assume an infinite number of conformations, since they are composed of many single bonds that are free to rotate. However, under normal biological conditions, proteins only assume a few of the most stable conformations, which are critical in determining the specificity of proteins' biological functions. In this framework, the folding mechanism holds a great deal of biological importance (Creighton, 1990; Whitford, 2005). This mechanism involves an orderly sequential process, which gives the polypeptide the lowest possible energy state. The folding process involves a reversible reaction that is described as follows:

$$US \rightleftharpoons^{k_1}_{k_2} NS \tag{1}$$

where the equilibrium is shifted to the right so that the native state (*NS*) is more stable than the unfolded state (*US*). Therefore, proteins are said to be in their native state when their conformation is adequately folded and assembled with operative structure and function. Typically, the native state is the most stable among all the protein conformations and corresponds to minimum free energy. The conformation stability is then identified in terms of the unfolding Gibbs free energy difference (Pace et al., 1997) as follows:

$$\Delta G^0 = -RT\ln K \tag{2}$$

where K is the unfolding kinetic constant, R is the universal gas constant, and T is the temperature.

An appropriate tertiary folded structure ensures the protein's biological activity. However, a protein may be reversibly unfolded in several ways (Lapidus, 2017) and chemical denaturants, e.g., guanidine hydrochloride and urea, are often used to promote its unfolding. Protein unfolding can also be fostered by acting on environmental conditions, e.g., increasing or decreasing temperature and pressure. Eventually, if proteins are exposed to viscous stresses, their unfolded state can become more energetically favoured and, hence, more likely to be populated by the protein (Jagannathan and Marqusee, 2013).

The unfolding process causes the protein to lose its compact conformation and, in some cases, its biological activity. Moreover, this process often promotes the formation of aggregates that are undesired for drug products (Devkate et al., 2016). The aggregation process may lead to soluble and/or insoluble aggregates which, under specific conditions, may precipitate (Mahler et al., 2009, 2005). Soluble aggregates can be reversible and have a low molecular mass. However, the soluble aggregates in a product should be no more than 5–10 % because it is impractical to eliminate them above this threshold value (Wang et al., 2012).

On the other hand, insoluble aggregates originate when protein aggregation exceeds the solubility limit. In such conditions, the aggregates become irreversible and precipitate out of the solution (Pham and Meng, 2020). The acceptable limit for insoluble aggregates correlates with the size of particulates detected in the protein solution upon reconstitution. Particles and protein aggregates in drug products are classified as visible particles if visible during visual inspection (Narhi et al., 2015), subvisible particles (SVP) if not visible during visual inspection (USP 788, 2006), and high molecular weight species. The advancement of particle detection techniques and improved characterisation technologies set the framework for the current acceptance criteria for particle count (Ibrahim et al., 2023). As set forth by the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP), injectable solutions must be "practically/essentially free of visible particles" (Den Engelsman et al., 2011). As for SVP, USP 788 prescribes limits of $\leq$ 6000/container and $\leq$ 600/container for sizes greater than 10 and 25 μm, respectively (USP 788, 2006). This criterium allows for low levels of particles, which also reduces risks of safety concerns due to insoluble particles.

2.3. Protein instability

Several stressors have been identified as protein instability promotors, including interfaces (Babinchak and Surewicz, 2020; Duerkop et al., 2018a; Perevozchikova et al., 2015). Since proteins are surface-active molecules (Li et al., 2021), they frequently adsorb to interfaces, which could lead to conformation changes or even denaturation. During the development and manufacturing of therapeutics, proteins are subjected to interfacial stresses at each stage, because of the almost ubiquitous presence of air–liquid, solid–liquid, and liquid–liquid interfaces. Therefore, an accurate analysis to ensure the stability of protein molecules must be put in place. Several works investigated the instability resulting from the adsorption of proteins to an air–liquid interface (Duerkop et al., 2018a; Leiske et al., 2016; Maa and Hsu, 1997; Rospiccio et al., 2021); excipients like surfactants can be incorporated to the formulations in such a way to suppress such interfacial stress. Biologics are also vulnerable to exposure to solid–liquid interfaces, which are constantly present during the drug manufacturing process (Li et al., 2019) and whose effect has been thoroughly examined (Arsiccio et al., 2020, 2018; Arsiccio and Pisano, 2020). As an example, it was proven that insulin could adsorb to several types of containers, including glass vials, plastic vials, and infusion bottles; this significantly reduced the dose available for the patients (Weisenfeld et al., 1968). Unfolding and aggregation can result from protein adsorption to solid surfaces. Moreover, when the denatured proteins desorb from the solid surface, they can trigger protein aggregation in the bulk solution. Protein adsorption may also be promoted by the tube walls through which the therapeutic fluid flows (Deiringer et al., 2023). The choice of material is crucial to avoid clogging problems in any following filtration units. In fact, membrane fouling can result in decreased permeate flow, prolonged processing durations, and even process failure. Other than physical degradation, exposure to solid surfaces can also result in chemical degradation and protein cleavage (Li et al., 2019). A final class is represented by the liquid–liquid interface, which often occurs in delivery devices such as glass syringes. It is common practice to use silicon oil as a lubricant to allow a smoother sliding of the plungers within the barrels (Li et al., 2021). In such a case, proteins are known to unfold and form oil-protein particles at the lubricant–liquid interface of the syringe (Jones et al., 2005).

In the proteins' instability landscape, viscous stresses were suggested to be an additional driving force to the protein unfolding (Di Stasio and De Cristofaro, 2010), as discussed in Section §3.

3. Influence of shear stress in industrial applications

3.1. Shear stress in unit operations

Drug manufacturing includes various operations, namely 'formulation, fill and finish'. First, the purified form of the drug product is formulated with selected excipients; then, it is filled into vials or syringes and is thus ready for packing, labelling and quality inspection before its distribution (Patro et al., 2002). The filling process is critical for preserving product quality among the various steps. Mixing, pumping, filtration, and filling are typically involved in the filling process, as shown in Fig. 2. The biotherapeutic formulations can be subjected to non-negligible shear stresses during these unit operations.

The formulated product may also be freeze-dried to increase biotherapeutic stability, improve shelf life, and facilitate storage. Recently, spray-freeze drying and spray-drying opened avenues for research to overcome some of the challenges associated with standard freeze-drying technologies (Sharma et al., 2021). Yet, shear stress can occur at some stages of the spray-freeze drying and spray-drying process, specifically at the atomization step. Fig. 3 displays an example of a nozzle used for atomization.

When processed through these lines, the product is exposed to high fluid dynamic shear and interfaces that can threaten protein stability (Jameel and Hershenshon, 2010; Thomas and Geer, 2011); careful control of the process is therefore needed as the product is generally intended for parenteral administration.

The shear stress exerted in each operating unit of these two processes tightly depends on some geometric parameters and processing conditions, e.g., flow rate and equipment size. However, to provide an idea of the order of magnitude of shear stress and expected exposure time, Table 1 summarises some relevant values referred to the various unit operations gleaned from the literature.

To account for the combined effect of shear rate and exposure time, some authors introduced a new variable, known as 'total shear' (Ogunyankin et al., 2019) or 'shear history', SH, (Bekard et al., 2011a; Jaspe and Hagen, 2006), although the general notion is expressed as:

$$SH = \gamma t_{exp} \tag{3}$$

where t_{exp} is the time of exposure, which makes SH dimensionless.

Several sensors were developed to experimentally evaluate the shear stress at the solid–liquid interface for the various unit operations (Schmirler et al., 2013). Direct sensors, such as floating-element devices, and indirect sensors, such as thermal or optical sensors, are the most popular (Naughton and Sheplak, 2002). Direct devices measure the wall shear stress's integrated force on a flush-mounted floating element, which may be connected to a displacement transducer. On the other hand, the floating element sensor has several drawbacks. It can cause measurement errors associated with sensor misalignment; moreover, it is sensitive to pressure gradients, vibration, acceleration, and thermal expansion effects (Winter, 1979). Furthermore, thermal sensors work on temperature transduction to voltage. They are designed to achieve fast response time and high sensitivity; however, obtaining a unique calibration between wall shear stress and heat transfer is hard, and measurement errors can occur because of the temperature drift. Optical sensors, finally, rely on the Doppler shift of light scattered by particles.

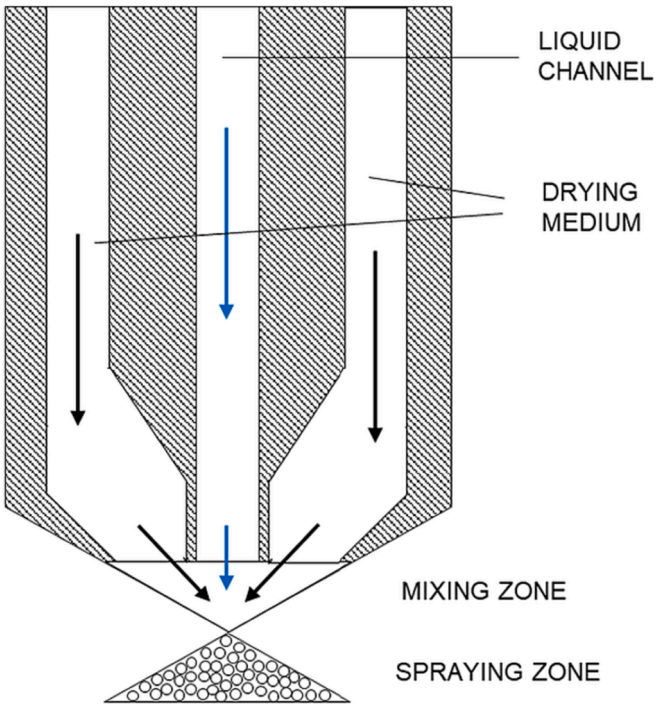

Fig. 3. Schematic diagram of an external two-fluid nozzle.

Table 1
Typical shear rate and relative exposure time for unit operations in pharmaceutical manufacturing lines.

Operation	Shear rate, s^{-1}	Exposure time	Reference
Mixing	Up to 200	Minutes to hours	(Ogunyankin et al., 2019)
Pumping	Up to 2,000	Milliseconds to seconds	(Nema and Ludwig, 2010)
Filtration	1,000 –10,000	Few seconds	(Bee et al., 2009)
Filling	Up to 20,000	Milliseconds	(Bee et al., 2009)
Atomization	10,000 –100,000	Milliseconds to seconds	(Maa and Prestrelski, 2000)

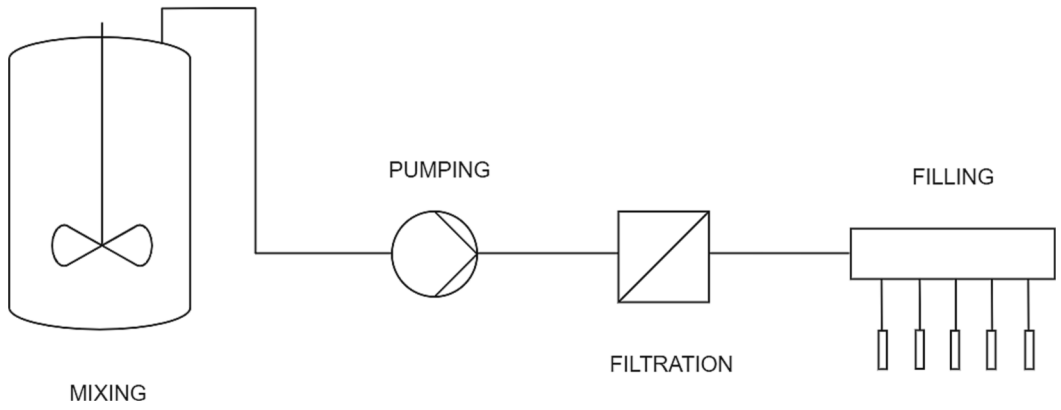

Fig. 2. Typical steps of a filling line for drug products.

Due to challenges in geometric design, their application is restricted.

An alternative to the direct and indirect measurement of the shear stress is represented by Computational Fluid Dynamics (CFD) modelling (Katritsis et al., 2007). This approach offers an inexpensive way to perform a qualitative fluid dynamics prediction that would be extremely difficult to achieve using experimental techniques. Indeed, CFD is experiencing considerable development in the biotechnological field, and applications are reported for upstream and downstream processing (Sharma et al., 2011). CFD provides full-field data and data at multiple locations, which are not achievable by probes, which instead provide point values (Pordal et al., 2002). This feature supports process monitoring and improvement. In addition, CFD is shown to be effective in enabling the identification of a design space (Rathore et al., 2012a), which refers to the combination of materials and operating conditions which assures quality for pharmaceuticals (U.S. Department of Health and Human Services Food and Drug Administration, 2009).

Furthermore, the complex nature of protein-based therapeutics poses unique challenges in the industrial field, which include the definition of appropriate scaling parameters to scale up the process from the laboratory unit. Similarly, scaling up from the Research & Development (R&D) stage to the pilot stage must be carefully controlled to avoid batch failures (Al-Chi et al., 2013). An ideal scale-down (and -up) approach would mimic conditions at a commercial (or laboratory) scale and accurately predict and characterise its performance (Levin, 2001; Moino et al., 2023a; Moscariello, 2016). In this light, potential scale-down methods to mimic shear stresses encountered in industrial unit operations will also be presented.

Hereafter, papers relevant to the effect of shear stress in common operations along a pharmaceutical manufacturing line will be presented, along with CFD details and relevant results.

3.1.1. Mixing

Mixing is a crucial step in drug production and occurs at various stages of the manufacturing process (Das et al., 2022). First, it is used to achieve homogeneous solutions of the drug substance and its excipients during the formulation stage (Crowley, 1999). In addition, mixing is crucial in ensuring the homogeneity of the drug product before the filling process (Das et al., 2022).

Mixing is generally conducted in a stirred tank (Piedmonte et al., 2018). Selecting the most appropriate impeller is crucial for maximising the process performance and can result in different shear stresses (Voll and Mirro, 2009). To provide an example, axial flow impellers are generally employed for shear-sensitive products due to milder shear conditions. On the other hand, magnetically driven bottom-mounted mixers may result in product aggregation when there is contact between the impeller and the drive unit (Brückl et al., 2016a); in the latter mixer configuration, cavitation and shear seem potential triggers of aggregation phenomena for monoclonal antibodies (Gikanga et al., 2015). Furthermore, single-use configurations are beginning to make inroads because of their reduced risk of contamination and increased flexibility (de Boulard and Kienle, 2022). Several single-use mixing systems are commercially available, generally categorized as mechanically and hydraulically driven systems (Eibl and Eibl, 2011). In particular, the availability of several types of single-use systems offers solutions with significantly low shear rates, thus making them suitable for shear-sensitive products (Junne and Neubauer, 2018).

Similarly, the choice of process parameters is critical. Typically, the mixing process requires high turbulence to ensure perfect mixing of the components. However, this can be harmful to shear-sensitive products, especially if high shear stresses are maintained for an extended time (Mcconville and Kessler, 2019). Several relationships have been developed to predict the shear stress expected around the impeller under different process conditions (Campesi et al., 2009; Metzner et al., 1961). Sanchez et al., (Sánchez Pérez et al., 2006) demonstrated that the average shear rate is a function of the rotational speed (N) only, under both laminar and turbulent conditions:

$$\gamma_{lam} = k_{lam} N_{lam} \tag{4}$$

$$\gamma_{turb} = k_{turb} N_{turb}^{3/2} \tag{5}$$

where k is a proportional constant.

Nonetheless, as shown in Table 1, biotherapeutics are subjected to a modest shear rate during mixing procedures, which usually lasts minutes to hours (Nesta et al., 2017; Ogunyankin et al., 2019). In a recent study, Chaubard et al. (Chaubard et al., 2010), focused on four mixing configurations; they applied Particle Image Velocimetry (PIV) technique to identify the shear stress distributions in the impeller area. Ultimately, they identified two configurations that appeared to show a good trade-off between acceptable mixing time and low shear stress.

It must be noted that, in addition to shear stress, biotherapeutics are exposed to solid surfaces during the mixing process, on which proteins could adsorb. As mentioned in Section §1, shear stress alone does not seem to cause protein aggregation (Thite et al., 2023); rather, its synergistic combination with interfacial stress represents the triggering phenomenon (Kopp et al., 2023; Nesta et al., 2017; Tavakoli-Keshe et al., 2014). In particular, Lin et al. found that shear stresses and interfacial stresses during mixing exposed aggregation-prone regions in the protein and resulted in protein aggregates (Lin et al., 2016).

Controlling shear stress during scale-up operations can become a complex undertaking (Babnik et al., 2020). Smaller versions of the commercial-scale tank are routinely used for scale-down process evaluations (Li et al., 2021), but it may be challenging to accurately scale up fill volume, impeller, and tank geometry. It is worth mentioning the work carried out by Sieck et al. (Sieck et al., 2013); they developed a scale-down model of hydrodynamic stress conditions similar to those of a production scale bioreactor and tested a monoclonal antibody's robustness to such hydrodynamic conditions. The throughput of specific monoclonal antibodies dropped at high levels of hydrodynamic stress; this decrease was considerably greater when mimicking the recurrent transit of cells through the impeller area. Another valuable alternative is represented by CFD, which allows the investigation of different process scenarios for a given mixing tank at varying parameters (Mishra et al., 2021; Rathore et al., 2012a). For reference, Fig. 4 displays the velocity trend in the impeller plane at different pump speeds. The velocity gradients generate shear stress, which results in higher stress at higher pump speeds.

In addition to enabling the evaluation of the shear stress distribution within a typical mixing unit, they permit the identification of design space to assure quality.

3.1.2. Pumping

Shear stresses in pumping systems are widely documented, ultimately through CFD simulations (Dreckmann et al., 2020; Song et al., 2003). They result from many factors, which include velocity, energy, pump efficiency, and tubing diameter. The pumping step is widely believed to impact product stability, which is why pump compatibility is chosen based on stability studies (Allmendinger et al., 2015). In this light, pumps can be classified as high-shear or low-shear pumps, and their selection is linked to product sensitivity.

Positive-displacement pumps and time-over-pressure fillers are two pumping systems used to handle biopharmaceuticals. Among the first category, the most used are piston pumps, rotary piston pumps, rolling-diaphragm (Karassik et al., 2001), and peristaltic pump fillers (Jameel and Hershenshon, 2010). Particularly, piston pumps and rolling-diaphragm fillers reduce the product's exposure to shear stress. Rolling-diaphragm pumps ensure the lack of seals or packing and show a low risk of particle formation (Nayak et al., 2011). On the other hand, peristaltic pumps exhibit more flexibility than piston pumps (Jørgensen and Lambert, 2008) and provide a gentle pumping action (Kovarcik, 2016), thus minimizing the stress on the product. However, they exhibit a lower accuracy (Saller et al., 2015), especially when handling highly

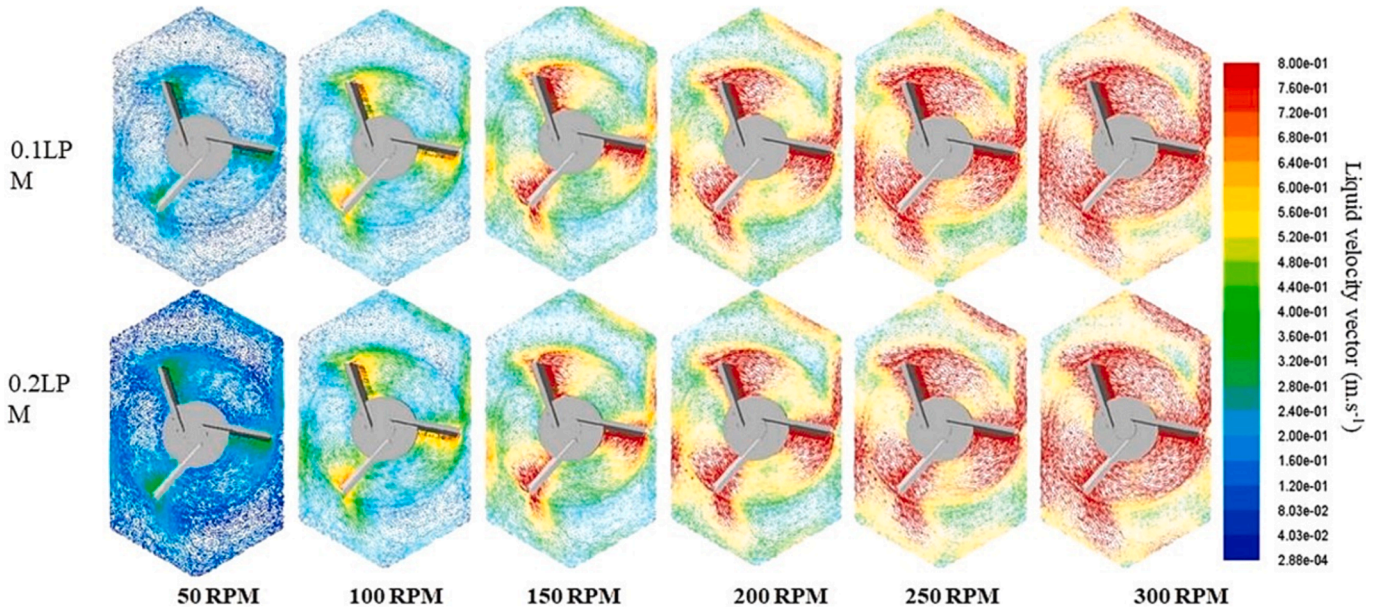

Fig. 4. Velocity plots in the impeller plane for different agitation rates at different aeration rates (0.1 and 0.2 LPM). Reprinted with permission from (Mishra et al., 2021).

viscous products. In the time-pressure system, the product, exiting from a pressurized manifold, passes through flow orifices, which help regulating the flow rate (Peterson, Eric Isberg, 2007). The opening time of the shut-off valves and the manifold pressure are the main parameters controlling the pumping step.

Various authors have investigated how shear stress might affect

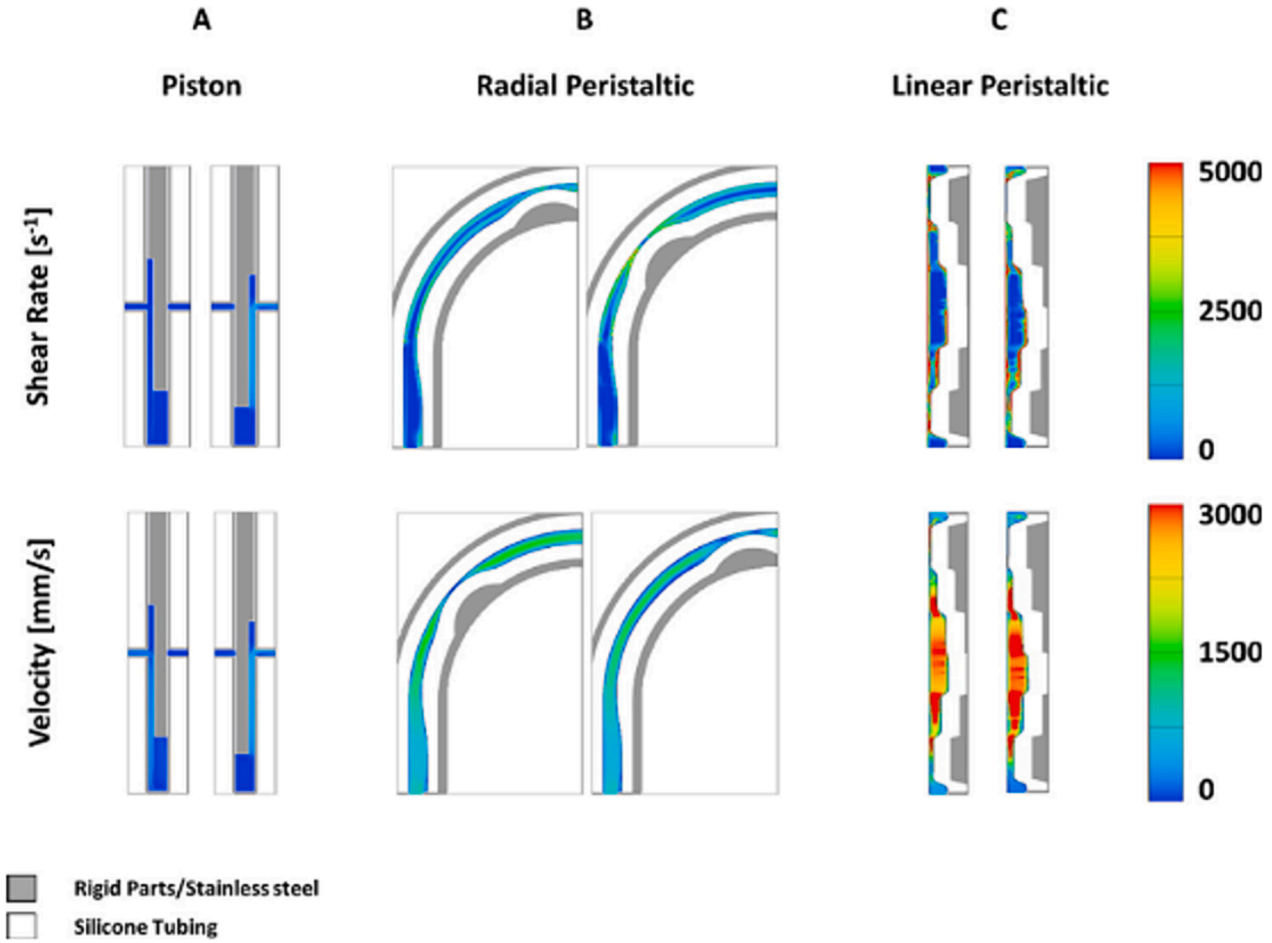

Fig. 5. CFD analysis for piston pump (A), radial peristaltic pump (B) and linear peristaltic pump (C). Reprinted with permission from (Dreckmann et al., 2020).

protein degradation in the pumping stage; once again, its ubiquitous role cannot be assessed easily, as other drivers for product degradation can occur simultaneously, including interfacial stress, foaming, physical grinding, and cavitation (Nayak et al., 2011). Physical grinding refers to the trapping of product between moving parts of the pump, while cavitation refers to the violent creation and implosion of microbubbles (Bee et al., 2009). Each filling system has pros and cons regarding the impact on protein stability based on pump size, filling volume, filling rates, and needle specifications (Das et al., 2022).

Tyagi et al. (2009) tested several piston pumps during filling and found that they resulted in variations in the amount of stainless-steel nanoparticles, which further served as nuclei for protein particle formation. In a further attempt (Roffi et al., 2021), the effects of filling pump type, pump speed, and protein concentration on particle formation were investigated by performing experiments at the laboratory scale and then scaling up at the industrial unit. According to the authors, SVP formation was not proportional to the exerted shear rate in rotary piston pumps. A stainless-steel rotary piston pump was then used on monoclonal antibodies to test its effect on SVP (Kalonia et al., 2018). It was found that product concentration and interface material were more likely responsible for the formation of SVP rather than shear rate or passage number. Furthermore, Duerkop et al. (2018b) demonstrated that the impact of isolated shear conditions on Human Serum Albumin (HSA) integrity was low, and aggregation occurred as a result of the cavitation. According to Wu & Randolph (2020), instead, cavitation within the pump, if present, plays a minor role in generating aggregates because the protein absorption initiates the aggregation in the pump chamber. Industrially relevant levels of shear rates seem, in fact, to be insufficient to cause protein denaturation in bulk solutions (Sediq et al., 2016). In a recent paper (Dreckmann et al., 2020), the effect of three different types of pumps (rotary piston, radial peristaltic and linear peristaltic pump) was investigated, and particle formation was detected. CFD was used to explore the fluid dynamics within these configurations and the resulting shear stresses are reported in Fig. 5.

In particular, the rotary piston pump resulted in the most notable protein aggregation even though the peristaltic pump operated at higher shear rates; according to the authors, this was because rotary piston pumps created a recirculation zone, which further enhanced protein degradation due to repetitive exposure of the product to solid surfaces. This result confirmed the tendency for rotary piston pumps to damage biopharmaceuticals, as previously reported (Bausch, 2008; Cromwell et al., 2006). It must be noted that the cause for aggregation was not attributed to the shear stress per se, which rather enhanced the degradation. Similarly, the direct contact with the product in the piston pump might lead to product aggregation, as suggested in (Rajan et al., 2021).

In a time-pressure pumping system, the estimated shear rate is relatively small (2×10^2 s^{-1}) (Nema and Ludwig, 2010) and its impact on drug product stability is considered minimal (Das et al., 2022; Lim et al., 2015).

A last category of pumping systems suitable for handling pharmaceuticals is represented by syringe pumps, which are easy to use but involve a product volume dependent on the syringe volume. However, they are generally used in microfluidics experiments (Hallow et al., 2008) rather than industrial applications.

Regarding the scale-down approach, Li et al. (Li et al., 2019) suggest performing laboratory-scale experiments using a pump with representative pumping parameters and rates. In this way, any potential risk associated with the pumping process can be identified (Li et al., 2019).

3.1.3. Filtration

Protein-based therapeutics often undergo filtration to prevent microbial and/or particulate contamination (Pillai et al., 2016). Filtration is a pressure-driven process aiming to separate solids from a mixture by forcing it through a semi-permeable membrane (Jornitz, 2020); the upstream pressure forces the fluid through the membrane pores and out the permeate.

In the realm of bioprocessing steps, filtration is generally classified as depth filtration, dead-end filtration, and cross-flow filtration (Affandy, 2013). During in-depth filtration, undesired particles are trapped as they flow through the fibrous membrane of the filter. In dead-end filtration, all the flows are directed through the membrane with material building up on the filter surface (Nathan et al., 2008). Ultimately, the flow is addressed across the membrane surface in cross-flow filtration, also known as Tangential Flow Filtration (TFF). This last technique holds great industrial importance; its principle is based on pumping the feed in a tangential direction to the membrane to avoid build-up and clogging of the membrane pores. Both flat sheets and hollow fibres membranes were reported in the literature, but the second one involves lower shear forces. It must be noted that high shear can result in better cleaning and improved flux but it is suggested to avoid it in bioprocessing (Bekard et al., 2011a). Moreover, this technique is increasingly used for the concentration, separation, or purification of biological material in the laboratory as well as industrial scale units (Baruah et al., 2005; Elias and Joshi, 1998). It benefits from running under mild operative conditions, i.e., low temperatures and pressures, and high throughputs, which are convenient for processing shear-sensitive products (Hussain, 2019). Ultrafiltration (UF) and microfiltration (MF) are two of the most widely used TFF processes. UF is designed to retain macromolecules (Elias and Joshi, 1998) and is industrially used to produce vaccines, plasma, and serum (Ligon, 2020). On the other hand, MF is designed to retain particulates (Fernandez-Cerezo et al., 2019) in the 0.25 to 10 μm size range (Nathan et al., 2008) and finds successful applications in the biotechnological industry.

Sterile filtration in filling operations is commonly performed using dead-end filtration through a membrane with a small filtration rating (Jornitz and Meltzer, 2008); this allows complete retainment of up to 10^7 colony-forming units/cm^2 of filter area. Following the FDA (Food and Drug Administration) and the USP (United States Pharmacopoeia) guidelines, sterilising filters should be product compatible (Kong, 2006) and made of non-fibre releasing materials with a nominal rating of 0.2 μm and 0.22 μm respectively (Meltzer and Jornitz, 2003; Ramstorp, 2003). These filter membranes are often made of Polyvinylidene Fluoride (PVDF) or Polyethersulfone (PES), whereas the housing is composed of a polymer, such as polycarbonate or polypropylene (Meyer and Coless, 2012). The choice of the proper filter is driven by some performance criteria, such as the total throughput, flow rate and yield loss associated with the filtration step (Priebe et al., 2003).

Pleated membrane configurations are generally used in pharmaceutical manufacturing as they allow a large membrane area to be packed into an element to increase filtration performance (Brown et al., 2009; Dippel et al., 2021). Capsule (Dixit, 2008) and cartridge filter (Kumar et al., 2015) types belong to this class. Disc configurations are also available but are generally used for filterability trials (Dixit, 2008; Giglia and Yavorsky, 2007).

Shear stress in the filtration unit generates by forcing the formulation through narrow channels, like the filter's pores. As a result, shear stress – and wall shear stress – is not constant but a function of the radial position across the membrane; moreover, it depends on geometric parameters such as the filter structure, i.e., the pore size. These high levels of shear stress are often generated at the interface of the filter membrane and the product; protein aggregation could therefore arise as a result of the interfacial stress (Li et al., 2019).

Determining the actual shear stress experienced by the formulation when it flows through a filtration unit is very challenging. In one of the earliest studies (Maa and Hsu, 1998), it was assumed that the pore could be approximated to a straight cylinder with a diameter equal to the pore size, i.e., 0.22 μm, and thus the average shear rate was calculated using **Eq. (6)**:

$$\bar{\gamma} = \frac{8Q}{3\pi r^3} \tag{6}$$

where Q is the volumetric flow rate and r the distance from the

centre.

Later, shear rates of $4.8 \times 10^5 - 4 \times 10^4$ were estimated for antibody solutions filtered through 0.2 μm sterilising membranes (Nema and Ludwig, 2010). Furthermore, as the drug product's concentration increases, the product's viscosity increases, which results in higher pressures and, thus, higher shear stress exerted on the product (Krause et al., 2018). The combined effect of shear and hydrophobic interface was instead thought to be responsible for protein aggregation in the study conducted by (Aimar and Bacchin, 2010); in particular, shear forces seemed to accelerate degradation. In a recent study, Zhan et al. (Zhan et al., 2020), found values of shear rates of $2 \times 10^3 - 1 \times 10^4$ s^{-1} in a TFF configuration for human embryonic kidney cells; the highest shear rates could cause cellular stress, resulting in apoptosis. Further experiments were conducted on the UF/DF unit operation by Callahan et al. (Callahan et al., 2014); the goal was to identify the dominant factor in protein particle formation among shear stress, impurities, interfacial interactions, or some combinations of those elements. It was demonstrated that the interfacial adsorption–desorption mechanism of the protein during the filtration operation was the principal cause of particle formation.

On the other hand, a few authors used CFD simulations to model and explore the filtration unit. Two different approaches can be adopted, named macro-scale and micro-scale approaches. The membrane is considered a porous medium in the macro-scale framework (Dippel et al., 2021; Velali et al., 2020). Several techniques were employed to reconstruct the pleat geometry in (Velali et al., 2020), as shown in Fig. 6; this allowed the investigation of the impact of the plastic cage on the flow resistance.

Nonetheless, such a macro-scale framework does not allow for monitoring local properties (i.e., shear stress arising from the flow through the pores). Therefore, a micro-scale approach is needed where the pore network is modelled. One of the most significant barriers to implementing this approach is modelling such a small yet complex structure. A few works were reported (Affandy, 2013) but further research should be conducted to shed more light on the shear stress resulting from the sterile filtration unit.

Different phenomena, such as product adsorption to the membrane, can occur besides exposure to shear stress (Bódalo et al., 2004). Furthermore, filters can shed particles or cause leachable to get into the product (Werner and Winter, 2015); this represents a major concern as foreign particles may remain in the final product, thus impacting product quality and resulting in immunogenicity reactions (Pillai et al., 2016).

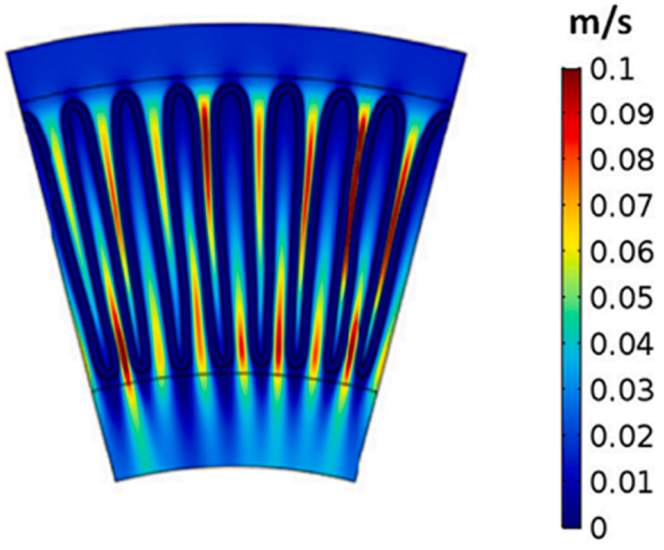

Fig. 6. Simulated velocity profiles in a 2D cross section for a pressure drop of 0.5 bar. Figure taken from (Velali et al., 2020) with modifications.

In addition, scale-down of filtration to bench scale poses unique challenges (Fernandez-Cerezo et al., 2019). An ideal scale-down approach should maintain flow path length and similar wall and entrance effect to help mimic the hydrodynamic shear characteristics. In parallel, pumping and piping flow effects should remain unchanged. Notable approaches were made by Fernandez-Cerezo et al. (2019) and Hussain (2019); their goal was to develop an ultra scale-down model in order to accurately predict the performance of large-scale TFF.

3.1.4. Filling

The filling is the final step in the overall drug manufacturing process. The drug is transferred from a filling needle to the container, usually a vial or a syringe for administration. Specifically, the product passes rapidly through the filling needle and other small-diameter filling parts (Carpenter et al., 2009). The filling needles are usually much smaller than the tubing that goes into them (Joyce and Witchey-Lakshmanan, 2013). Filling speed is often very high to decrease the overall process time (Peterson, Eric Isberg, 2007). Several authors have investigated the injection forces of filling needles with various fluids. As general guidelines, the higher the product's viscosity, the greater the forces involved (Allmendinger et al., 2014; Krayukhina et al., 2020).

Proteins are simultaneously exposed to interfacial (by contact with the solid needle surface) and shear stresses during filling. Because of interfacial stresses, the material used during the filling step can greatly affect product stability (Rathore and Rajan, 2008). Filling is made possible by the use of a dosage pumping system, the details and implications of which were given in Section §3.1.2. The filling technology must be chosen based on compatibility with the product and the various process parameters, which include filling rates, pump size and volume, and needle size and configuration (Jameel and Hershenshon, 2010).

The maximum shear stress experienced by a model product through a needle can be determined using the well-known analytical equations for laminar and turbulent flow (Moino et al., 2023a). Filling an antibody solution into vials through a 5 mm needle would expose the product to a shear lower than 10^3 s^{-1} (Nema and Ludwig, 2010); if 20-gauge needles of 10 cm are used, instead, a shear rate of 2×10^4 s^{-1} would be exerted on the protein for about 50 ms given a flow rate of 0.5 mL s^{-1} (Bee et al., 2009). Although the experimental maximum shear and exposure time were far more than the calculated one, no relevant aggregation was detected in their work. Later on, Nesta et al. (Nesta et al., 2017) simulated the passage of monoclonal antibodies through a 27-gauge, syringe-guided needle, and once again, no aggregation phenomena were encountered. Nevertheless, their calculations show that with the reduction of the needle diameter, the filling needle shear rate increases, but so does also the shear stress. Furthermore, for non-Newtonian solutions, shear rates in the range of $3 \times 10^4 - 1.6 \times 10^5$ s^{-1} for injection rates between 0.5 mL and 2.0 mL/10 s were calculated for 0.21 mm diameter needles (Allmendinger et al., 2014).

As far as the scale-down strategy, combined with the dosing system previously reported in Section §3.1.2, the filling needle size should be chosen to represent the manufacturing scale (Li et al., 2019).

3.1.5. Atomization

Protein therapeutics-based formulations may show instability issues such as aggregation and denaturation during storage. To overcome these barriers, spray-freeze drying and spray-drying are promising methods for their stabilization (Kanojia et al., 2017; Poozesh and Bilgili, 2019). These two technologies share the steps of droplet formation, i.e., atomization, and final drying. In the case of spray-freeze drying, there is an intermediate freezing step, the monitoring and optimization of which (Moino et al., 2021) is critical to prevent freezing-induced degradation. The drug product formulation includes defined components to preserve the protein structure from freezing- and drying-induced stresses (Butreddy et al., 2021).

Considering the atomization step, shear stresses arise because of the passage through a nozzle (Ameri and Maa, 2006). In the case of a two-fluid nozzle atomizer, for instance, the average shear rate can be estimated based on the spray dryer nozzle properties as follows (Ghandi et al., 2012):

$$\overline{\gamma} = \frac{2(u_{av} - u_{liq})}{D_i} \quad (7)$$

where D_i is the inner nozzle diameter, u_{liq} is the liquid velocity at the point of atomization and u_{av} is the average velocity at the mixing point. In a thorough analysis, shear rates were estimated in the order of 10^5 s^{-1} (Morgan et al., 2020), spanning from milliseconds to seconds (Adali et al., 2020). Furthermore, another work reports shear rates estimated through mathematical modelling in the range of $10^4 - 10^5$ s^{-1} (Maa and Prestrelski, 2000).

Such shear stresses were assumed to be responsible for product degradation when combined with the air–water interface, which occurs as a result of the atomization (Adler and Lee, 1999; Maa et al., 1998; Maa and Hsu, 1997). When applied to vaccine-excipient liquid mixtures, furthermore, shear stress was thought to potentially reduce or lose antigen activity (Kanojia et al., 2017). Nonetheless, a recent work (Dao et al., 2022) claims that the primary detrimental factors during the atomization step are believed to be exposure to a high surface area-to-volume ratio and turbulent flow. The nozzle choice also appears to play a pivotal role, and different losses in activity were observed under different nozzle configurations (Grasmeijer, 2015).

Nonetheless, modulating the atomization pressure, solution flow rate and viscosity or using stabilizers might help overcome the issues related to the atomization process (Maa and Prestrelski, 2000).

Finally, during the final drying stage, as the phases dehydrate and shrink at different rates, shear stresses are generated and could threaten protein stability (Heller et al., 1997).

Protein stabilization cycles can be scaled up for different purposes, either for both product quality and drying time, or for product quality only. This operation presents significant challenges (Patel and Pikal, 2011), and it appears that mathematical modelling is typically required (Pisano et al., 2013). For more information concerning scale-up issues during spray drying, refer to Poozesh and Bilgili (Poozesh and Bilgili, 2019).

3.2. *Potential impact of interfacial and shear stress combination on the finished product*

Shear stress was initially believed to impact the catalytic activity of protein therapeutics (Elias and Joshi, 1998). Downstream the proposed literature analysis (Section §3.1), however, it seems that the primary source of damage occurring in most bioprocessing steps is the synergistic presence of shear stress and interfacial stress; if proteins still maintain their tertiary structure even under extreme shear rate conditions (10^5 s^{-1}) (Jaspe and Hagen, 2006), it is rather its combination with interfacial stress the potential cause for product instability (Kopp et al., 2023). Indeed, shear stress can promote product turnover close to interfaces which accelerates aggregation (Aimar and Bacchin, 2010; Thite et al., 2023).

Product stability throughout the entire processing of protein-based therapeutics must be ensured (Rathore and Rajan, 2008) and, therefore, different strategies can be adopted to mitigate the impact of stressing conditions on the product. The precise choice depends on multiple factors, including the product's specificity and operating conditions. However, some general guidelines can be drawn for each unit step.

In the case of mixing, for instance, the impeller and the placement must be chosen with care, and low-shear mixers must be preferred (Chaubard et al., 2010; Converti et al., 1996). It may also be possible to inhibit agitation–induced aggregation by carefully choosing excipients (Serno et al., 2010).

In the case of shear-sensitive products, it might be necessary to optimise pumping (and filling) parameters to minimise the impact on the product, including using a lower pumping rate or larger nozzle size (Jameel and Hershenshon, 2010). If the challenge is severe, the pump must be chosen to limit both the resultant shear stress and the exposure time to that shear (Dreckmann et al., 2020), thus preferring low-shear pumps (Jameel and Hershenshon, 2010).

The concomitant effects of exposure to solid–liquid or air–liquid interfaces, as well as shear, can compromise product quality (Li et al., 2021). Given the complex interplay of interfacial and stress-induced denaturation, it is challenging to draw some general guidelines. Nonetheless, single-pass filtration should be preferred as it significantly reduces the overall exposure of the protein to interfaces as well as the shear stress within the pump and filter. As regards atomization, gas and liquid feed rates can be adjusted to minimise product activity loss (Maa and Prestrelski, 2000; Morgan et al., 2020).

Furthermore, in the industrial realm, experiments can be carried out to investigate product stability under different process scenarios and thus ease the translation to manufacturing; in this light, identifying appropriate scale-down (and scale-up) methods is paramount. To transfer the filling process from the manufacturing to the laboratory unit, the pump, filling speed, and tubing size should be characterised so that they result in shear rates comparable to those encountered at the manufacturing scale (Jameel and Hershenshon, 2010).

In addition, it is crucial to highlight the importance of performing experiments at the laboratory level using lab chips because, as suggested by (Ashton et al., 2009), the shear-sensitivity of protein-based therapeutics might be product-dependent.

4. Lab chips for shear stress investigation

Considerable efforts have been made to develop potential devices to study the effect of shear stress on specific products. They differ based on the type of device and the technique used to characterise the product upon stressing. It is worth noting that they are not intended to mimic manufacturing unit operations, i.e., scale-down devices, but were designed to generate defined shear stress and assess its impact on the product stability.

The literature describing shear-induced degradation experiments on proteins goes back to the early 1970s. Forced degradation studies are crucial in developing protein-based therapeutics (Hawe et al., 2012). The devices hereafter presented differ in the product chosen for the analysis and in the properties studied to quantify the damage; moreover, the type or duration of protein stressing varies significantly among the various experiments. The devices are generally classified into capillary

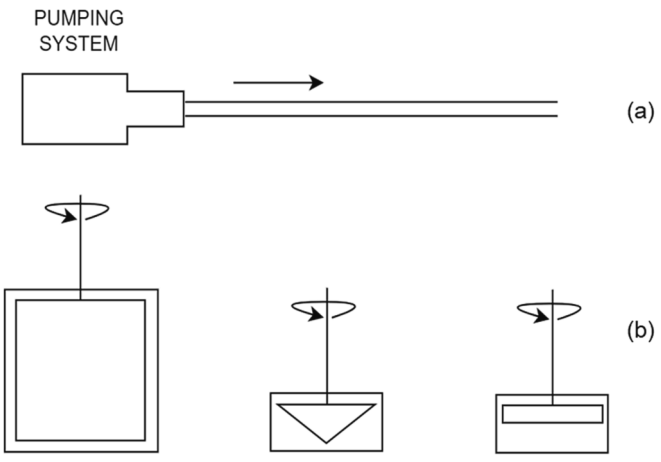

Fig. 7. Traditional scheme for (a) capillary devices and (b) rotational devices (concentric cylinder, cone-and-plate and parallel plate device).

and rotational, and their working principle is shown in Fig. 7.

If performing experiments with simple interfacial stresses is relatively easy, exploring the effect of pure shear is not straightforward. Indeed, in practice, it is not possible to isolate shear stress from other stress phenomena. This is precisely the reason for the confusion generated by old work that attributed the cause of product damage to shear, without considering the synergistic effect of shear and interfacial stress. Nonetheless, devices that have been developed more recently attempted to minimise other forms of stress, which include solid–liquid and air–liquid interfaces (Thomas and Geer, 2011). According to Hudson et al. (2015), the ideal shear device should involve a small product volume, allow a large dynamic range of shear stresses to be investigated and limit the air–liquid interface.

Given these premises, capillary and rotational devices are further discussed in the following sections.

4.1. Capillary devices

The product is generally forced through a conduit of known dimension in capillary devices by applying a pressure difference between its inlet and outlet. The generated flow is of the Poiseuille type, with a parabolic velocity profile reaching the maximum value at the centerline (White, 2006); therefore, the shear is maximum at the wall boundaries and reduces gradually towards the conduit centre. Even though the shear stress is not constant, it is common practice to refer to an average shear stress (Moino et al., 2023a, 2023b).

In the analysed designs, the driving force for the motion of the fluid is either provided by a finger pump (Charm and Wong, 1970a), a piston (Murphy et al., 2020), a syringe (Dobson et al., 2017), or a high-pressure liquid pump (Ogunyankin et al., 2019). In general, they allow the application of high shear rates (up to 2.5×10^5 s^{-1}) over short residence times. If needed, recirculation of the formulation can be used to increase the product's exposure to shear stresses; nonetheless, this leads to an increased risk of interfacial denaturation at the solid–liquid interface. A critical advantage of using the capillary device in place of the rotational one lies in the lower product consumption.

One of the first documented attempts at stressing products in capillary devices was made by Charm & Wong (Charm and Wong, 1970a); the activity of catalase flowing through a Teflon capillary tube was tested as a function of the applied shear stress and the relative exposure time. Loss in activity was found at a relatively low shear rate, i.e., 67 s^{-1}, and long exposure time, i.e., 90 min, showing a strong correlation between shear history and protein damage. Later on, a further capillary device was developed (Thomas and Dunnill, 1979). A piston forced catalase solutions through the capillary device built from stainless-steel capillary tubes. The authors found no significant activity loss at shear rates up to 10^6 s^{-1}. These conflicting results raised the question of whether the enzymes in the earlier study were denaturating through an interaction with an air–liquid interface or solid surfaces, rather than as a consequence of shear. In this light, Jaspe and Hagen (2006) investigated the stability of cytochrome when flowing through a narrow capillary (d = 0.15 mm), exploiting fluorescence spectroscopy to detect any degree of unfolding. They found no evidence that even high shear rates alone could destabilize the folded protein. A further device called PSA (Protein Shear stress Application) was designed and patented by Heizelmann et al. (2008); the goal was to subject molecules in solution to shear stress and determine by analytical processes the changes in the stability characteristics. Tests were carried out at constant shear and over different residence times; it was found that as the shear stress increased, aggregated particles and turbidity increased considerably. In another work, a capillary rheometer was exploited to apply high shear rates for short periods; solutions of monoclonal antibodies were driven through the capillary using a piston. DLS was used to analyse the sheared samples, but no aggregation was detected for shear rates up to 2.5×10^5 s^{-1} (Bee et al., 2009). Hudson et al. (2015) developed a miniaturised capillary rheometer to assess the fluid viscosity; it could utilise a small amount of product, i.e., a few microliters of sample. UV–vis spectrophotometer was employed to determine the concentration of the product (mAb).

Another remarkable investigation was conducted by Nesta et al. (2017) on mAbs stressed by a syringe pump up to 3×10^5 s^{-1}; the work aimed at assessing whether surface interaction, shear stress, a combination of those, or other events, i.e., cavitation, were causative for protein aggregation. No visible aggregates were detected, showing that a more likely driver for product degradation was the exposure to the solid surface rather than the high shear rate. Dobson et al. (Dobson et al., 2017) developed a low-volume flow device able to deconvolute the effects of shearing (resulting from the flow within a capillary) and extensional flow (resulting from a rapid constriction). Two syringes drove the fluid motion to avoid exerting high shear stress on the product. They concluded that extensional flow could trigger the aggregation of BSA. This, together with previously published articles (Simon et al., 2011), suggests that the ability of shear and extensional flow to induce aggregation is protein-dependent. Another valuable work was carried out by (Ogunyankin et al., 2019), who developed two small-scale tools to isolate interfacial and shear stress. In particular, the interfacial stress was generated by the contact with uniform bubbles which passed through the solution for finite periods. On the other hand, shear stress was created using a small-scale high-pressure pump connected to fine stainless-steel tubing. The quality attributes assessed after applying the stress focused on detecting physical aggregation by HPLC. Recently, Murphy et al. (Murphy et al., 2020) developed a novel capillary device to stress the product and then, analyse its microstructure through small-angle neutron scattering (SANS). Tests on mAb at 25 mg/L showed that nano-structural changes were expected at a shear rate higher than 1×10^4 s^{-1}.

A summary of the most relevant experiments involving capillary devices is reported in Table 2, where essential details of the tests are reported for clarity.

4.2. Rotational devices

Rotational devices generally impart shear stress on the formulation across a narrow gap and are composed of a rotating and a stationary part (Bekard et al., 2011a). They include cone and plate, parallel plate, and concentric cylinder viscometers. The generated flow is of the Taylor - Couette type, with a linear velocity profile reaching the maximum in correspondence to the moving wall (White, 2006). Extensive research has been devoted to the analysis of the effect of shear on proteins stressed in rotational devices, since their design minimises any end effects and, if the gap width is far lower than the radius of the inner cylinder, the shear rate can be considered constant for the entire solution (Bekard et al., 2011a). On the other hand, they require a relatively high product volume and the air-sample interface can be an issue (Hudson et al., 2015).

Various designs have been developed to generate shear flow, which include the Couette flow-cell, cone and plate, and concentric cylinder. In the Couette flow-cell and the concentric cylinder layout, the average shear rate can be calculated as follows:

$$\overline{\gamma} = \frac{4\omega\left(\frac{R_i}{R_o}\right)^2 \ln\left(\frac{R_i}{R_o}\right)}{\left(1-\left(\frac{R_i}{R_o}\right)^2\right)^2} \tag{8}$$

where ω is the angular velocity of the rotating inner tubing, R_i and R_o are the inner and outer cylinder radii, respectively (Edwards et al., 2010). In the cone and plate design, instead, the average shear rate depends on the cone angle θ:

Table 2
Main details for relevant protein degradation studies using capillary devices.

Product	Shear rate	Focus	Technique	Reference
Catalase	67 s^{-1}	Activity	Rheogoniometer	(Charm and Wong, 1970a)
Catalase	4.6×10^4 s^{-1}	Unfolding	Absorbance	(Thomas and Dunnill, 1979)
Horse cytochrome *c*	1×10^5 s^{-1}	Unfolding	Fluorescence	(Jaspe and Hagen, 2006)
mAb	6.4×10^4 s^{-1}	Aggregation	PCS, turbidity	(Heinzelmann et al., 2008)
mAb	2.5×10^5 s^{-1}	Aggregation	DLS, TEM, SEC	(Bee et al., 2009)
mAb	$10 - 3 \times 10^3$ s^{-1}	Aggregation	UV–vis	(Hudson et al., 2015)
mAb	3×10^5 s^{-1}	Aggregation	SEC-MALS, DLS, MFI	(Nesta et al., 2017)
BSA	1×10^4 s^{-1}	Aggregation	DLS, NTA, TEM, FCS	(Dobson et al., 2017)
mAb	1×10^5 s^{-1}	Unfolding aggregation	MFI, HPLC-SEC, DSF, IA	(Ogunyankin et al., 2019)
mAb	1×10^4 s^{-1}	Aggregation	SANS	(Murphy et al., 2020)

$$\bar{\gamma} = \frac{\omega}{\sin(\theta)} \tag{9}$$

One of the first attempts at shear-induced degradation in rotational devices dates back to the 1970s. Charm and Wong adopted a cone-and-plate device and stressed fibrinogen up to 290 s^{-1}. They found that shearing could be the major responsible for fibrinogen degradation in the circulation. Rennet and catalase were the subject of further research conducted by Charm and Lai (1971); they were stressed up to 91.5 s^{-1} in a cone-and-plate device, and activity loss was detected in a rheogoniometer.

Peterson et al. stressed (up to 8.210^3 s^{-1}) some glycoproteins in a cone-and-plate device; the product was characterised in terms of particle count and size, and particle formation was detected (Peterson et al., 1987). Ikeda et al. (1991) exposed platelets to shear stress up to 6.7×10^3 s^{-1} using a cone-and-plate viscometer, and aggregation phenomena were monitored through an aggregometer. Works on rotational devices continue with Maa and Hsu (1996), who designed a novel concentric-cylinder device and stressed deoxyribonuclease and growth hormone at 1.5×10^3 rpm; various techniques were adopted, which included SDS-PAGE electrophoresis to determine protein fragmentation. They found no significant change in the sheared deoxyribonuclease, whilst unfolding and fragmentation occurred for the growth hormone.

A cone-and-plate design was adopted in a subsequent study (Van Der Veen et al., 2004); α-amylase was subjected to shear stress up to 120 s^{-1} and the relative activity was determined. It was found that hardly any inactivation could occur at low shear stress; however, increasing the shear stress resulted in significant irreversible inactivation of α-amylase.

Biddlecombe et al. (2007) designed a rotating-disk shear device to assess the impact of high shear rates on protein stability at the solid–liquid interface; it provided an air-free environment and allowed sampling while the device was in operation. The authors stressed 1.0 and 0.5 mg/mL immunoglobulin in phosphate-buffered saline (PBS) up to 3.4×10^4 s^{-1}; high shear rates resulted in significant protein aggregation and precipitation levels. A Couette flow cell was used by Ashton et al. to investigate the shear-induced unfolding of lysozyme in situ with Raman spectroscopy and PIV (Ashton et al., 2009); reversible conformational changes were monitored. Afterwards, Bee et al. (2009) stressed monoclonal antibody formulations (100 mg/mL) up to $20,000$ s^{-1} for up to 300 ms in a parallel-plate rheometer; SEC analysis was adopted to characterise aggregate formation. It was found that shear exposure alone during bioprocessing should not cause aggregation; air-bubble entrainment, adsorption to solid surfaces, pump cavitation stresses are rather believed to be the primary causes. In another thorough study (Bekard and Dunstan, 2009), a Couette cell was exploited to study the effect of shear on insulin in situ and in real-time; using Tyr fluorescence and CD, it was found that aggregates could be expected at relatively low shear rates, i.e., 200 s^{-1}. In addition, the authors proved that the size of insulin aggregates decreased with an increasing shear rate. In a further study on a Couette cell (Bekard et al., 2011b), poly-L-lysine was stressed at various shear rates for 1 h and in-line CD analysis was conducted to probe unfolding; it was found that the α-helical-PLL structure unfolds in simple shear flow and the extent of unfolding depends on monomer size, shear rate, and duration of shear application. A few years later, Tavakoli-Keshe et al. (2014) developed a shear device capable of generating a high-shear environment and tested it on modified monoclonal antibodies; the device was composed of a round device chamber containing a stainless-steel disk and the air–liquid interface was eliminated, creating instead a well-controlled shear and interface environment. The relative stability of monoclonal antibodies was measured, and molecular modelling techniques were employed to investigate the proteins' secondary and tertiary structures upon stressing. It resulted that the proposed technique could be used as an orthogonal method for antibody screening. Brückl et al. developed a Couette device and stressed ghGh and IgG1 in free solution, where the interfacial effects are negligible (Brückl et al., 2016b); shear stress was unlikely to unfold the proteins up to shear rates of at least 1×10^4 s^{-1}. In another Couette device experiment, instead, aggregates were detected for human insulin even at low shear rates (McBride et al., 2015). In the work of Nesta et al. (2017), already mentioned in the previous section, a parallel-plate rheometer was used to stress monoclonal antibodies up to $20,000$ s^{-1} for relatively high residence time. They detected an increase in particles throughout all size ranges induced by the applied shear stress that, according to the authors, exacerbated the degradation pathway of the surface-stressed formulation. Another remarkable device is the one developed by (Morimoto et al., 2017); the researchers established a novel Rheo-NMR (Nuclear Magnetic Resonance) approach that allowed to observe protein dynamics during shear and trace atomic-level structural change. Shear rates of $510 - 950$ s^{-1} applied to ubiquitine resulted in the formation of amyloid fibrils. Similarly, in a further work from the same research group (Iwakawa et al., 2021), shear rates of $290 - 540$ s^{-1} were applied to SOD1 (amyotrophic lateral sclerosis-related) protein in the Rheo-NMR and aggregation through amyloid formation was monitored.

The details of these shear-induced rotational devices degradation tests are summarized in Table 3.

5. Conclusions

Interest in the impact of shear stress on protein stability began many decades ago, and still more and more papers are being published to try to discern its effects. Downstream of this literature review, the authors believe that early attempts misinterpreted the results and incorrectly attributed the main cause of protein degradation to mere shear stress. In fact, the primary role appears to be played by interfacial stress, possibly amplified by shear stress that promotes product turnover in areas in contact with the interface. Finding a shear stress threshold for protein denaturation is far from simple: it depends not only on the product, but also on the method of determination (and thus the extent of the interface). Protein-based therapeutic products are often processed through production lines that may include mixing, pumping, filtration, filling, and atomization. Because shear stresses commonly originate during these steps, a more critical understanding of its impact on product

Table 3
Main details for relevant protein degradation studies using rotational devices.

Product	Shear rate / Rotational velocity	Focus	Technique	Reference
Fibrinogen	290 s^{-1}	Degradation	Thrombin addition	(Charm and Wong, 1970b)
Catalase	91.5 s^{-1}	Activity	Rheogoniometer	(Charm and Lai, 1971)
Rennet	91.5 s^{-1}	Activity	Rheogoniometer	(Charm and Lai, 1971)
Glycoprotein	8.2×10^3 s^{-1}	Unfolding	Particle size, count	(Peterson et al., 1987)
Platelet	6.7×10^3 s^{-1}	Aggregation	Aggregometer	(Ikeda et al., 1991)
Deoxyribonuclease	1.5×10^3 rpm	Aggregation	SDS-PAGE	(Maa and Hsu, 1996)
Growth hormone	1.5×10^3 rpm	Aggregation	SDS-PAGE	(Maa and Hsu, 1996)
α-amylase	120 s^{-1}	Activity	Modified DNS	(Van Der Veen et al., 2004)
Immunoglobulin	3.4×10^4 s^{-1}	Aggregation	UV–vis	(Biddlecombe et al., 2007)
Lysozyme	7×10^2 s^{-1}	Aggregation	Raman	(Ashton et al., 2009)
mAb	2×10^4 s^{-1}	Aggregation	SEC	(Bee et al., 2009)
Insulin	600 s^{-1}	Unfolding	Fluorescence, CD	(Bekard and Dunstan, 2009)
Polylysine	715 s^{-1}	Unfolding	CD	(Bekard and Dunstan, 2009)
Modified mAb	9×10^3 rpm	Aggregation	SE-HPLC, DSC	(Tavakoli-Keshe et al., 2014)
rhGH, IgG1	1×10^4 s^{-1}	Unfolding	CD, LD, fluorescence	(Brückl et al., 2016b)
Human insulin	150 s^{-1}	Aggregation	Turbidity, absorbance, AFM, CD	(McBride et al., 2015)
mAb	2×10^4 s^{-1}	Aggregation	SEC-MALS, DLS, MFI	(Nesta et al., 2017)
Ubiquitine	510 −950 s^{-1}	Aggregation	NMR	(Morimoto et al., 2017)
SOD1	290 −540 s^{-1}	Aggregation	NMR	(Iwakawa et al., 2021)

stability has been gained through screening the available literature. The importance of laboratory chips for investigating shear stresses should be emphasized, as they allow the product to be stressed to a defined extent and study the consequences on the product behaviour. Again, a broad spectrum of results was observed for different devices, showing not only that shear susceptibility depends on the product, but more importantly that the presence of interfacial stress is the main cause of protein degradation. In a future perspective, molecular dynamics could be adopted to gain further insights into product behaviour in shear flow. In addition, we foster technological advancements in designing lab chips for controlled shear stress experiments under conditions that limit the influence of interfaces so as to deconvolute shear stress-induced and interfacial denaturation of proteins.

CRediT authorship contribution statement

Camilla Moino: Writing – original draft, Writing – review & editing. **Fiora Artusio:** Writing – original draft, Writing – review & editing. **Roberto Pisano:** Writing – original draft, Writing – review & editing.

Declaration of competing interest

Camilla Moino holds a Doctorate studentship and collaborates with GSK as part of her PhD training.

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