

IgG Particle Formation during Filling Pump Operation: A Case Study of Heterogeneous Nucleation on Stainless Steel Nanoparticles

ANIL K. TYAGI,¹ THEODORE W. RANDOLPH,² AICHUN DONG,³ KEVIN M. MALONEY,⁴ CARL HITSCHERICH JR.,⁴ JOHN F. CARPENTER¹

¹Center for Pharmaceutical Biotechnology, Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado 80262

²Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado 80309

³Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, Colorado 80639

⁴Protein Pharmaceutical Development, Biogen Idec Inc., 14 Cambridge Center, Cambridge, Massachusetts 02142

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ABSTRACT: This study investigated factors associated with vial filling with a positive displacement piston pump leading to formation of protein particles in a formulation of an IgG. We hypothesized that nanoparticles shed from the pump's solution-contact surfaces nucleated protein aggregation and particle formation. Vials of IgG formulation filled at a clinical manufacturing site contained a few visible particles and about 100,000 particles (1.5–3 μm) per mL. In laboratory studies with the same model (National Instruments FUS-10) of pump, pumping of 20 mg/mL IgG formulation resulted in about 300,000 particles (1.5–3 μm) per mL. Pumping of protein-free formulation resulted in 13,000 particles (1.5–15 μm) per mL. More than 99% of the particles were 0.25–0.95 μm in size. Mixing of protein-free pumped solution with an equal volume of 40 mg/mL IgG resulted in 300,000 particles (1.5–15 μm) per mL. Also, mixing IgG formulation with 30,000/mL stainless steel nanoparticles resulted in formation of 30,000 protein microparticles (1.5–15 μm) per mL. Infrared spectroscopy showed that secondary structure of IgG in microparticles formed by pumping or mixing with steel nanoparticles was minimally perturbed. Our results document that nanoparticles of foreign materials shed by pumps can serve as heterogeneous nuclei for formation of protein microparticles. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 98:94–104, 2009

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INTRODUCTION

There are growing concerns about safety problems associated with protein aggregates in therapeutic protein products.¹ Aggregates can cause an immune response in patients that potentially can neutralize the drug product and perhaps even

Correspondence to: John F. Carpenter (Telephone: +303-315-6075; Fax: +303-315-6281; E-mail: john.carpenter@uchsc.edu)

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the endogenous version of the therapeutic protein.¹ The consequences of these reactions can range from rendering a product ineffective in patients to permanent harm and even death.¹ Although the potential for protein aggregates to cause immunogenicity is well recognized, it is not clear which types of aggregates are required to trigger the response. However, there are some hypotheses based on insights from fundamental principles of immunology and from development of vaccine products. It has been proposed that relatively large aggregates (i.e., composed of numerous protein molecules), particularly those containing protein molecules with natively like structure, are most likely to be the most potent in eliciting an immune response.¹

Because aggregate-induced immunogenicity cannot be predicted, it is critical to assure that aggregate levels within a product are kept to a minimum. This is an extremely difficult goal to achieve because proteins can aggregate at any step during initial fermentation, upstream and downstream processing, fill/finish operations, shipping, storage and delivery to the patient.² There are numerous published and unpublished studies investigating approaches to minimize protein aggregation by optimizing solution conditions during processing and in the final formulation.³ Also, there are approaches used to reduce aggregate levels in protein solutions with processing steps such as preparative size exclusion chromatography, filtration and high-pressure disaggregation.^{2,4} Most of this work focuses on aggregation processes that lead to the conversion of a sufficient mass of protein into aggregates such that the loss of native protein is easily measurable (e.g., >1% aggregated).

However, there are other types of aggregates that are often frustratingly difficult to detect, understand and control. This category of degradation product consists of relatively large aggregates (e.g., >0.2 μm in diameter) that are referred to as "particles". They can form during bioprocessing steps and/or in the final product container. Usually, this problem is first identified because a small number of visible particles are observed, but relatively large numbers of subvisible particles also can be present. The particles often constitute a miniscule fraction of the protein molecules, such that loss of native soluble protein molecules is virtually undetectable by standard methods such as filtration and size exclusion chromatography. Also, unlike most aggregation processes, many times particle formation does

not lead to a further increase in the level of protein aggregates over time. Complicating matters further, in some cases protein particle formation appears to be random across dose containers in a given lot and/or from lot-to-lot during manufacturing.

Despite the apparent wide-spread occurrence of problems with protein particle formation, there are few relevant published reports on this phenomenon. In one, Chi et al. described investigations of particle formation in glass vials of a protein product, recombinant human platelet-activating factor acetylhydrolase.⁵ They found that microparticles of glass were responsible for heterogeneous nucleation of protein particle. Seeding samples with glass nanoparticles nucleated protein aggregates, but subsequent growth of aggregates was limited. Thus, the relatively small numbers of visible protein particles observed in product vials could be directly related to the small number of glass particles present in each vial. And the apparent random appearance of protein particles in product vials in a given lot could be due to the variability of the number of glass particles per vial. Also, of relevance to the potential immunogenicity of aggregates, it is important to note the protein molecules in the particles retained substantial natively like structure, based on optical spectroscopy.⁵

A recent report from Cromwell et al.² described particle formation in an IgG formulation during vial filling operations. The formulation formed particles during processing with a rotary piston pump. The cause for particle formation was not identified. Processing with a rolling diaphragm pump, however, did not result in particle formation. Therefore, the practical solution to this manufacturing issue was to avoid using a rotary piston pump and to fill the product with a rolling diaphragm pump. Unfortunately, often constraints of manufacturing sites do not allow for the option to switch to a different type of filling pump.

There is speculation that heterogeneous nucleation may be a major cause of this type of problem. Micro- and nanoparticles of foreign materials, which are shed from bioprocessing equipment (e.g., pumps, tubing, filters, etc.) and containers/closures (e.g., glass, rubber, silicone oil, etc.),^{5,6} could stimulate protein aggregation and particle formation. Presumably any solid material with which the protein solution is in contact potentially could be a source of foreign particles.

Larger particles (e.g., $>0.2 \mu\text{m}$) are removed from the protein solution during the sterile filtration step that is used when the protein solution is transferred into the holding container for the filling unit.² There is not further filtration of the solution as it is filled into individual vials or syringes.² For intravenous administration, an in-line filter could be employed to remove larger particles. But for subcutaneous and intramuscular injection, filtration of the protein solution is not currently used. Thus, with these routes of administration, particles that form during filling operations and in the final product container will be delivered to the patient.

In the current study we tested the heterogeneous nucleation hypothesis as part of our investigation of the cause(s) of protein particle formation during the vial filling operation for a therapeutic monoclonal antibody product. In preliminary studies, a formulation of the IgG, which had been optimized for physical and chemical stability in aqueous solution, was found to form a few visible particles within 24 h of vial filling with a positive displacement piston pump (Filamatic[®] type FUS-10 filling pump). Filling with either a peristaltic or a rolling diaphragm pump did not result in the formation of visible particles. The particles formed during pumping with the positive displacement piston pump were observed in each vial in a given filling run. Thus, it did not appear that they could be attributed to factors such as occasional sloughing off of a protein film that might accumulate at the base of the cylinder in piston pumps. Also, the particles were observed when the formulation was pumped into different container types, suggesting that heterogeneous nucleation of protein particles by foreign materials derived from the containers was not involved.

Our hypothesis was that micro- and/or nanoparticles shed from the solution-contact surfaces in the pump were nucleating protein aggregation and microparticle formation. In order to obtain quantitative results, we used a Coulter Counter to determine the number of subvisible particles formed during the experiments. We first quantified particle formation during pumping with a Filamatic[®] type FUS-10 filling piston pump. Next, to test our hypothesis directly, we pumped protein-free buffer and measured the level of particles. Then this buffer was mixed with unpumped protein solution and particle levels were determined. This approach eliminated potential contributions from mechanical manip-

ulations of the protein solution (e.g., shear) to pumping-induced particle formation. As a further test of our hypothesis, the IgG formulation was mixed with stainless steel nanoparticles to determine if protein microparticle formation could be induced artificially. To gain insight into the effects of microparticle formation on protein structure, we used infrared spectroscopy to compare the secondary structure the native IgG to that for the protein in microparticles formed during pumping or by seeding with stainless steel nanoparticles.

MATERIALS AND METHODS

Materials

The pharmaceutical grade IgG used in this study was produced at Biogen Idec (Cambridge, MA). The *pI* of the main fraction of the protein was 8.0. The IgG product was an aqueous solution containing 20 mg/mL protein formulated in an isotonic phosphate buffer (pH 6) containing 0.02% polysorbate 80 (hereafter referred to as "formulation"). Vials of the formulation were obtained that had been filled with the 5.3 mL of the formulation at a clinical manufacturing site. A National Instrument Filmatic[®] pump (model FUS-10 piston/cylinder) was used to fill the vials at this site.

Nanoparticles of 316L stainless steel were obtained from Argonide Corporation (Sanford, FL). As described by the supplier, these particles were nonporous and the average primary particle size was 100 nm. NaCl, dibasic sodium phosphate, hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific (Hanover Park, IL). Monobasic and dibasic potassium phosphate were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All chemicals were of reagent grade or higher quality. Buffer solutions were prepared with ultrapure water collected from a Milli-Q[®] Ultrapure Water Filtration System (Millipore, Billerica, MA).

Effect of Pumping Operation on Protein Particle Formation

A National Instrument Filmatic[®] pump (model FUS-10 piston/cylinder) was purchased from National Instrument Company, Inc. (Baltimore, MD) and used throughout the study. The FUS-10 piston rod and cylinder are made from 316L

stainless steel. The piston head is made of Kynar[®] polyvinylidene fluoride. The O-ring located on the piston head is made of silicone and is used to create the seal between the piston and the cylinder.

The filler pump was operated as described in the manufacturer's manual. The line speed was 39 cycles per min. Prior to pumping, the protein formulation was filtered through a 0.2 μm filter (Whatman model GD/X polyethersulfone membrane). In initial experiments the pump was set to deliver 2 mL/cycle for 12 cycles (24 mL total) into a single test tube. Alternatively, a single pump cycle of 5.3 mL was employed to pump the protein formulation into a test tube, mimicking the process used at the clinical filling site. In other experiments, protein-free formulation or buffer was pumped, and then the pumped solution was mixed with formulation containing 40 mg/mL IgG. With each pumping protocol, triplicate samples were pumped and incubated at room temperature, prior to counting the particles present in the solution. Unless otherwise indicated, results presented are for samples that were incubated for 10 h at room temperature before particle counting.

A Coulter Counter Model Z1 (Beckman Coulter, Inc., Fullerton, CA) was used to determine the number of subvisible particles in solution. With this instrument, particles could be detected in the 1.5–30 μm size range. Results were obtained for five different size ranges of particles: 1.5–3, 3–6, 6–8, 8–15 and 15–30 μm . Isotone (Coulter Balanced Electrolyte Solution) was used for sample dilution before particle counting. A 200 μL sample was mixed with 20 mL of Isotone solution and incubated for 20 min at room temperature before the measurements were made. The instrument was calibrated with particle standards from Beckman Coulter, Inc.

Elemental Analysis of Particles Shed from FUS-10

Samples were pumped through the FUS-10 pump, incubated under identical conditions as above and analyzed at the Department of Geological Sciences, University of Colorado, Boulder using an inductively coupled plasma optical emission spectrometer (ARL 3410 + ICP-OES). Nitric acid was added to the samples to dissolve suspended metals. NIST-traceable standards were used to calibrate the spectrometer and samples were analyzed as previously described.⁷

Heterogeneous Nucleation of IgG Aggregation by Stainless Steel Nanoparticles

To test the hypothesis that stainless steel nanoparticles can serve as heterogeneous nuclei for IgG aggregation, the IgG formulation (filtered through 0.2 μm filter) was seeded with 316L stainless steel nanoparticles (100 nm primary particle size). Buffer (10 mM potassium phosphate, pH 7.0, with 150 mM NaCl) containing 0.25ng/mL of nanoparticles (an amount estimated to contain 60,000 particles/mL based on information from the supplier) was mixed with an equal volume of IgG formulation with 40 mg/mL protein. The resulting mixture, containing approximately 30,000 nanoparticles/ml and 20 mg/ml protein, was incubated at room temperature. Triplicate samples were prepared and assayed for particle content by Coulter Counter.

SEC-HPLC Analysis

Size exclusion HPLC (SEC-HPLC) was utilized to quantify levels of monomeric protein and to examine protein solutions for soluble aggregates. Prior to analysis, solutions were centrifuged at 10,000g (4°C) for 20 min. A 20 μL aliquot of the supernatant was removed, and diluted to a final protein concentration of 2 mg/mL with buffer (10 mM Potassium phosphate and 140 mM sodium chloride at pH 6.0), and a 25 μL aliquot was assayed by SEC-HPLC. A Hewlett Packard 1090 HPLC system and a Tosohaas TSK 3000SW SEC column were employed. A filtered and degassed mobile phase containing 10 mM Potassium phosphate and 140 mM sodium chloride at pH 6.0 was used at a flow rate of 0.6 mL/min. UV absorbance 280 nm was monitored. Area under the IgG peak was used to calculate the percent of soluble protein remaining. The average monomer peak areas of filtered formulation was determined in triplicate samples and used as the control value.

Secondary Structural Characterization

To assess the effect of microparticle formation on IgG secondary structure, infrared (IR) spectroscopy was used to compare the secondary structures of native and microparticulate IgG. Infrared spectra were acquired using an ABB Bomem (Quebec, Canada) MB-series Fourier transform infrared spectrometer. Samples were loaded into

a BioCell™ (Bio Tools, Jupiter, FL) with CaF₂ windows with a 6.5- μm fixed pathlength well. A 128-scan interferogram was collected for each spectrum in single beam mode with a 4 cm^{-1} resolution. Spectra for solutions or suspensions of IgG and for a protein-free buffer blank were acquired. Protein absorbance spectra were obtained by subtraction of the spectrum for the buffer blank according to previously described criteria and water subtraction procedures.^{8,9} Water vapor signals, if present, in the spectrum of protein were removed by subtracting the spectrum of gaseous water. Second-derivative spectra were obtained with a 7-point Savitsky–Golay derivative function.¹⁰ Second-derivative spectra were baseline corrected as previously described.¹¹ Final spectra were treated with a $2 \times$ FFT interpolate function and plotted with SigmaPlot 8.0 software (Systat Software, Inc., Chicago, IL).

To study the effect of pumping, the formulation with 20 mg/mL IgG was filtered through a 0.2 μm filter. The filtrate was pumped into a single glass test tube using FUS-10 pump. The pump was operated to deliver 2 mL/cycle, and the line speed was 39 cycles per min. The pumped sample was incubated for 24 h at room temperature prior to analysis. The protein adsorbed on the particles was pelleted by centrifugation and resuspended in protein-free buffer at 15 mg/mL. To study effects of protein interaction with stainless steel nanoparticles, the IgG formulation (with 40 mg/mL IgG) was mixed with an equal volume of buffer (10 mM potassium phosphate pH 7.0, 150 mM NaCl) containing 2 mg/mL solution of steel nanospheres. The mixture was incubated for 1, 3 and 24 h at room temperature before samples were processed as described above and the IR spectra were acquired.

RESULTS AND DISCUSSION

The Filmatic® model FUS-10 positive displacement piston pump used in the current study has been used for vial and syringe filling in commercial manufacturing facilities. During operation of this pump, protein molecules are exposed to several potential stresses that could promote protein aggregation and particle formation. Adsorption onto the solution-contact surfaces of the pump may cause conformational perturbation of protein molecules, which upon desorption can result in protein aggregates and particles in solution.^{12–21} Also, shearing, although not a direct cause of protein unfolding,²² may facilitate mass

transfer of adsorbed protein molecules and/or aggregates into the bulk solution. Finally, nano- and microparticles shed from the contact surfaces of the pump may serve as heterogeneous nuclei for protein aggregation. Our hypothesis for this study is that this latter stress is dominant in pumping-induced microparticle formation for the IgG solution. The following experiments characterize the pumping-induced microparticle formation and test directly our hypothesis for the mechanism for this phenomenon.

Effect of Pumping Operation on Protein Microparticle Formation

The IgG formulation was filled into vials at a clinical manufacturing site with a Filmatic® FUS-10 pump. Analysis in our laboratory documented that the formulation contained more than 100,000 particles/mL, most of which were in the 1.5–3 μm size range (Fig. 1A). Particles were not detected in the 15–30 μm size range.

After the particle-containing formulation was filtered through 0.2 μm filter, particles were undetectable by the Coulter Counter (data not shown). The filtered IgG formulation was used for the pumping studies conducted in our laboratory. In the initial experiment, 12 pumping cycles of 2 mL each with the Filmatic® FUS-10 filler were used to pump 24 mL of protein solution into a single test tube. This pumping operation, followed by incubation of the pumped solution at room temperature, resulted in the generation of more than 300,000 particles/mL, mostly in the 1.5–3 μm size range (Fig. 1A and B). Particles were not detected in the 15–30 μm size range. Although a large number of particles was generated by the pumping process, SEC-HPLC analysis of the pumped solution showed an insignificant reduction ($0.8 \pm 0.9\%$, mean \pm SD, $n = 3$) in IgG monomer level after pumping and 10 h incubation at room temperature. Soluble aggregates were not detected by SEC-HPLC (data not shown).

As described in more detail below, two observations support the conclusion that the particles formed during pumping contained protein. First, infrared spectra of collected particles have a prominent amide I absorbance that is due to protein. Second, pumping of solutions that did not contain protein resulted in a 20-fold smaller number of particles generated than observed when the IgG formulation was pumped (see below).

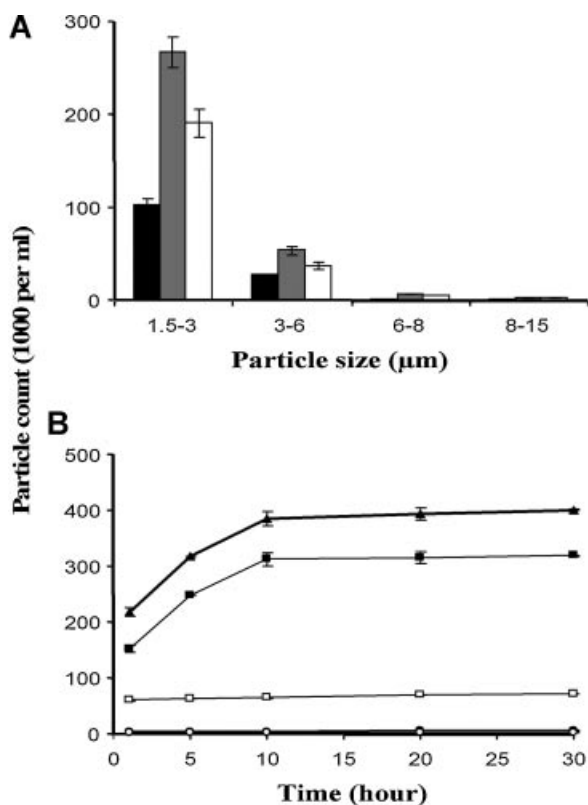


Figure 1. (A) A comparison of particle formation in IgG formulation during pumping through FUS-10 pump at different fill volumes. Black bars: IgG formulation pumped at manufacturing site into a single vial at 5.3 mL/cycle. Gray bars: IgG formulation first filtered through 0.2 µm filter and then a 24 mL volume was pumped into a single tube at 2 mL/cycle. White bars: IgG formulation first filtered through 0.2 µm filter and then a 5.3 mL volume was pumped into a single tube at 5.3 mL/cycle. (B) Particle formation monitored as a function of incubation time for incubation at room temperature, in size ranges of (■) 1.5–3 µm, (□) 3–6 µm, (●) 6–8 µm, (○) 8–15 µm and (▲) 1.5–15 µm. Error bars are standard deviations for triplicate samples.

In an earlier study on recombinant human platelet-activating factor acetylhydrolase it was found that increasing the concentration of Pluronic F-68 to greater than 0.05% (w/v) greatly inhibited heterogeneous nucleation of protein aggregation by glass nanoparticles, because of inhibition of adsorption of protein molecules to the glass surface.⁵ To determine if this type of formulation approach would be useful for reducing pumping-induced microparticle formation of the IgG formulation, we increased the polysorbate concentration from 0.02% to 0.2% and repeated the pumping experiment (12 cycles of 2 mL each with the FUS-10) with filtered IgG formulation.

This formulation adjustment did not result in a significant reduction in the number of microparticles formed during pumping (data not shown).

There was a higher particle count in the IgG formulation after pumping in our lab compared to that observed in samples pumped in the same model of Filmatic[®] filler at a clinical filling site. One reason could be that in our initial experiments we pumped the IgG formulation for several cycles into a single test tube. In contrast, at the commercial site a single cycle of 5.3 mL was used to pump the IgG formulation into each vial. To test the effect of pumping protocol, we pumped 5.3 mL in a single cycle and compared the results to those obtained by pumping 24 mL into a single test tube, using 12 cycles of 2 mL each (Fig. 1A). Using a single pumping cycle generated only about 20% fewer microparticles than when 12 cycles were used. This observation indicates that the protein microparticle formation occurs during each pumping cycle.

The number of microparticles generated with a single pumping cycle in our lab was still substantially higher than that measured in the formulation from clinical vials. Speculatively, the formation of a greater number of particles may be due to the use of a new pump, which has not been subjected to any surface treatments (e.g., so-called “passivation”) or “breaking-in” period. Presumably the clinical filling pump had been treated and broken-in. But even so, the clinical pump generated a large number of protein microparticles each time a vial of the IgG formulation was filled.

Effects of Pumping Protein-Free Formulation and Mixing with Protein Formulation

Next, we conducted an experiment that allowed us to test directly our hypothesis that the dominant factor causing protein particle formation during pumping was heterogeneous nucleation of protein aggregates/microparticles by micro- or nanoparticles of materials shed from the pump. Furthermore, this experiment tested for foreign material-induced protein aggregation in the absence of other stresses potentially arising during pumping such as shear and exposure to liquid–solid interfaces at the surfaces of the piston/cylinder. First, we pumped the formulation that did not contain protein (protein-free formulation) with the FUS-10 for 12 cycles of 2 mL/cycle into a single

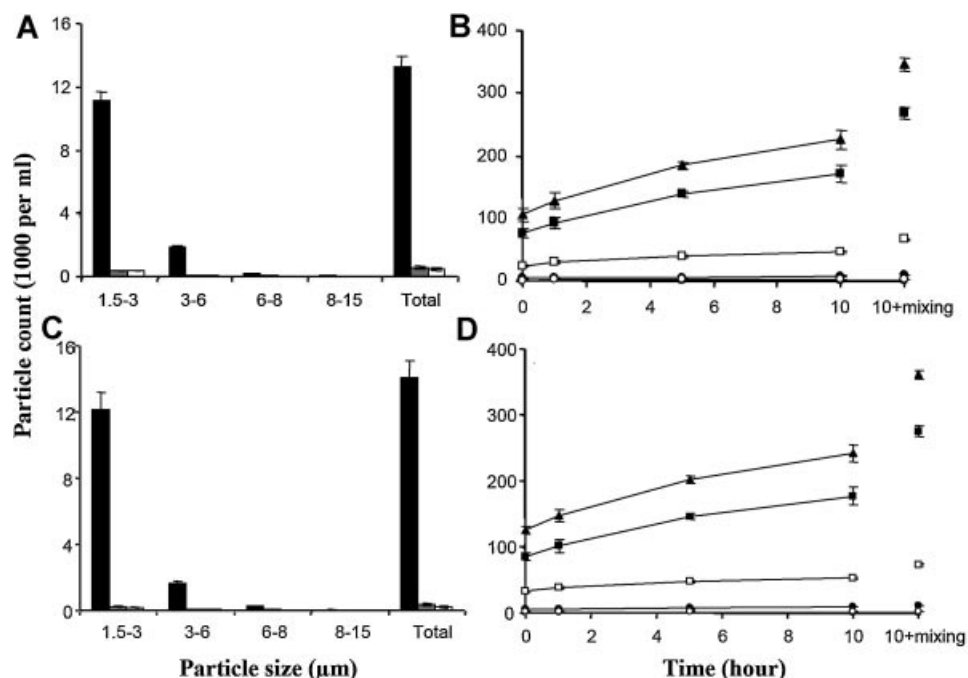


Figure 2. (A) Total number of particles in solutions. Black bars: protein-free formulation pumped through FUS-10 pump. Gray bars: IgG formulation at 40 mg/mL protein concentration filtered through 0.2 μm filter. White bars: protein-free formulation not pumped. (B) Particle formation monitored as a function of incubation time at room temperature, in size ranges of (■) 1.5–3 μm , (□) 3–6 μm , (●) 6–8 μm , (○) 8–15 μm and (▲) 1.5–15 μm . IgG formulation at 40 mg/mL protein (unpumped) was mixed with protein-free formulation that had been pumped through FUS-10 pump. (C) Total number of particles in solutions. Black bars: 10 mM potassium phosphate pH 7 containing 150 mM NaCl pumped through FUS-10 pump. Gray bars: IgG formulation at 40 mg/mL protein concentration filtered through 0.2 μm filter. White bars: 10 mM potassium phosphate pH 7 containing 150 mM NaCl not pumped. (D) Particle formation monitored as a function of incubation time at room temperature, in size ranges of (■) 1.5–3 μm , (□) 3–6 μm , (●) 6–8 μm , (○) 8–15 μm and (▲) 1.5–15 μm . IgG formulation at 40 mg/mL protein (unpumped) was mixed with 10 mM potassium phosphate pH 7 containing 150 mM NaCl that had been pumped through FUS-10 pump and the resulting mixture was incubated at room temperature. Error bars are standard deviations for triplicate samples.

test tube. Prior to pumping of this solution, particles were not detected with the Coulter Counter, but pumping resulted in about 13,000 particles/mL in the 1.5–6 μm size range (Fig. 2A). The pumped protein-free formulation was then mixed with an equal volume of formulation containing with 40 mg/mL IgG, which had been filtered through a 0.2 μm filter. Prior to mixing with the pumped solution, the protein-containing formulation did not have detectable micro-particles (Fig. 2A). After mixing the two solutions, the mixture was incubated at room temperature. Particle counts were made immediately and after 1, 5 and 10 h. Also, after 10 h the solution was gently agitated and particles were counted. Most

of the particles detected were in the 1.5–3 μm size range, and there were more than 300,000 particles per ml after 10 h of incubation and remixing. To test the effects of pH, buffer type and NaCl on pumping-induced particle formation, the experiment was repeated. Almost identical results were obtained when 40 mg/mL IgG formulation was mixed with pumped protein-free buffers containing: (1) 10 mM potassium phosphate, pH 7, with 150 mM NaCl (Fig. 2C and D); (2) 10 mM potassium phosphate, pH 7 without salt (data not shown); or (3) 10 mM potassium phosphate, pH 9, with 150 mM NaCl (data not shown) or without salt (data not shown). In control experiments, in which protein-free solutions were not

pumped prior to mixing with 40 mg/mL IgG, microparticles were not detected by the Coulter Counter (data not shown).

Therefore, although pumping of protein-free solutions resulted in only about 13,000 detectable microparticles ($\geq 1.5 \mu\text{m}$) per mL, pumped solutions had the capacity to induce almost 20-fold greater number of microparticles when mixed with the protein-containing solution. These results suggest that the pump was shedding a large number of particles that were smaller than the $1.5 \mu\text{m}$ detection limit for the Coulter Counter, which due to protein adsorption subsequently grew in size into a detectable size range.

The size distribution of particles in the pumped protein-free buffer in a $0.04\text{--}2000 \mu\text{m}$ size range was measured with a Beckman LS230 particle sizer (Fig. 3). More than 99% of the detected particles were in the range of $0.25\text{--}0.95 \mu\text{m}$, with the remainder in the size range of $1\text{--}3 \mu\text{m}$. Elemental analysis by ICP-OES of the pumped protein-free buffer (10 mM potassium phosphate, pH 7, with 150 mM NaCl) documented the presence of Si (0.8 ppm), Fe (0.8 ppm), Ni (0.1 ppm) and Cr (0.1 ppm). These results are consistent with the shedding of stainless steel from the piston/cylinder during pumping operation. These elements were not detected by ICP-OES in the buffer prior to pumping. Taken together, the results document that submicron stainless steel nanoparticles shed by the pump served as

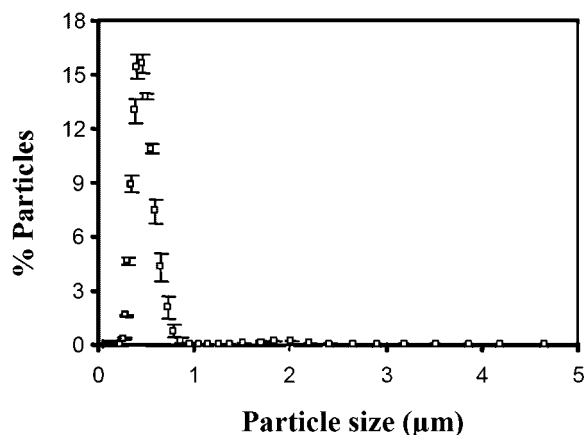


Figure 3. Particle size distribution in pumped buffer. Ten millimolar potassium phosphate buffer at pH 7.0 containing 150 mM NaCl with no protein was pumped through FUS-10 pump. The samples were incubated at room temperature for 10 h before the particle size measurements were made. Error bars are standard deviations for duplicate samples.

heterogeneous nuclei for the formation of IgG microparticles during pumping.

Heterogeneous Nucleation of IgG Microparticles by Stainless Steel Nanoparticles

To further test our hypothesis, the capacity of 316L stainless steel nanoparticles (100 nm primary particle size) to nucleate IgG microparticles was tested. Buffer (10 mM potassium phosphate, pH 7.0, with 150 mM NaCl) containing 0.25ng/ml of nanoparticles (an amount estimated to contain 60,000 particles/mL) was mixed with an equal volume of IgG formulation with 40 mg/mL protein. The resulting mixture, containing approximately 30,000 particles/mL and 20 mg/mL protein, was incubated at room temperature. After 20 h, there were almost 30,000 microparticles in the $1.5\text{--}15 \mu\text{m}$ size range (Fig. 4). There was not a substantial further increase in the number of particles after 20 h of incubation (data not shown). Based on SEC analysis, the reduction in the IgG monomer levels was $3.9 \pm 0.9\%$ and $4.2 \pm 1.3\%$ (mean \pm SD, $n = 3$), respectively, after 1 and 24 h of incubation. Soluble aggregates were not detected by SEC-HPLC (data not shown). Overall, these results further document that stainless steel nanoparticles can serve as heterogeneous nuclei for formation of IgG microparticles. The size of the particles

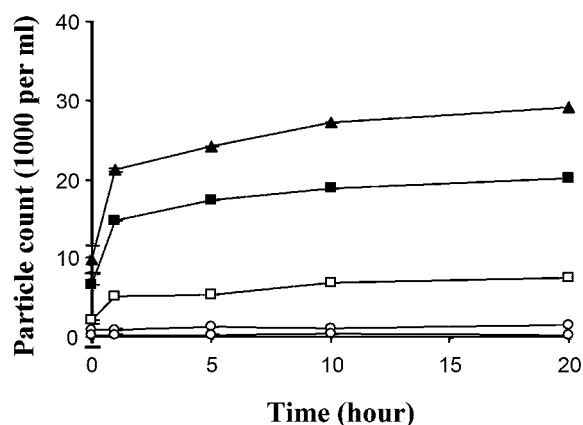


Figure 4. Particle formation in IgG formulation seeded with 100 nm stainless steel (316L) particles monitored as a function of incubation time at room temperature in size ranges of (■) $1.5\text{--}3 \mu\text{m}$, (□) $3\text{--}6 \mu\text{m}$, (●) $6\text{--}8 \mu\text{m}$, (○) $8\text{--}15 \mu\text{m}$ and (▲) $1.5\text{--}15 \mu\text{m}$. Error bars are standard deviations for triplicate samples.

formed indicated that multiple layers of protein molecules are coated onto the steel nanoparticles.

Structural Characterization of IgG Microparticles

To assess the effect of microparticle formation on IgG secondary structure, IR spectroscopy was used to compare the secondary structure of the native protein in solution to that in microparticles. Second-derivative spectra of IgG in the conformationally sensitive amide I region are shown in Figure 5 for native IgG in aqueous solution, for IgG in pumping-induced microparticles (Fig. 5A) and IgG in microparticles nucleated by stainless steel nanoparticles

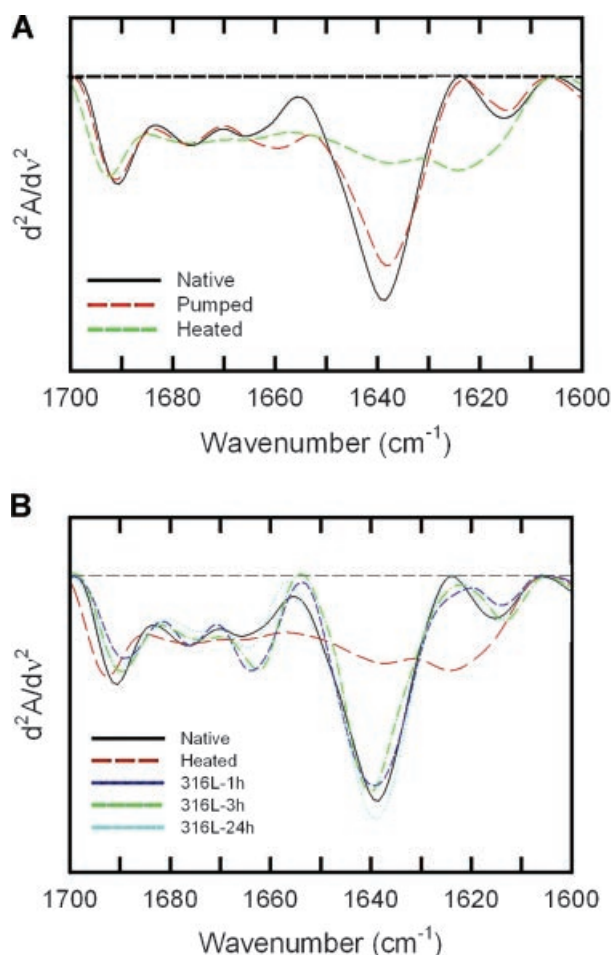


Figure 5. (A) Second-derivative amide I spectra of IgG in the native state, IgG in microparticles from pumped IgG solution and aggregates from heat-treated IgG sample. (B) Second-derivative spectra of IgG in the native state, aggregates from heat-treated IgG sample and for IgG after mixing with 100 nm 316L stainless steel nanoparticles and incubation for 1, 3, and 24 h.

(Fig. 5B). For comparison, the IR spectrum for IgG that had been heat denatured and precipitated is also shown (Fig. 5). The spectrum for IgG in its native state exhibits a pair of β -sheet bands, a strong lower wave-number band centered near 1639 cm^{-1} and a weak higher wave-number band centered near 1691 cm^{-1} , and two β -turn bands at 1677 and 1666 cm^{-1} .^{8,9} Compared to the spectrum of the native protein, the spectra of IgG in pumping-induced microparticles or in microparticles induced by stainless steel nanoparticles have slight changes in intensity in the main β -sheet band at ca. 1639 cm^{-1} , and a small increase in intensity at ca. 1662 cm^{-1} . In contrast, in the spectrum for the heat denatured and precipitated IgG, the intensity of the main β -sheet at ca. 1639 cm^{-1} is greatly reduced and there is a prominent new band for nonnative intermolecular β -sheet at ca. 1623 cm^{-1} . These results document that the secondary structure of IgG in microparticles was slightly altered compared to the gross structural perturbation associated with heat-induced denaturation and precipitation. Thus, heterogeneous nucleation of IgG aggregates and microparticles by foreign materials did not result in major alteration of the protein's secondary structure. Previously it has been shown that heterogeneous nucleation of particles of recombinant human platelet-activating factor acetylhydrolase by glass nanoparticles did not result in perturbation of protein conformation as assessed by infrared and UV spectroscopy.⁵

CONCLUSIONS

Our results documented that formation of IgG particles during vial filling occurred because the pump shed nanoparticles of stainless steel that served as heterogeneous nuclei for protein microparticles. This process could be mimicked by spiking stainless steel nanoparticles into the IgG formulation. In the IgG microparticles formed by both methods, the secondary structure of the protein was only slightly perturbed. These types of protein aggregates fit into the category that is hypothesized to be particularly immunogenic because there is a large number of protein molecules per microparticle and the native protein structure is not significantly perturbed.¹ Even though the total mass of protein in the microparticles in a given dose of a therapeutic protein product might be relatively small, these types of protein aggregates may play an important role in

inducing immunogenicity. However, there has not been published evidence that such particles of therapeutic proteins do indeed induce an immune response in patients or in an animal model. Even with this caveat, it is critical during formulation and process development that protein micro-particle levels are quantified and that efforts are made to minimize their formation. As part of this work, compatibility of the formulation with the fill/finish process and the primary container/closure system must be established.

For subvisible particle analysis, it appears that most manufacturers of therapeutic proteins follow guidelines in USP <788>, which were originally developed for small molecule parenteral drug products. These guidelines state that parenteral products should contain less than 6000 particles per container $\geq 10 \mu\text{m}$ and less than 600 particles per container $\geq 25 \mu\text{m}$. In the current study, in samples with hundreds of thousands of 1.5–3 μm particles, there were only a few thousand or less particles measured in the 8–15 μm size range. Also, in a published study²³ that measured a range of subvisible particles sizes in a therapeutic protein formulation, it was found that particles $\geq 10 \mu\text{m}$ constituted a minute fraction of the total subvisible particles. Samples of the therapeutic cytokine formulated with human serum albumin were analyzed for subvisible particles by light obscuration.²³ Samples contained more than 40,000 particles, with >90% in the 1–2 μm size range, ca. 7–9% 2–10 μm and <0.01% that were larger than 10 μm . Therefore, based on these two examples, it seems that only counting particles $\geq 10 \mu\text{m}$ could lead to failure to realize that the product contains smaller subvisible particles that are much more numerous.

Microparticles that are formed due to heterogeneous nucleation by foreign materials from filling pumps and/or the container/closure system will be delivered to the patient in products administered by subcutaneous or intramuscular injection. Filters are not currently used clinically to remove particles from products delivered by syringes. Development and use of such a filtration system might reduce the potential, but currently unproven, risk for immunogenicity in patients caused by microparticles.

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