Control of Protein Particle Formation During Ultrafiltration/Diafiltration Through Interfacial Protection

DANIEL J. CALLAHAN, BRADFORD STANLEY, YULING LI

Department of Purification Process Sciences, MedImmune, Gaithersburg, Maryland 20878

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ABSTRACT: This study investigates the mechanism of protein particle formation during ultrafiltration/diafiltration (UF/DF), finding that agitation drives particle formation by promoting protein-interface adsorption and desorption. Low conductivity and the presence of surfactant reduced the level of particle formation in small-scale stirring studies, and the same trends were observed in pumping and UF/DF. Polysorbate 80 (PS80) and hydroxypropyl-β-cyclodextrin (HPβCD) reduced particle formation in UF/DF by factors of 15 and 4, respectively. Measurements of conformational stability, colloidal stability, and surface tension demonstrated that PS80 protects against particle formation by preventing protein-interface adsorption, low conductivity improves the colloidal stability of the protein, and the mechanism of action of HPβCD remains unclear. This work demonstrates that interfacial adsorption–desorption of the protein during UF/DF is the principal cause of particle formation, that the level of surfactant-free particle formation depends on the colloidal stability of the protein, and that the inclusion of surfactant greatly reduces in-process particle formation during UF/DF. © 2014 The Authors. *Journal of Pharmaceutical Sciences* published by Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:862–869, 2014
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INTRODUCTION

The complex molecular architecture of protein pharmaceuticals leads to a propensity toward the formation of aggregates ranging from dimers to micron-sized particles. Although particles typically account for a small mass percentage and often do not impact drug potency, protein aggregates and particles may elicit immunogenic responses.[1–3] Therefore, controlling protein particle formation is a major focus in the development of biopharmaceuticals.

There are multiple pathways to protein particle formation.[4] In the simplest pathway, an aggregation-prone protein forms higher order oligomers that in some cases grow to micron-sized particles. Proteins may also undergo conformational changes that increase their susceptibility to particle formation, either because of intrinsic conformational instability or in response to an external stress. Proteins adhere to a wide range of surfaces, including air–liquid and liquid–solid interfaces, and the accumulation of proteins on these surfaces can lead to particle formation, in some cases following a surface-induced conformational change. Foreign particles can also serve as nucleation sites for the formation of protein particles.

The growing understanding of these pathways to particle formation has led to a successful approach to mitigating particle formation in which the formulation composition is selected to maximize the conformational stability of the protein and provide interfacial protection. The purification process must also be optimized to remove potential particle-nucleating impurities. However, the purification process itself presents a variety of stresses that can lead to in-process particle formation. Although in-process particle formation is sometimes disregarded with the expectation that these particles will be removed by filtration, we argue that it is essential to understand and limit in-process particle formation to establish a well-controlled process, reduce the particle burden for filtration, and reduce the risk that prefiltration stresses could lead to postfiltration particle formation.

Multiple authors have noted the formation of protein aggregates during ultrafiltration/diafiltration (UF/DF) operation,[5–7] and we have observed millions of micron-sized particles per milliliter in the UF/DF products of multiple proteins prior to final filtration. The complexity of the UF/DF unit operation leads to several hypotheses for the principal mechanism of UF/DF-induced particle formation: (1) shear stress or other characteristics of the fluid flow, (2) impurities leached into the product stream from contact surfaces during extended periods of recirculation, (3) interfacial interactions, and (4) pump-specific stresses such as cavitation and tubing spallation.

First, we consider the possibility of shear stress-driven particle formation. Many studies have demonstrated that exposing a protein to shear stress can lead to protein aggregation, although the mechanism of this effect remains unclear. Some argue that the shear stress itself induces a conformational change that results in protein aggregation,[8] whereas others propose that the associated fluid flow increases reactive collisions in the bulk or facilitates protein transport to and from aggregation-inducing interfaces.[9] Early studies on enzyme activity reported a significant reduction in activity upon shear,[10] suggesting that shear disrupted the structure and thus the function of the enzymes under study, but shear did not induce a conformational change in cytochrome C.[11] Another set of studies showed that shear by a rotating disk led to protein aggregation with the amount of monomer loss depending on the shear rate,[12] that this aggregation did not involve covalent modifications, and that the

Correspondence to: Yuling Li (Telephone: +301-398-4845; Fax: +301-398-9433; E-mail: LiYu@medimmune.com)

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level of aggregation was reduced by the addition of surfactant and increased by an increase in the surface roughness of the rotating disk.[13] These results suggest that shear induces protein aggregation by enhancing the transport of the protein to and from interfaces rather than by directly affecting the protein. This agrees with other studies showing that shear alone does not lead to the aggregation of monoclonal antibodies,[14] that shear induces aggregation through the air–liquid interface,[15] and that polysorbate prevents antibody precipitation under shear.[16] One study suggested that altering the flow parameters of UF/DF operation could limit particle formation by changing the shear stress experienced by the protein.[7] Although the modified parameters successfully limited aggregation and particle formation, the maximal reduction was only two-fold, suggesting that shear itself may not be the main driver of particle formation in UF/DF.

A second pathway to protein particle formation that may be involved in UF/DF-induced particle formation is nucleation on foreign particles present in solution, also referred to as heterogeneous nucleation.[4,17] For example, filling pump-induced particle formation was related to the shedding of steel nanoparticles that acted as nucleation sites for protein particle formation.[18] In another case, protein precipitation was induced by soluble tungsten, an impurity that can be present in prefilled syringes.[19] During UF/DF operation, the repeated flow of the protein solution over various materials including the membrane, tubing, and retentate tank could result in impurities leaching into the product stream and promoting particle formation, with the portion of the tubing that is repeatedly stressed by the pump head as a particularly likely source of such impurities.

Third, proteins come into contact with a variety of interfaces during UF/DF operation, including solid–liquid and air–liquid interfaces, and interfacial adsorption could lead to protein particle formation. Protein-interface adsorption is often based on hydrophobic interactions, but electrostatic interactions also play an important role.[4] Surface-induced particle formation may involve a conformational rearrangement of the protein to maximize its area of interaction with the surface or to minimize the energy of that interaction,[4,20] or the surface may instead promote protein–protein association without any conformational event.[16] Surfactants in biopharmaceutical formulations protect against particle formation by competing with protein molecules for interfacial adsorption, preventing protein adsorption to interfaces.[21] For example, surfactants protect proteins from exposure to the air–liquid interface in static vials during long-term storage, and also provide protection during short time-scale perturbations of that interface such as during product shipping. Surfactants can also exert effects through direct interactions with the protein.[22] Proteins commonly adsorb on solid interfaces, and solid materials such as stoppers and tubing have been implicated in particle formation.[23,24] UF/DF operation presents multiple solid interfaces (tank, tubing, and membrane surfaces) as well as an air–liquid interface, suggesting that interfacial interactions could play a role in UF/DF-driven particle formation.

Fourth, pump-specific stresses are often implicated in protein particle formation, and pump-driven particle formation has been suggested as a route to particle formation in UF/DF.[6] Pumping itself represents a complex stress encompassing shear stress, surface interactions, potential introduction of impurities through leaching or tubing spallation, and potential cavitation. Filling pumps have been implicated in protein particle formation,[25] and pump-induced cavitation has been suggested as the cause of UF/DF-induced protein aggregation.[6]

The goal of this study is to identify which of the possible mechanisms described above is the dominant factor in protein particle formation during UF/DF operation: shear stress, impurities, interfacial interactions, or some combination of these factors present in the pump or the overall unit operation. We accomplish this by breaking the UF/DF unit operation into its constituent parts, examining the effects of solution conditions and additives on agitation-induced particle formation, and studying the biophysical properties of the protein in these solutions. The results of this work indicate a primarily interfacially driven mechanism of particle formation in UF/DF and suggest several viable particle mitigation strategies.

MATERIALS AND METHODS

Proteins

This study used the four in-house IgG molecules mAb A, B, C, and D (A, B, and C are IgG 1 and D is IgG 2), and the proteins lysozyme, ovalbumin, and α-chymotrypsinogen (Sigma, St. Louis, Missouri). The proteins were dialyzed or diafiltered into the appropriate buffers for experimentation, and in all cases were filtered through a 0.22 μm filter immediately prior to the experiment. Except where noted, data in this manuscript were collected at a protein concentration of 5 mg/mL.

Particle Measurement

For all experiments, particle formation was monitored using Micro-Flow Imaging™ (model DPA-4200; Protein Simple, Santa Clara, California). Undiluted 1 mL samples were loaded on the instrument, and 600 μL were measured (or $>10^6$ particles) at a flow rate of 150 μL/min. The data reported here represent the total number of particles per milliliter with an estimated circular diameter of more than 1 μm (1 μm is the lower limit of particle size that can be detected by this instrument).

UF/DF Recirculation

Ultrafiltration/diafiltration recirculation experiments were conducted using a semiautomated tangential flow filtration (TFF) system including a tandem peristaltic pump (SciLog, Madison, Wisconsin) and a Minimate™ TFF capsule with a 50 cm^2 Omega™ (modified polyethersulfone) membrane (Pall, Port Washington, New York). All UF/DF experiments used a 1 foot section of STA-PURE® tubing (Thermo Scientific, Waltham, Massachusetts) at the pump head and platinum-cured silicon tubing elsewhere. To ensure that the mixing in the retentate tank was consistent across experiments, the retentate tank was placed on a magnetic stir plate (Corning PC-410D; Corning, New York) and stirred at 300 rpm. In recirculation experiments, 100 mL of 5 mg/mL mAb was placed in the retentate vessel and both the retentate and permeate were routed back to the tank to maintain a constant volume throughout the process.

Stirring in Vials

Small-scale stirring experiments were conducted with 3 mL of protein solution in 3 mL glass vials (Schott AG #6800-0316, Mainz, Germany) with headspace, with Teflon stoppers (West