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No Touching! Abrasion of Adsorbed Protein Is the Root Cause of Subvisible Particle Formation During Stirring

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ABSTRACT

This study addressed the effect of contact sliding during stirring of a monoclonal antibody solution on protein aggregation, in particular, in the nanometer and micrometer size range. An overhead stirring set-up was designed in which the presence and magnitude of the contact between the stir bar and the container could be manipulated. A solution of 0.1 mg/mL of a monoclonal antibody (IgG) in phosphate buffered saline was stirred at 300 rpm at room temperature. At different time points, samples were taken and analyzed by nanoparticle tracking analysis, flow imaging microscopy, and size-exclusion chromatography. In contrast to non–contact-stirred and unstirred samples, the contact-stirred sample contained several-fold more particles and showed a significant loss of monomer. No increase in oligomer content was detected. The number of particles formed was proportional to the contact area and the magnitude of the normal pressure between the stir bar and the glass container. Extrinsic 9-(2,2-dicyanovinyl) julolidine fluorescence indicated a conformational change for contact-stirred protein samples. Presence of polysorbate 20 inhibited the formation of micron-sized aggregates. We suggest a model in which abrasion of the potentially destabilized, adsorbed protein leads to aggregation and renewal of the surface for adsorption of a fresh protein layer.

Introduction

Therapeutic proteins have gained a paramount place in modern pharmaceuticals, accounting for more than 70% of the total revenue generated by the 10 best-selling pharmaceuticals.[1] The share of therapeutic proteins in the pharmaceuticals under development is also getting bigger while the probability of regulatory approval from entering clinical development is 32% for therapeutic proteins versus 13% for small-molecule drugs.[2,3] In this category of pharmaceuticals, monoclonal antibodies (mAbs) account for almost half of the sales.[4] The benefits of therapeutic mAbs have been proven to be of great value in many life-threatening diseases including cancer and inflammatory and immune diseases, such as rheumatoid arthritis.[5,6]

Despite this important role of therapeutic proteins in pharmacotherapy, their marginal stability remains an important challenge in formulation, storage, shipping, and delivery of these drugs. Stability issues very often imply aggregation of proteins, which could lead to immunogenicity [7,8] and/or reduced efficacy of the drug.[9] Therefore, regulatory authorities have developed guidelines for quality control of protein drug formulations, which often contain upper limits for the concentration of visible and subvisible particles (including protein aggregates) present in parenteral drug products.[10-12]

Many external factors that cause the aggregation of therapeutic proteins have been identified.[13,14] Among those, mechanical stresses in the form of shaking and stirring of liquid protein formulations[15-21] have been shown to potentially induce considerable amounts of protein aggregates. These types of stress factors are encountered commonly at different stages from manufacturing process up to bedside administration to the patient. Recently, Kiese et al.[15] showed that stirring of a liquid IgG1 formulation results in large numbers of micron-sized aggregates, whereas the consequence of shaking was limited to formation of (high-molecular-weight) oligomers and was dependent on presence of an air-filled

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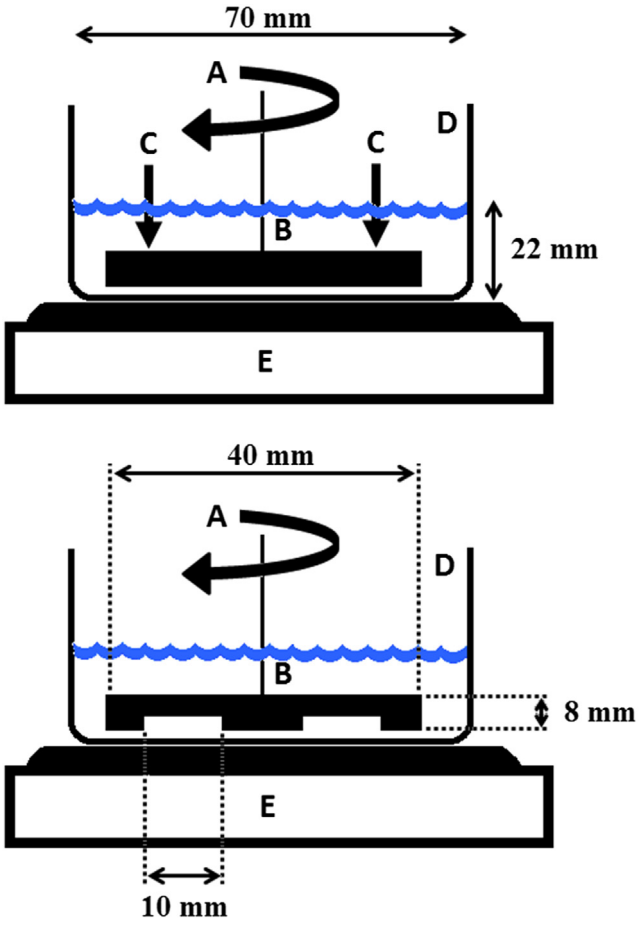

Figure 1. Scheme of the experimental set-up for the overhead stirring system used in this study. The rotor (a) turned the stir bar (b) with a rotation speed of 300 rpm, whereas the normal force (c) applied to the bottom of the glass container (d) during the contact mode was monitored by the balance (e). Stirring in the absence of any contact was performed by rotating the stir bar at a distance of ~5 mm from the bottom of the container. Bottom figure: the system with a reduced contact area by using a stir bar with cavities on its lower surface.

headspace in a bottle. It was also shown that polysorbate 20 had a protective effect on antibody formulations against aggregation. The authors have listed several parameters, including shear, interfacial effects, and cavitation, that could potentially lead to severe aggregation of protein during stirring. Bee et al.[22] ruled out the effect of high shear force on the aggregation of an antibody. There are studies that have addressed the effects of other parameters on protein aggregation; however, the exact underlying mechanism of stirring stress–induced aggregation is still not fully understood.

In a typical stirring-stress study, stirring involves a constant contact sliding of the surface of a stir bar against the solid surface of a container. Such contact would disturb a critical solid–liquid interface that is a target for protein adsorption. Interestingly, however, to our knowledge, the effect of the contact sliding of the solid surfaces, as present during the stirring, on aggregation of proteins has not been investigated.

In this study, we addressed the effect of contact sliding during stirring of a liquid mAb protein formulation on aggregation of the protein. For this purpose, a stirring configuration has been designed in which the presence, magnitude, and normal pressure of the contact between the solid surfaces can be manipulated. The aggregation of the antibody was monitored by size-exclusion chromatography (SEC), nanoparticle tracking analysis (NTA), and flow imaging microscopy. Our results indicate that contact sliding–triggered abrasion of adsorbed protein is the key to the formation of micron-sized aggregates induced by stirring.

Materials and Methods

Materials

Phosphate buffered saline (PBS; 8.2 g/L NaCl, 3.1 g/L Na_2H-$PO_4.12H_2O$, 0.3 g/L $NaH_2PO_4.2H_2O$, pH 7.4) was obtained from Braun (B. Braun Melsungen AG, Germany) and filtered by using a 0.22-μm polyethersulfone-based syringe-driven filter unit (Millex GP, Millipore, Carrigtwohill, Ireland). Polysorbate 20, sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium azide, sodium sulfate, and 9-(2,2-dicyanovinyl) julolidine (DCVJ) were obtained from Sigma (Sigma-Aldrich, Steinheim, Germany). Ethanol was purchased from Biosolve (Biosolve B.V., Valkenswaard, The Netherlands). Ultrapure water (18.2 MΩ cm) was dispensed by using a Purelab Ultra water purification system (ELGA LabWater, Marlow, UK). A monoclonal human IgG1 (IgG), formulated at 65 mg/mL in 10-mM sodium citrate buffer containing 5% sucrose at pH 6.0 as described before[23-25] was used as a model protein. This stock solution was diluted to a concentration of 0.1 mg/mL IgG in PBS, either with or without 0.01% (w/v) polysorbate 20. In addition, a solution of 0.5-mg/mL IgG in PBS without polysorbate 20 was also prepared. Furthermore, a monoclonal human antibody of the IgG1 subclass (IgG-BI), kindly provided by Boehringer Ingelheim (Biberach, Germany), myoglobin (from equine skeletal muscle) and bovine serum albumin (BSA), both purchased from Sigma (Sigma-Aldrich), were used as model proteins in a small set of experiments.

Mechanical Stress Conditions

The stirring stress was generated at room temperature by magnetic stirring or an in-house–designed overhead stirring system. For the magnetic stirring system, a Teflon-coated stir bar with a diameter of 8 mm and a length of 40 mm, a flat-bottom crystallizing glass (diameter of 70 mm, cat. no. 2131141; Duran; Schott, Mainz, Germany) and a magnetic stirrer (RCT basic IKAMAG; IKA-Werke GmbH, Staufen, Germany), operating at a constant rate of 300 rpm, were used. The overhead stirring system (Fig. 1) was used to study the effects of surface contact, contact area, and force generated by the stir bar on the glass surface. For this series of experiments, a rod was used to fix an in-house–designed stir bar made of polyether ether ketone. The shape and dimensions of this bar were similar to the one used with magnetic stirring experiments. The effect of surface contact was investigated by stirring 80 mL of IgG solution at 300 rpm while the stir bar contact slid over the glass surface. A balance placed underneath the glass container was used to monitor the force applied by the stir bar (force calculated through multiplication of the displayed mass and gravitational force), which was in this case 5.88 N. Similarly, noncontact stirring was performed by stirring with 5 mm space between the stir bar and the surface of the glass container. As negative control, an equal amount of IgG solution was left for 270 min unstirred in the same type of crystallizing glass at room temperature. An additional control consisted of stirring with contact sliding (or magnetic stirring) of protein-free PBS.

All the previously mentioned stirring conditions were applied for polysorbate-free IgG solutions. In addition, the contact sliding stirring experiments were performed with polysorbate-containing IgG solutions.

To investigate the effect of the contact area, a modified stir bar with the same dimensions, but half the contact area, was used while the normal force on contact was reduced to 2.94 N to keep the applied pressure the same. In addition, the stir bar with full