SUBVISIBLE PARTICLES IN THERAPEUTIC PROTEIN PRODUCTS AND
POTENTIAL BIOLOGICAL CONSEQUENCES

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Therapeutic proteins offer treatment options for a variety of chronic medical conditions including cancer, diabetes, rheumatoid arthritis etc. However, all therapeutic proteins have an inherent potential to cause immune responses. Moreover, product quality attributes like protein aggregates even in low concentration are implicated in eliciting an immune response. Specifically proteinaceous particles in the size range of few nm-µm even in significant concentrations represent less than 0.1% of the total protein in solution. These particles can form during any stage of drug product development from purification, storage, fill-finish operations to even delivery to patients. Hence it’s a challenge to keep the concentration of particles in solution at a minimum. Presently particles in the size range of 2-100 µm are routinely monitored. In this work, we have evaluated particles smaller than 2 µm for their contribution to the formation of larger micron sized particles upon exposure of the protein to various pharmaceutically relevant stress conditions. Thereafter we determined particle concentrations that may inadvertently be delivered to patients, during an IV infusion of a therapeutic protein and further evaluated the factors contributing to these particles. Lastly, we examined the biological consequences of administering these particles during IV infusion of therapeutic proteins. Towards this we monitored the activation of various processes of the innate and adaptive immune system such as cytokine release in whole blood, activation of the toll-like receptors, upregulation of dendritic cells and T cell proliferation in
response to particle enriched or particle free fractions of IV solution of infliximab – a therapeutic antibody prescribed for numerous indications including Crohn’s disease and rheumatoid arthritis.

The form and content of this abstract are approved. I recommend its publication.

Approved: John F. Carpenter
I dedicate this work to all those who dreamt this dream with me.

To chachaji, chachi, Shantanu, mom, dad, dadi, Divya, Chiraag, Syna, mummy and papa
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Proteins therapeutics: their benefits and drawbacks

Protein therapeutics offer effective treatment options for a large number of medical conditions such as cancer, diabetes, crohn’s disease, rheumatoid arthritis, conditions relating to endogenous deficiencies in hormones or enzymes etc. Over the years, the field of protein therapeutics has seen the advent of wide variety of recombinant protein molecules that include cytokines, growth factors, hormones, and antibodies. Some of these molecules perform life-saving functions as replacement therapy in case of deficiency of the endogenous counterpart. For example hemophilia treatment with recombinant human (rh) factor VIII, replacement with rh growth hormone in the event of inherent deficiency or AIDS related wasting, and use of recombinant cytokines and colony stimulating factors in case of AIDS treatment, bone marrow transplantation, chemotherapy and leukemia treatment. Monoclonal antibodies (mAb), a class of therapeutic proteins that function via specific binding and blocking the function of molecules or organisms by either targeting them for destruction or stimulating specific signaling pathways are the fastest growing class of protein therapeutics. This group of therapeutics will continue to grow further as more disease signaling pathways are identified. Genetic engineering has also enabled the development of fusion proteins, wherein genes for the two proteins of interest are ligated and the resultant protein expression generates the fusion protein that combines the benefits of each of the molecules. For examples, albinterferon marketed as albuferon is a combination of albumin and interferon α-2b. Fusion with albumin helps prolong the half-life of the therapeutic protein to about 6 days compared to a few hours for interferon α-2b alone. Etanercept is another example of a fusion
protein where tumor necrosis factor (TNF) receptor is fused with the Fc region of the human antibody protein IgG1 enabling binding to excess TNF in the plasma on one end and supporting secondary immune response by binding to the Fcγ receptor on the other end.\textsuperscript{1,2}

Antibodies with their binding specificity have an advantage over the small molecule counterparts. This advantage can be further harnessed to enhance the efficacy of small molecule therapeutics by conjugating them to the constant region of the antibody - a class of molecules known as antibody-drug conjugates (ADCs). There are currently two ADC products on the market brentuximab vedotin (Adcetris\textsuperscript{®}) prescribed for Hodgkin lymphoma and ado-trastuzumab emtansine (Kadcyla\textsuperscript{®}) prescribed for treatment of breast cancer. More than 35 ADCs are in different phases of clinical trials making ADCs an increasingly important subclass of antibody-related therapeutics.\textsuperscript{3} Moreover the field is constantly evolving with therapeutic options like bispecific antibodies that combine specificities of two antibodies and can simultaneously address different antigens or epitopes. Blinatumomab marketed as Blincyto\textsuperscript{®} is one such example. It combines the binding sites for CD3 site for T cells and a CD19 site for the target B cells enabling the activation of cytotoxic T cell against the target malignant B cell.\textsuperscript{4}

With such indispensable benefits offered by therapeutic proteins, there are also some drawbacks. Therapeutic proteins are a very expensive treatment option. They cannot be administered orally due to denaturation in the gut and most importantly all therapeutic proteins have a potential to cause immunogenic responses in patients. Immunogenicity may range in severity as altered efficacy of the protein therpeutic to even patients turning non-responsive to the therapy due to the formation of neutralizing antibodies (nAbs).\textsuperscript{1} Formation of neutralizing antibodies can have serious consequences beyond patients turning non-
responsive. For example, incidences of pure red cell aplasia among patients that developed nAbs in response to one formulation of recombinant erythropoietin.\(^5\) In certain cases nAbs can also cross react and neutralize the endogenous protein. These responses could be from factors such as primary sequence or glycosylation patterns, route of administration, excipients and/or aggregates in formulation, dosing regimen, patient characteristics and even some unknown factors.\(^5\) To date we are still working to understand the mechanistic details of immunogenic responses against therapeutic proteins.\(^6\)

**Mechanisms of activation of immune response**

B cells can be activated by two immunological mechanisms – T cell independent (Ti) or T cell dependent (Td) activation of B cells. Ti activation of B cells may occur when costimulation from T cells is not required to launch an antibody response. It is speculated that immune response to protein aggregates may be elicited by this mechanism wherein the antigen can effectively cross-link the B cell receptor and induce an immune response via polyclonal activation of splenic marginal zone (MZ) B cells. However, stimulation of splenic MZ B cells results in IgM production and this signal may not be sufficient to activate class switching to IgG subclasses that elicit the immune response in humans.\(^7\) Computational analysis to evaluate mechanisms of immunogenicity due to protein aggregates also supports the hypothesis that Ti B cell activation has a low probability of occurrence under physiological conditions.\(^8\) *In vivo* and *in vitro* data in support of this statement have been recently reviewed by Moussa *et al* where they have documented few instances of Ti activation of B cells, but most cases of immunogenicity to protein aggregates in mice model and in *in vitro* assays using human cells occur via Td activation of B cells.\(^9\)
The mechanism of Td B cell activation, also referred to as the classical immune response is presented in Fig.1. Our immune system has developed ‘tolerance’ - a mechanism that overlooks endogenous proteins and responds only to foreign or ‘non-self’ proteins.\textsuperscript{10} Breakdown of this immune tolerance is usually a reason for immunogenicity to recombinant proteins and is often observed in the presence of impurities or aggregates in the drug product. These aggregates may only be slightly different from the native protein but that may be sufficient for the immune system to identify the protein as non-self.\textsuperscript{5}

Figure 1: Interactions of innate and adaptive immunity. When pathogens invade the body, the innate immune system is activated first and participates in the initial attack against the pathogens. Among the cells involved in innate immunity, dendritic cells act as antigen-presenting cells and migrate from the infected tissue to the regional lymph nodes where they present the antigens to T cells. Subsequently, the adaptive immune system is activated, and antibody production and killer T cells are induced. The resulting antibodies and killer T cells specifically attack the pathogens.\textsuperscript{11} (Adapted from reference 11)
Figure 2: The APC—T cell synapses: three signal model for T cell activation. In the lymph node, signal 1 activates the T cell through interaction of the T cell receptor (TCR) with antigen presented in the context of MHC molecules. MHC-I binds the CD8 TCR; MHC-II interacts with the CD4. Costimulation in the form of CD80/CD86 (also known as B7.1 and B7.2) and CD28 ligation provides the necessary second signal, promoting T cell function and survival. The CD40–CD40L (CD154) interaction is important during costimulation, as it further activates the APC, supporting upregulation of CD80/86. Cytokines, either pro- or anti-inflammatory in nature, provide signal 3, which dictates the differentiation status of the T cell.\textsuperscript{12} (Adapted from reference 13)

The speculation is that the repetitive arrays of protein monomers formed in an aggregate may have the potential to present epitopes similar to antigens and can activate the cascade of events ultimately resulting in the formation of antibodies.\textsuperscript{13} Upon administration, aggregates in the drug product may interact with various antigen-presenting cells of the innate immune system. Dendritic cells (DCs), the strongest activators of naïve T cells are need two signals for activation a) the release of pro-inflammatory cytokines and b) the
epitopes presented by the aggregated protein which may be similar to the repetitive arrays shared by most pathogens - also known as pathogen associated molecular patterns (PAMPs). Activated DCs have enhanced expressions of MHC and various surface receptors such as CD83, (maturation marker for DCs), CD80, CD86, CD40 etc. DCs upon activation can phagocytose the aggregates and present the protein fragments via the MHC-II to other immune cells, especially naïve T cells. Activation of naïve T cells by DCs also requires multiple signals – a) recognition of the MHC-II on DCs by the TCR (T cell receptor), b) recognition of co-stimulatory molecules CD80 and CD86 on DCs by CD28 on T cells and c) secretion of inflammatory cytokines such as IL-12, IL-6, TGF-β (Fig.2). Upon activation, the naïve T cells can proliferate and differentiate into CD4+ (helper) and CD8+ (cytotoxic) T cells. The CD4+ T helper cells in turn activate the B cells, which generate the antibodies specific to the antigens or in this case the therapeutic protein aggregates. In cases of immunogenicity, these antibodies could bind to the therapeutic protein but result in no clinical effect, or they could bind and neutralize the therapeutic protein in its native form, and may even bind to and neutralize the endogenous counterpart of the therapeutic protein.5,14 This neutralizing action can be fatal if the endogenous protein or its replacement therapeutic protein performs a non-redundant function as in the case of enzyme replacement therapies. Moreover the activation of the immune system can also lead to formation of memory cells that can compromise the therapy at an enhanced rate during subsequent dosing.15

**Infusion reactions**

Most monoclonal antibodies are delivered via intravenous infusion. This route of administration may also cause infusion related hypersensitivity reactions. In extreme cases adverse events may include anaphylactic shock. Some of the moderate symptoms of infusion
reactions include headache, dizziness, and tightness in throat that usually decrease subsequent infusion cycles.\textsuperscript{16,17} These reactions are frequently observed among patients receiving mAb therapy and so immunosuppressive agents like anti-histamines, acetaminophens or corticosteroids are coadministered during IV infusion.\textsuperscript{18} Premedication has helped reduce the occurrence of infusion reactions but to date our knowledge about the immunological mechanism of infusion reactions remains limited. Hypersensitivity reactions are classified into 4 categories and mAbs are known to elicit all 4 types of reactions.

![Cytokines in the inflammatory response. TGFβ and IL-6 are placed at the fulcrum of this equilibrium, because they can be either pro-inflammatory or anti-inflammatory, depending on the route of administration and the specific response examined. The balance is shifted to the right, indicating that the anti-inflammatory cytokines are dominant.

- IFNγ
- GM-CSF
- IL-8 and other chemokines
- IL-1
- IL-6
- IL-10
- IL-15
- IL-16
- IL-17
- IL-18
- TGFβ
- IL-1RA
- sTNF-R Monoclonal antibody to TNF
- IL-4
- IL-8
- IL-11
- IL-13
- IL-18BP

Granulocyte–macrophage colony-stimulating factor (GM-CSF); interferon γ (IFNγ); interleukin-1 (IL-1); interleukin-1 receptor antagonist (IL-1Ra); transforming growth factor (TGFβ); tumor necrosis factor α (TNFα).\textsuperscript{19} (Adapted from reference 19)
The inflammatory cytokines are considered to play an important role in eliciting the symptoms for infusion reactions. The speculation is that mAbs upon binding to their molecular targets may lead to the release of inflammatory cytokines like TNF-α and IL-6 (Fig.3). These reactions could also be caused by direct effect on immune cells or could be due to mast cell sensitization facilitated by antigen mediated IgE activation. In addition, various inflammatory cytokines are secreted at every step in the signaling pathway leading up to immunogenic responses (Fig.1).

**Linking the innate and the adaptive immune system**

Supporting the above discussion, there have been suggestions to use secretion of inflammatory cytokines as an early indicator of immunogenicity. Studies have assessed the impact of protein aggregates on various stages of immune system activation. For example, Joubert et al have shown activation of the innate system in response to protein aggregates via interaction with TLRs, Fcγ receptors and complement system. Studies have reported enhancement in the DC activity such as antigen presentation capacity, co-stimulatory activity and, cytokine secretion profiles in the presence of protein particles in the micron size range. These studies have also shown an enhanced T cell activation and numerous studies have reported higher antibody titers against aggregated protein compared to native protein. Pisal et al have even shown a four-fold higher T cell activation upon subsequent administration of native Factor VIII showing evidence for the formation of memory cells.

**Protein aggregates in immunogenicity**

Of the numerous factors that can lead to immunogenic reactions, the focus of this work is the aggregates in therapeutic protein solutions, in particular protein particles in the subvisible size range (60 nm to 100 μm). Production of neutralizing antibodies (nAbs) and
subsequent loss of efficacy was observed in patients receiving β-interferon products in phase III clinical trials. This was attributed to the presence of aggregates; since improvements in manufacturing, purification and storage procedures that decreased aggregate formation also reduced the incidences of nAb generation in patients in this study. Similar immunogenic responses against protein aggregates have also been reported for growth hormone, interferon-α, factor VIII and some IgG products.£15,24,31-34

Figure 4: Approximate size range of analytical methods for size determination of subvisible and visible (protein) particles. £35 (Adapted from reference 27)
Subvisible particles in protein solutions

In the last few years emphasis has been on aggregates/particles in the subvisible range (50 nm to 100 µm) and their role in immunogenic responses. Numerous analytical techniques such as microflow imaging, flowcam, coulter counter, resonant mass measurement and nanoparticle tracking analysis have been developed in the recent years to enable quantification of subvisible particles in various size ranges (Fig. 4). With such focused effort towards understanding the impact of subvisible particles on product quality, there is still a debate in the literature if there is enough evidence to suggest the contribution of subvisible particles on product stability and immunogenicity. The argument being that particles are ubiquitous in protein solutions and a much higher concentration than that observed in marketed products is required to stage an immune response. However, Barnard et al compared 3 marketed formulations of β-interferon – Rebif®, Betaseron® and Avonex® and found > 100,000 particles/mL in the size range of 1-50 µm. As such the regulatory agencies like the U.S. Food and Drug Administration (FDA) have advised in favor of the precautionary principle suggesting that an attribute be considered a risk factor if there is a probability of that attribute to be associated with potential for harm, even if the link to clinical performance has not been clearly identified. They further advise rigorous monitoring of the risk factor i.e. subvisible particles in case of this discussion until their potential for harm (immunogenicity) is well established.

Traditionally protein aggregates are divided into categories based on attributes such as size, reversibility, secondary/tertiary structure perturbations, covalent modifications and morphology of aggregates. Aggregates of different sizes are also categorized as soluble/insoluble aggregates and there is a large amount of literature discussing the
mechanism of protein molecules in native state forming soluble and insoluble aggregates.\textsuperscript{40-46}

However little is known about the mechanism of formation of subvisible particles from native protein molecules. Few studies have investigated the mechanism of formation of subvisible particles for different proteins and concluded that this mechanism is specific to the protein, to its solution conditions as well as to the stress condition it may be exposed to.\textsuperscript{47,48}

**Subvisible particle formation during some pharmaceutically relevant stress conditions**

Subvisible particles, based on the size range of detection of analytical techniques can be further classified as nano- and microparticles (Fig. 4). In Chapter 2 we have investigated the potential roles of nanoparticles in the formation of microparticles under some pharmaceutically relevant stress conditions. Intravenous immunoglobulin (IVIG) used as the model protein for this study was exposed to quiescent incubation at 25° C, quiescent incubation at 50° C, freeze-thawing or agitation. Exposure of IVIG solutions to the interface-associated stresses of freeze-thawing or agitation resulted in relatively large increases in microparticle concentrations, which depended directly on the levels of initially-present, preexisting nanoparticles. Thus, nanoparticle agglomeration appears to play a role in microparticle formation under these stresses. In contrast, increases in microparticle concentrations during quiescent incubation at elevated temperatures (50° C) were independent of the initial nanoparticle concentrations in solution. In addition, studies with fluorescently-labeled IVIG protein molecules and flow cytometry showed that protein monomers can be incorporated into microparticles. Overall, the results suggest that agglomeration of nanoparticles and addition of monomers contributes to the formation of microparticles of IVIG during exposure to stresses such as agitation and freeze-thawing.
**Subvisible particles generated during delivery to patients via IV infusion**

In addition to exposure to various pharmaceutical operations during manufacturing, these protein particles can also form downstream - during delivery to patients; where maintaining stability may be beyond the domain of formulation scientists. Most mAbs currently on the market are formulated for delivery via intravenous infusion. IV infusion, which is controlled by an infusion pump, allows for complete systemic drug absorption and easy monitoring of plasma drug concentrations. This route of administration is better suited for injecting large volumes over extended period of time as in the case of fluid/electrolyte replacements. In case of therapeutic proteins, drugs that have low lipid solubility, those that may cause irritation upon injection or those that have a narrow therapeutic index where drug concentration needs to be tightly monitored are usually administered via IV infusion.\(^\text{49}\) However, administering intravenously has its own challenges; requiring a trained professional to find the correct vein while maintaining aseptic conditions during needle insertion and removal. Moreover, factors related to the drug product and its formulation are known to cause inflammation of the veins (phlebitis) and hypersensitivity/infusion reactions, in addition to the immunogenic responses observed among patients against therapeutic protein products.\(^\text{50,51}\) Moreover, exposure of the therapeutic protein to various surfaces of the IV tubes and the in-line filters, the air-water interface in the drip chamber (Fig.5) and the extensive dilution of the protein formulation into the IV diluent can increase the aggregation propensity of the therapeutic protein.

In addition, the package inserts that are the main source of directions for handling and administration of protein therapeutics do not recommend any filtration during administration of most mAb therapies currently in the market (Fig.6).
In chapter 3, we determined the subvisible particle levels in IV solutions and after the solutions were processed with an IV administration setup that mimicked the clinical administration. IV saline in bags manufactured by both Hospira and Baxter contained 1,600-8,000 microparticles/mL and $4.73 \times 10^6$ nanoparticles/mL in solution. For IVIG diluted into the IV saline there were 3,700-23,000 microparticles/mL and $18-240 \times 10^6$ nanoparticles/mL.
During processing of the solution through the IV system, in-line filters removed most microparticles. However there were still $1-21 \times 10^6$ nanoparticles/mL in IV saline and $7-83 \times 10^6$ nanoparticles/mL in IVIG diluted in saline. Finally, in samples processed through in-line filters we found relatively large microparticles (20-60 µm) that were composed of protein or polycarbonate. These particles resulted from shedding of polycarbonate and sloughing off of protein films downstream from the filter membrane. Overall, the results document that even with in-line filters in place, high levels of subvisible particles are delivered to patients and there is a need for improved, more effective filters and IV solutions with lower particle levels.
Infusion reactions and immunogenic responses against IV solution of Remicade®

Having determined that a substantial level of nanoparticles were generated that could inadvertently be delivered to patients during IV infusion of IVIG, we wanted to test this for another marketed product Remicade® (infliximab). Remicade®, an anti-TNF-α monoclonal antibody (mAb) is prescribed for the treatment of Crohn’s disease, ulcerative colitis, psoriasis, psoriatic arthritis, ankylosing spondylitis and rheumatoid arthritis. Among patients that receive Remicade®, 10% - 25% of the patients have adverse infusion related reactions and approximately 30% of the patients have immunogenic responses in the clinic. In chapter 4, we have discussed the hypothesis that particles in intravenous (IV) solution of Remicade® that may be delivered to patients may have a role in eliciting these infusion related reactions and immunogenic responses. In this study we determined the concentration of subvisible particles in IV solution of Remicade® and their contribution in activating various pathways of the innate and the adaptive immune system. To this end, IV solution was processed through the infusion system as is done in the clinic either with or without an in-line filter connected to the infusion system. This IV solution was further treated with an ultracentrifugation step to generate a particle enriched fraction and a particle free supernatant fraction. These samples along with the no centrifugation sample were tested for activation of the innate immune system via a) cytokine release in whole blood and in b) peripheral blood mononuclear cell (PBMC) cultures, and c) activation of the Toll like receptors (TLRs). Activation of the adaptive immune system was monitored via a) upregulation of surface receptors on dendritic cells (DCs) and b) T cell proliferation in response to IV solution of Remicade®. Our results indicate that subvisible particles in solution activate the immune
system but there are extrinsic factors potentially contributed by the in-line filters or other process parameters that also contribute to immune system activation.
Introduction

Subvisible particle levels in therapeutic protein products are critical quality attributes, potentially affecting patient health and safety. Importantly, even when present at relatively low levels (e.g., < 0.1% of total protein mass) these particles may cause immunogenic responses and/or infusion related hypersensitivity reactions in patients. For example, a recent article authored by FDA researchers reported 49 cases of anaphylaxis including 7 fatalities among patients receiving peginesatide, a commercial pegylated erythropoietin peptide mimetic. The product was found to comply with USP <788> requirements for particle content as measured by the traditional light obscuration method. However, using more sensitive micro flow imaging (MFI) and nanoparticle tracking analysis (NTA) techniques, higher concentrations of particles in the nano- and micrometer size range were observed in the immunogenic marketed formulation compared to those found in the non-immunogenic product used in the clinical studies.\textsuperscript{52} In further support of the role of subvisible particles in immunogenicity, numerous \textit{in vivo} and a few \textit{in vitro} studies have shown that small amounts of these particles can activate various pathways of the innate and adaptive immune system.\textsuperscript{15,24,34,53-56}

In addition, analysis of subvisible particles provides a sensitive tool to monitor and characterize aggregation in protein products. For example, stresses incurred during one cycle of freeze thawing or during filling operations for monoclonal antibodies (mAb) did not result
in the formation of soluble aggregates that could be detected by size-exclusion chromatography (SEC) analysis, but thousands of microparticles per mL were detected by particle counting methods.\textsuperscript{57,58} Recently, several industry scientists have discussed case studies showing that monitoring subvisible particles enabled them to devise particle control strategies and make better decisions in support of formulation and process development, and ensure higher product quality.\textsuperscript{59}

Subvisible particles are ubiquitous in protein solutions.\textsuperscript{36} Freeze thawing, oxidation, elevated temperatures, light exposure, agitation and mechanical shock -- stresses to which the protein product may be exposed to during purification, production, storage, transport and delivery to patients -- can cause formation of subvisible particles.\textsuperscript{47,48,57,60-62} However, detailed mechanisms by which subvisible particles form are unknown. Some researchers have investigated agitation-induced microparticle formation and concluded that the mechanisms are protein dependent with no clear relationship between levels of soluble oligomers and microparticle formation.\textsuperscript{47,48} Particularly, the contribution of nanoparticles in microparticle formation was not investigated. In a recent study, however, Bai; \textit{et al.} observed a direct relation between initial nanoparticle concentrations in solution and subsequent microparticle formation during isothermal incubation of interferon-\(\beta\)-1a.\textsuperscript{61}

The goal of the current study was to investigate the potential roles of nanoparticles in the formation of microparticles during incubation at elevated temperature, freeze-thawing or agitation. We manipulated the pre-incubation concentrations of micro- and nanoparticles in IVIG solutions using ultracentrifugation or freeze-thawing. Subsequent stress-induced particle formation was monitored using nanoparticle tracking analysis (NTA) and microflow imaging (MFI). In addition, fluorescently labeled IVIG molecules were incubated with
unlabeled protein during agitation, and flow cytometry was used to determine the incorporation fluorescent IVIG molecules into microparticles.

Materials and methods

Materials

Gammagard® (IVIG), 100 mg/mL (Lot# LE12N107AB, Expiry: May 2016) manufactured by Baxter Healthcare Corporation (Westlake Village CA.) was used as the model protein in this study. Glycine, sodium phosphate, sodium chloride and Tween 20 were purchased from Fisher Scientific (Fair Lawn, NJ). Alexa Fluor® 488 was purchased from Invitrogen (Eugene, OR).

Sample preparation

Freeze thaw with and without prior ultracentrifugation

IVIG (1 mg/mL) was formulated in 200 mM glycine, pH 4.2. 25 mL aliquots of the formulation were ultracentrifuged at 112,000g for 3 hours, and 20 mL of the supernatant was decanted and used as a particle-free IVIG control solution. IVIG solutions (non-centrifuged and centrifuged) were frozen by immersion in liquid nitrogen and thawed in a water bath at room temperature. Nano- and microparticle concentrations in freeze-thawed solutions were characterized using NTA and MFI, respectively. For each determination of nano- and microparticles, three independent replicates were used.

Freeze-thawing prior to agitation

To generate high levels of particles, a 25 mL aliquot of 1 mg/mL IVIG was agitated end-over-end at 40 RPM for 48 hours in a 50 mL conical centrifuge tube. This IVIG solution was then centrifuged for 3 hours at 112,000g, and 20 mL of the supernatant was subjected to
either freeze thawing (procedure mentioned above) and/or end-over-end agitation (40 RPM for eight hours in 50 mL polypropylene tubes) and changes in nano- and microparticle concentrations were characterized using NTA and MFI, respectively, for three independent replicates.

**Generating varying levels of nanoparticles in IVIG solutions**

Empirically, it was determined that centrifugation for 45 minutes at 2350g (30 mL in nalgene tubes) protein solution in the removed most microparticles, 45 minutes at 30,000g (30 mL in nalgene tubes) removed some nanoparticles and centrifugation for 3 hours at 112,000g (25 mL in Beckman polycarbonate tubes) removed most nanoparticles. Hence to selectively remove particles of varying sizes, samples of IVIG solution that had been agitated end-over-end for 48 hours were centrifuged at either 2350g or 30,000g using Sorvall RC6plus centrifuge or at 112,000g using a Beckman optima LE-80K ultracentrifuge with Beckman SW 28 rotor. After centrifugation these samples were then split into three aliquots in 20 mL glass vials for which were incubated quiescently at ambient temperature for five days or at 50° C for five days, or in 50 mL polypropylene tubes which were frozen in liquid nitrogen and thawed at ambient temperature, or agitated end-over-end for 8 hours at ambient temperature. Levels of nano- and microparticle concentrations in the various samples were measured using NTA and MFI, respectively.

**Particle characterization**

Microparticles (particles greater than 1 μm) were characterized using a Protein Simple (Ottawa, ON, Canada) 4200 MFI system with a 100 μm flow cell. Sample volumes of
0.5 mL were characterized, of which 0.1 mL was used as purge volume, 0.05 mL was used for background optimization and 0.35 mL was used for particle analysis.

Nanoparticles (particles between 60-1000 nm) were characterized using a Nanosight (Amesbury, United Kingdom) LM 20 system with the LM 12B sample chamber or the NS 300 system with a LM 14 viewing unit; equipped with a 405 nm laser light source. A sample volume of 0.5 mL was injected using a silicone oil-free 1 mL plastic syringe (National Scientific Company, TN). Sample videos recorded for 60 seconds were processed using NTA 2.3 Build 127 software. For analysis a screen gain of 1 and a detection threshold of 10 were used.

**Particle analysis using fluorescently labeled IVIG and flow cytometry**

IVIG was fluorescently labeled with Alexa Fluor® 488 according to the procedure mentioned in the manufacturer’s protocol. The labeled protein was then dialyzed into the formulation buffer (200 mM glycine, pH 4.2), with three exchanges of the buffer. Next, the labeled IVIG solution was centrifuged at 112,000g for 3 hours to remove most micro- and nanoparticles that might have formed during the labeling and dialysis processes. To monitor the potential role of IVIG molecules in microparticle formation, labeled IVIG was spiked in a 1:100 (v/v) ratio into unlabeled IVIG. The unlabeled IVIG solution was previously agitated for 48 hours and centrifuged at either 2350g or 112,000g before spiking in the labeled IVIG solution. The IVIG solutions spiked with labeled IVIG were then agitated for eight hours and subsequent particle formation was monitored by flow cytometry. Fluorescence signal from microparticles containing fluorescently labeled IVIG was monitored using a BD FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) equipped with a 488 nm blue laser. Sample volumes of 200 µL were analyzed for 1 minute at a flow rate of
12 μL per min. The gain settings and amplitude were optimized for this sample such that forward scatter (FSC) signal was recorded on a linear scale with the gain setting of E01, amplitude of 9.99 and a threshold of 52. For side scatter (SSC), a gain setting of 535 was used with amplitude setting of 1.0 on a linear scale while the fluorescence signal (FL1) was set at a gain of 651 on a logarithmic scale.

**Results**

Freeze thawing IVIG with or without prior centrifugation

![Figure 7](image.png)

Figure 7: Total particle concentration following freeze-thawing of non-ultracentrifuged and ultracentrifuged IVIG samples. (a) Particles ≥ 1 µm; and (b) particles between 60-1000 nm. Error bars represent standard deviation from three independent replicates.

To investigate the role of pre-existing particles on subsequent particle formation, particle-free IVIG solution was prepared by centrifugation as described above. The non-centrifuged sample was used as the IVIG solution with pre-existing particles. Both solutions were exposed freeze-thawing stress and consequent particle concentrations were compared. Freeze-thawing the particle-free IVIG solution resulted in a much smaller increase in microparticle levels than when the particle containing solution was freeze-thawed (Fig.7a).
The increases in the nanoparticle levels caused by freeze-thawing were similar to the increase in microparticle levels for the two IVIG solutions (Fig. 7b).

**Agitation with or without prior freeze-thawing**

To determine the impact of particles generated during freeze-thawing on subsequent agitation-induced particle formation, IVIG exposed to one cycle of freeze-thawing was incubated with agitation for 8 hours. The control sample was not freeze-thawed prior to agitation. Agitation of the freeze-thawed IVIG solution resulted in a greater increase in microparticle levels than that observed in the control solution. (Fig. 8a). However, after an apparent lag phase, microparticle counts did increase in the control solution during agitation. Also, although the freeze-thawed IVIG solution initially had higher nanoparticle levels than the control solution, the rates of increases in nanoparticle concentrations were similar for the two IVIG solutions (Fig. 8b).
Generating varying levels of nanoparticles

To monitor further the role of nanoparticles in microparticle formation, varying levels of nanoparticles were generated in IVIG solution using agitation and different subsequent centrifugation protocols. Agitation for 48 hours generated sufficient nano- and microparticles to saturate the detection limit of the particle analyzers (Table.1). Centrifugation at 2350g removed most microparticles, leaving a high concentration of nanoparticles in solution. Centrifugation at 30,000g removed microparticles and an intermediate level of nanoparticles. Centrifugation at 112,000g removed microparticles and most nanoparticles. These IVIG solutions were then exposed to either quiescent incubation at 25° C or 50° C, freeze thawing or agitation.

Table 1: Particle concentration in IVIG solution after 48 hours of agitation and subsequent centrifugation

<table>
<thead>
<tr>
<th></th>
<th>Particles 60-1000 nm (E6/mL)</th>
<th>Particles ≥ 1 µm (#/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation for 48 hours</td>
<td>6.44 ± 0.36 **</td>
<td>917,823 ± 259,867</td>
</tr>
<tr>
<td>Centrifugation at 2350 g</td>
<td>10.035 ± 6.979</td>
<td>1,936 ± 1,986</td>
</tr>
<tr>
<td>Centrifugation at 30000 g</td>
<td>0.4711 ± 0.139</td>
<td>1,314 ± 1,440</td>
</tr>
<tr>
<td>Centrifugation at 112,000 g</td>
<td>0.069 ± 1.27</td>
<td>137 ± 81</td>
</tr>
</tbody>
</table>

*Measurement error represents standard deviation from measurement of three independent samples

**Above the limit of detection
IVIG incubation at 25° C or 50° C in the presence of varying levels of nanoparticles

Figure 9: Total particle concentration for isothermal incubation of IVIG solution processed through centrifugation at various speeds (with varying levels of nanoparticles). (a) Incubation at 25° C (b) incubation at 50° C. Error bars represents standard deviation from three independent replicates.

During incubation at 25° C, there were only slight increases in microparticle concentrations in the IVIG solutions with varying initial levels of nanoparticles (Fig.9a). During incubation at 50° C, the magnitude of increase in microparticle levels was greater than that observed at 25° C (Fig.9b), but there was no discernable effect of the initial levels of nanoparticles. There were no significant changes in the nanoparticle concentrations over 5 days of incubation during incubation at either 25° C or at 50° C (Table 2).

Freeze thawing IVIG in the presence of varying levels of nanoparticles

The solutions of IVIG processed by the different centrifugation procedures were also freeze-thawed. This stress caused increases in microparticle levels that were directly proportional to the initial levels of nanoparticles present in solution (Fig.10 and Table.3). Freeze-thawing of the IVIG samples that had been subjected to centrifugation at 30,000g or at 112,000g resulted in similar increases in the levels of nanoparticles. There were no discernable changes in nanoparticle levels during freeze-thawing of the sample processed at
2350g, but even prior to freeze-thawing the levels were very high and at the upper limit for NTA measurement.

Table 2. Particle concentration between 60-1000 nm (E6/mL) in IVIG solution centrifuged at various g forces and incubated at either 25° C or at 50° C

<table>
<thead>
<tr>
<th></th>
<th>2350g</th>
<th>30000g</th>
<th>112000g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation at 25° C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>8.5</td>
<td>0.64</td>
<td>0.29</td>
</tr>
<tr>
<td>Day 1</td>
<td>8.88 ± 3.3</td>
<td>0.32 ± 0.155</td>
<td>1.96 ± 1.15</td>
</tr>
<tr>
<td>Day 2</td>
<td>7.9 ± 1.97</td>
<td>0.292 ± 0.269</td>
<td>1.93 ± 1.13</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.45 ± 0.28</td>
<td>0.425 ± 0.186</td>
<td>0.63 ± 0.22</td>
</tr>
<tr>
<td>Day 4</td>
<td>6.51 ± 0.73</td>
<td>0.5485 ± 0.1</td>
<td>1.33 ± 0.62</td>
</tr>
<tr>
<td>Day 5</td>
<td>4.5 ± 1.66</td>
<td>1.76 ± 2.1</td>
<td>1.18 ± 0.41</td>
</tr>
</tbody>
</table>

| **Incubation at 50° C** |          |          |          |
| Day 0                | 24.08    | 4.1      | 1.28     |
| Day 1                | 29.3 ± 14.84 | 4.49 ± 0.275 | 0.713 ± 0.26 |
| Day 2                | 31.57 ± 6.68 | 4.19 ± 1.59 | 1.02 ± 0.685 |
| Day 3                | 34.65 ± 5.49 | 2.35 ± 2.28 | 0.372 ± 0.141 |
| Day 4                | 38.9 ± 3.711 | 1.9 ± 0.236 | 0.084 ± 0.0577 |
| Day 5                | 37.30 ± 3.13 | 1.80 ± 0.822 | 0.762 ± 0.475 |

*Measurement error represents standard deviation from measurement of three independent samples

**Above the limit of detection

Agitation in the presence of varying levels of nanoparticles

The IVIG solutions processed by the different centrifugation procedures were also subjected to stress by agitation (Fig. 11a). In the solution processed at 2350g, which was depleted of microparticles but contained high levels of nanoparticles, microparticles increased to high levels during 8 hours of agitation. In contrast, in the IVIG solutions centrifuged at 30,000g or 112,000g, which were depleted in microparticles and had low levels of nanoparticles, there were much lower levels of microparticles formed. And there was an apparent lag phase (0-4 hours) before microparticles started to increase in
concentration. It appears that sufficient levels of nanoparticles must form before microparticles start to form, presumably via agglomeration of nanoparticles.

Figure 10: Total particle concentrations ≥ 1 µm in IVIG solution processed through centrifugation at various speeds (with varying levels of nanoparticles) exposed to one cycle of freeze-thawing. Error bars represents standard deviation from three independent replicates.

Nanoparticle concentrations increased significantly during eight hours of agitation in IVIG samples that had been centrifuged at 30,000g or 112,000g (Table.3). In the IVIG sample centrifuged at 2350g, even prior to agitation the nanoparticle levels were near the upper limit for the NTA instrument, and levels remained high during agitation.
Figure 11: Total particle concentrations in IVIG solution processed through centrifugation at various speeds (with varying levels of nanoparticles) exposed to (a) up to 8 hours of agitation (b) up to 8 hours of agitation in the presence of Tween 20. Error bars represent standard deviation from three independent replicates.

Nonionic surfactants are often used to reduce agitation-induced aggregation of therapeutic proteins. To test for such stabilization in our system, the IVIG solution that had
been centrifuged at 2350g was agitated with and without polysorbate. The presence of polysorbate 20 inhibited agitation-induced increases in microparticle concentrations (Fig. 11b). Nanoparticle counts could not be obtained because the levels of all samples exceeded the upper limit for the NTA instrument.

Table 3. Particle concentration between 60-1000 nm (E6/mL) in IVIG solution centrifuged at various g forces and exposed to either freeze thawing, agitation or agitation in the presence of polysorbate 20

<table>
<thead>
<tr>
<th></th>
<th>2350g</th>
<th>30000g</th>
<th>112000g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2543 ± 156</td>
<td>47 ± 0.7</td>
<td>23 ± 20</td>
</tr>
<tr>
<td>Freeze-Thawing</td>
<td>1990 ± 0.569</td>
<td>285 ± 2.68</td>
<td>150 ± 0.595</td>
</tr>
<tr>
<td>Agitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>1003</td>
<td>47</td>
<td>6.9</td>
</tr>
<tr>
<td>0.75 hr</td>
<td>892 ± 282</td>
<td>289 ± 103</td>
<td>80 ± 44</td>
</tr>
<tr>
<td>1.5 hr</td>
<td>873 ± 133</td>
<td>487 ± 103</td>
<td>285 ± 64</td>
</tr>
<tr>
<td>2 hr</td>
<td>855 ± 202</td>
<td>566 ± 57</td>
<td>301 ± 84</td>
</tr>
<tr>
<td>4 hr</td>
<td>685 ± 231</td>
<td>708 ± 71</td>
<td>582 ± 164</td>
</tr>
<tr>
<td>6 hr</td>
<td>801 ± 153</td>
<td>748 ± 154</td>
<td>676 ± 149</td>
</tr>
<tr>
<td>8 hr</td>
<td>813 ± 339</td>
<td>748 ± 207</td>
<td>603 ± 143</td>
</tr>
<tr>
<td>Agitation with Tween 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>n/a a</td>
<td>Not determined b</td>
<td>Not determined b</td>
</tr>
<tr>
<td>4 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 hr</td>
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</tbody>
</table>

*Measurement error represents standard deviation from measurement of three independent samples

a Samples above the limit of detection of the instrument

b Samples not tested for this treatment
Monitoring microparticle growth using fluorescence activated cell sorting assay (FACS)

![Fluorescence intensity (FL1-H) as a function of forward scatter (FSC-H) for one replicate of IVIG sample centrifuged at 112,000g (top panel) and IVIG sample centrifuged at 2350g (bottom panel), spiked with fluorescently labeled IVIG and agitated for 0, 4 and 8 hours. Numbers in the box are particle counts from one replicate for control sample (a,b); and mean and standard deviation from three replicates for 4 hours (c,d); and 8 hours (e,f); of agitation.](image)

Figure 12: Fluorescence intensity (FL1-H) as a function of forward scatter (FSC-H) for one replicate of IVIG sample centrifuged at 112,000g (top panel) and IVIG sample centrifuged at 2350g (bottom panel), spiked with fluorescently labeled IVIG and agitated for 0, 4 and 8 hours. Numbers in the box are particle counts from one replicate for control sample (a,b); and mean and standard deviation from three replicates for 4 hours (c,d); and 8 hours (e,f); of agitation.

To further investigate the mechanism(s) of microparticle formation, fluorescently labeled IVIG was spiked into IVIG samples processed by centrifugation at 2350g or at 112,000g. At the spiking level used, fluorescently labeled IVIG molecules account for only 1% (v/v) of the total IVIG in solution. As noted above, IVIG samples centrifuged at 2350g are depleted in microparticles but retain nanoparticles, whereas samples processed at
112,000g are depleted in both micro- and nanoparticles. The samples were then agitated, and samples were removed at specific time points for analysis with flow cytometry. This method enabled monitoring of the forward scattering signal (related to particle size) as a function of fluorescent intensity for particles greater than 0.5 µm (Fig.12). Before agitation, no microparticles were detected in IVIG samples centrifuged at either 2350g or at 112,000g (Fig.12a and 12b). After four hours of agitation, particles with relatively low scattering intensity and high fluorescence intensity started to populate the upper left quadrant in plots for both IVIG samples (Fig.12c and 12d), indicating the incorporation of labeled IVIG into particles > 0.5 micron in size. After eight hours of agitation larger particles having a high fluorescence intensity started to populate the upper right quadrant of the data plots (Fig.12e and 12f). At both time points, microparticle concentrations were higher in the IVIG sample that had been centrifuged at 2350g suggesting that microparticles form more efficiently in the presence of pre-existing nanoparticles. Importantly, in the samples that had been depleted of micro- and nanoparticles by centrifugation at 112,000g, fluorescent microparticles still formed. In both sample types, the fluorescent signal indicated that labeled IVIG monomers were incorporated into the microparticles. This incorporation might occur directly into microparticles and/or by first being incorporated into nanoparticles which then agglomerated into microparticles.

**Discussion**

For therapeutic protein products, microparticle formation can occur during any step from manufacturing, shipping, storage and delivery to patients. In order to develop effective control strategies for microparticles -- as required by regulatory authorities -- it is critical to employ the appropriate analytical methods and to understand key factors that
contribute to particle formation. In the current study with the model therapeutic protein IVIG, we found that the presence of pre-existing nanoparticles fostered more rapid formation of microparticles during agitation and freeze-thawing. In contrast, in IVIG solutions depleted of nanoparticles, much lower levels of microparticles were formed during freeze-thawing; and during agitation there was a time period in which microparticles were not formed. But we also observed that new nanoparticles can form during these stresses, and these nanoparticles presumably contribute to the formation of microparticles. Microparticles appear to form primarily due to agglomeration of nanoparticles during the stresses. And based on our results with fluorescently-labeled IVIG and flow cytometry, monomers from solution also can be incorporated into microparticles; and presumably into newly formed nanoparticles.

Whether or not similar mechanisms will contribute to microparticle formation in a given process for a given protein depends on numerous factors including: the protein’s physicochemical properties; the solution composition; the specific details of the stresses to which the protein is exposed to; and if foreign micro- and nanoparticles are being shed into the protein solution. Therefore, it is essential for each step in a product’s life history that robust particle analytical methods, such as NTA and MFI, are employed routinely to investigate particle formation. And, as part of a quality-by-design study, relevant process and product parameters should be varied and appropriate analytical methods for micro- and nanoparticles must be used. Otherwise, the causes and control for particle formation will not be properly understood.

Often protein particle formation is associated with interfacial stresses to solutions, and many processes to which protein solutions are exposed to include interfacial stresses. In the current study, it was observed that during quiescent incubation at elevated temperature
(50°C) the initial concentrations of nanoparticles did not affect microparticle formation rates. However, nanoparticles were found to promote microparticle formation in IVIG solutions during freeze-thawing and agitation, stresses that include exposure to interfaces.

During freeze-thawing, multiple stresses occur that may affect protein particle formation and agglomeration. Ice crystal formation produces a relatively large amount interfacial surface area. Protein molecules can adsorb to the ice-water interface, resulting in formation of aggregates and particles, which are then observed upon thawing. In addition, existing particles may accumulate at the ice-water interfaces, further increasing the propensity for particle agglomerate. Furthermore, ice formation during freezing leads to greatly increased concentrations of solutes and particles in the remaining liquid phase, also potentially increasing particle agglomeration rates. Overall, during freeze-thawing both formation of new particles and agglomeration of existing particles may occur.

Similarly during agitation, it is the adsorption of protein molecules onto the air-water interface that largely contributes to protein particle formation. Consistent with this mechanism is the observation in the current and many earlier studies that inclusion of a non-ionic surfactant in the formulation can substantially inhibit agitation-induced protein aggregation and particle formation. In the absence of surfactant, protein molecules that adsorb onto the air-water interface may unfold and associate to form a gel or film. Protein particles in the bulk solution at the start of an agitation stress may also adsorb onto the interface and contribute to the formation of the layer of gelled protein. Mechanical stresses and rupture of the interfacial layer can lead to more particles in the bulk. During agitation, compression and expansion of the air-water interface and bursting of air bubbles can mechanically rupture the interfacial gel. In our experiments, IVIG samples depleted
of nanoparticles prior to agitation did not form microparticles for a substantial period of time. These results suggest that, at least for this system, the adsorption of nanoparticles onto the air-water interface and their resultant agglomeration is a key step in agitation-induced microparticles.

Overall the results from the current study and other recent studies on subvisible particle formation\textsuperscript{47,48,59,61} have important practical implications for therapeutic protein product development and quality assurance. For example, consider a fill-finish operation for a therapeutic protein formulation that occurs after frozen bulk drug substance is thawed. It is important to realize that the manner in which the frozen drug substance was prepared and handled, as well as the thawing regime, solution handling and formulation steps, can greatly affect the levels of micro- and nanoparticles present in the protein solution. Of course, just prior to the filling operation, sterile filtration with a 0.2 µm filter will remove most particles but nanoparticles smaller than 200 nm will still be present and new particles might form due to the filtration operation itself. During the filling pump operation, nanoparticles can be shed from the pump materials and/or tubing used in peristaltic pumps, and protein molecules can readily adsorb to these particles.\textsuperscript{58} Protein molecules also adsorb to surfaces of the pumping systems and can slough off as particles. Agglomeration of particles in the filtered bulk and new particle formation can be caused by the combination of stresses to which a protein formulation is exposed to during the fill-finish operation.

This hypothetical example only points out some of the potential contributors to particle formation. But this non-exhaustive list serves to make the point that essentially every step can affect particle formation and that each product and process must be studied rigorously. Only by carefully studying nano- and microparticle levels with appropriate
instruments will the relative contributions (and potential synergistic interactions) of all of these factors be discerned and product quality, relative to particle levels, be optimized.

In conclusion, agglomeration of pre-existing nanoparticles as well as formation of new particles can contribute to protein microparticle formation during freeze-thawing and agitation. Such interfacial stresses to protein solutions are ubiquitous in a product’s life history. Also, the mass percent of protein in quantifiable levels of subvisible particles might constitute much less than 0.1%. Thus, important differences in particle levels may be observed, even though mass loss of monomer may not be detectable by methods such as size exclusion chromatography (SEC). This situation may account for the observed lack of correlation between protein aggregation levels measured by SEC and subvisible particle concentrations.57

Overall, for a given protein and formulation it is not possible to predict quantitatively the effects of all of stresses on particle formation, and SEC may not be sensitive enough to detect the loss of monomer leading to subvisible particles. Therefore, it is critical that both nano- and microparticles be measured rigorously during quality-by-design studies. Also, the potential roles of nanoparticles in the formation of microparticles should be understood and appreciated for their important impacts on product quality and manufacturing robustness.
CHAPTER III
MICRO- AND NANOPARTICLES DELIVERED IN INTRAVENOUS SALINE AND IN INTRAVENOUS SOLUTION OF A THERAPEUTIC ANTIBODY PRODUCT

Introduction

Therapeutic protein products are commonly administered parenterally, which includes intramuscular, subcutaneous or intravenous (IV) routes of administration. As of 2015, 30 of the 47 monoclonal antibodies (mAbs) on the market were formulated for delivery by IV infusion. Administering therapeutic proteins intravenously can lead to hypersensitivity/infusion reactions in patients, as well as immunogenicity. Hypersensitivity/infusion reactions usually occur within 72 hours of administration with symptoms ranging in severity from mild headaches, flushing and itching, to tightness in throat, dizziness and even anaphylactic shock. For intravenous immunoglobulin (IVIG), which contains IgG molecules from pooled plasma of human donors and is the model protein for this study, incidences of infusion reactions vary from 1 to 81% of patients or infusion cycles. In most cases, these reactions are frequent among patients during initial treatment and decrease over subsequent infusion cycles. Immunogenic reactions caused by therapeutic proteins may compromise drug product efficacy because of the production of neutralizing antibodies against the therapeutic protein. More than 20% of the patients experience loss of efficacy during treatment with different mAb products including infliximab, daclizumab, adalimumab, abciximab and efalizumab.

Protein aggregates and particles resulting from physicochemical instability of drug products represent important factors in eliciting adverse infusion reactions and/or immune responses. In an IV infusion system, numerous factors can lead to protein
aggregation. Foremost, a diluent incompatible with a therapeutic protein product could cause protein aggregation. Packaging inserts for some mAbs including abciximab, alemtuzumab and rituximab recommend using either 0.9% saline or 5% dextrose as a diluent. But for mAbs such as panitumumab, pertuzumab and trastuzumab, saline is the only recommended diluent, and for trastuzumab it is specifically noted that dextrose should not be used as a diluent. Demuele et al characterized trastuzumab diluted into saline or dextrose, and found much higher aggregate formation in the 5% dextrose solution.  

Furthermore, because of the high level of dilution occurring when IV solutions are prepared, protein stability provided by the product formulation is greatly reduced, increasing the likelihood of protein aggregation. For example, Kumru et al found that upon dilution into IV saline, an IgG4 mAb formed soluble aggregates and subvisible particles, which were measured by microflow imaging (MFI) and nanoparticle tracking analysis (NTA). Other studies have reported no changes in protein stability over at least 24 hours of incubation after dilution in saline for cetuximab, panitumumab, trastuzumab, pertuzumab and infliximab. However, in these cases either light obscuration or an enzyme-linked immunosorbent assay was used to determine the presence of aggregates or changes in protein concentration, respectively. Such techniques are typically not sensitive enough to detect levels of damage that may account for less than 0.1% of the total protein concentration, but can still be associated with formation of significant numbers of protein particles.  

During processing through an IV infusion system, protein drug products are exposed to a number of surfaces – IV bags, IV tubes, in-line filter units etc. IV bags are usually made of polyvinyl chloride (PVC) or polyolefin (PO). The IV infusion sets are composed of PVC tubing and silicone tubing in the section on which the pump operates. The connectors are
made of polycarbonate. The housing chamber for most filters and the framework on which
the filter membrane rests are also made of polycarbonate; and there is typically a tube
downstream from the filter membrane made of PVC that connects to the needle. Protein
molecules readily adsorb onto such solid surfaces at the liquid-solid interface. For example,
factor VIII diluted in saline and stored for 48 hours in an IV bag made of PVC resulted in
reduction in protein activity, which was attributed to adsorption of the protein molecules onto
the bag surface.\textsuperscript{95} With all of the solid-liquid interfaces mentioned above, along with the air-
water interface in the drip chamber and the IV bag, adsorption of protein molecules can result
in the formation of surface films. The sloughing off and/or mechanical rupture of such films
leads to subvisible particles in solution.\textsuperscript{67,96} Protein particle formation in IV bags due to
protein adsorption to interfaces was also suggested by Kumru, \textit{et al.}, and they observed a
reduction in protein particles in the presence of polysorbate 20, an effect attributed to
inhibition of protein adsorption to the bag walls and to the air-water interface.\textsuperscript{62}

We hypothesized that dilution of formulation excipients and exposure to various
interfaces in the infusion system will cause protein aggregation in the form of micro- and
nanoparticles. In the current study, we started by characterizing IV saline for its micro- and
nanoparticle concentration. IV saline bags of two sample volumes (100 mL and 250 mL)
from two manufacturers (Hospira and Baxter) were examined for particle concentration. In-
line filters manufactured by Baxter (pore size - 0.2 µm and 1.2 µm) and CareFusion (pore
size - 1.2 µm) were also tested for their efficiency in filtering out these particles. Thereafter,
we characterized micro- and nanoparticles formed by a model therapeutic protein, IVIG,
upon dilution into IV saline and during processing through a conventional infusion system.
The IV system was composed of IV saline bag, IV tube, infusion pump and filter units that
are used routinely for clinical administration of therapeutic protein products. Finally, an automated Raman microscope was used to identify some of the microparticles observed in solutions of IVIG that had been processed through the infusion system.

Materials and Methods

Materials

The IV administration arrangement employed an Alaris 8100 pump module and an Alaris 8015 pump controller. IV tubes (CareFusion, serial # 2426-0500), in-line filters (CareFusion: 1.2 µm pore size, serial # 20128E, Baxter: 1.2 µm pore size, serial # 2C1103, and 0.2 µm pore size, serial # 2C8671), and IV saline bags (Hospira, Lake Forest, IL, 100 mL serial # NDC 0409-7984-23 and 250 mL serial # NDC 0409-7983-02), (Baxter, 100 mL serial # NDC 0338-0049-48 and 250 mL serial # NDC 0338-0049-02) were purchased from various medical equipment suppliers and distributors. Gammagard ® (100 mg/mL IVIG, Lot# LE12N107AB, Expiry: May 2016) manufactured by Baxter Healthcare Corporation (Westlake Village CA,) was used as a model protein.

Methods

Processing of samples through IV system

In the lab, we mimicked the infusion system and the protocol used by the Outpatient Infusion Center at the University of Colorado Hospital (Fig.1). First, 10 mL of saline was removed from the injection port of the IV bag using a siliconized plastic BD syringe. IVIG was then diluted in this saline and introduced into the IV bag for a final protein concentration of 0.4 mg/mL. Using a non-siliconized syringe, 10 mL of the IVIG-saline solution was withdrawn from the bag for particle concentration analysis in the initial IVIG-saline sample. The tube was primed using the roller clamp to control the flow rate, ensuring no air bubbles
were generated during this step. The primed IV tube was then connected to the infusion pump and the flow was set to 140 mL per hour. The solution was then pumped through the tube without an in-line filter in place. Thereafter, three different in-line filters (a 1.2 µm CareFusion filter, a 1.2 µm Baxter filter and a 0.2 µm Baxter filter) were sequentially attached to the IV tube to collect filtered IVIG-saline samples. Five measurements were conducted on the initial sample collected from the injection port of the IV bag to determine the micro- and nanoparticle concentrations. For samples processed through the infusion system, particle concentrations were determined after analyzing five aliquots for each of the samples - IVIG processed through the IV tube with – a) no filter; b) 1.2 µm CareFusion (CF) filter; c) 1.2 µm Baxter (BX) filter; or d) 0.2 µm Baxter filter.

For characterization of IV saline without protein, a non-siliconized syringe was used to remove saline from the IV bag for initial particle counts. Then the same procedure described for IVIG was followed to obtain solutions processed through the IV system without and with in-line filters.

**Particle characterization**

A Protein Simple (Ottawa, ON, Canada) 4200 MFI system with a 100 µm flow cell was used to characterize particles ≥ 1 µm in size. A minimum sample volume of 0.5 mL was required, of which 0.1 mL was used as purge volume, 0.05 mL was used for background optimization and 0.35 mL was used for particle analysis.

A benchtop B3 series FlowCAM (FC) (Fluid Imaging Technologies) instrument equipped with a 100 µm multi-use flow cell was used to characterize particles ≥ 2 µm in size. The instrument used a 10X magnification lens and was controlled by the visual spreadsheet
software version 3.1.10. A minimum sample volume of 500 µL was introduced in the sample chamber of which 300 µL was analyzed at a flow rate of 0.08 mL/min.

Submicron particles (≥ 60 nm) were characterized using a Nanosight Model NS 300 (Malvern Instrument Ltd., Amesbury, UK) equipped with a 488 nm laser, CMOS camera and an integrated syringe pump. NTA 2.3 (software build 033) was used for analysis. The sample chamber was manually primed with the sample using a 1 mL silicone oil-free plastic syringe (National Scientific Company, TN, USA), and then the syringe was connected to the syringe pump. Sample analysis was done at a flow rate of 10 and camera setting of 12. Video analysis was done at a detection threshold of 12.

**IVIG particle concentration as a function of IV bag volume processed through IV system**

IVIG (100 mg) diluted in a 250 mL saline IV bag was processed through the IV system with an in-line filter in place. Three individual IV bags were analyzed for each of the three in-line filters (a 1.2 µm CareFusion filter, a 1.2 µm Baxter filter or a 0.2 µm Baxter filter). Samples were collected as 5 sequential aliquots of 50 mL each, and particle concentrations were analyzed in each aliquot; i.e., as a function of sample volume processed through the IV system. Particle concentrations presented for IVIG-saline solution in Fig.18 are from MFI analysis and the particle images presented in Fig.19 are from FlowCAM analysis.

**Particle identification using G3-ID**

Particles from saline or IVIG-saline solution processed through the IV system with a 0.2 µm in-line filter were collected on custom fabricated quartz filter membrane (pore size 10-12 µm). The collected particles were characterized using a Malvern Morphologi G3-ID
automated Raman spectroscopy microscope. The instrument was equipped with a diode laser (785 nm) (Malvern Instruments, Ltd, UK), a RamanRxn 1 spectrometer (Kaiser Optical Systems, Inc, USA) and a CFI 60 bright field/dark field microscope (Nikon Corporation, Japan).

Before sample filtration, the filter membrane was cleaned and scanned for any particulate content. Thereafter a total of 5 mL of sample in 500 µL aliquots was passed through the filter membrane. The membrane was then rinsed with 10 mL filtered water and allowed to dry under vacuum for 20 minutes before analysis on the Morphologi G3-ID. Particles were identified by comparing the Raman spectra obtained from Morphologi G3-ID to those in the BioRad - KnowItAll Raman spectral library.

**Protein adsorption to the infusion system during IV infusion**

Protein adsorption to IV bags was investigated in two ways, using either coomassie stain or using fluorescently labeled IVIG solution. For the study with coomassie dye, IV bags incubated with IVIG-saline solution were first rinsed with water, and then incubated with the coomassie dye for 24 hours. After which the IV bags were gently rinsed with water and imaged on a gel reading plate. To determine whether protein adsorbed to the surface of the bags, the images from these bags were compared to the IV bags incubated with saline alone. For the study with fluorescently labeled IVIG solution, labeled IVIG at a concentration of 1 mg/mL was labeled with Alexa Fluor® 488 C5 Maleimide in 50 mM sodium phosphate buffer at pH 7.0. IVIG solution was incubated overnight with 50 µm dye at room temperature away from direct light. This was followed by three buffer exchanges with 0.2 M glycine pH 4.2 to remove excess free dye. Labeled IVIG was incubated with IV bag or the tube downstream from the filter membrane for ~ 4 hours. Thereafter, these surfaces were rinsed
with water to remove any excess protein that did not adsorb onto the surfaces. Images for protein adsorption to the infusion system were captured using a Nikon Digital eclipse C1, (Nikon Inc., Melville, NY) microscope with a 488 nm green excitation fluorescent filter.

**Particle desorption during saline flush after IVIG-Saline solution was processed through the infusion system**

IVIG labeled with Alexa-fluor 488 was spiked into IV solution of IVIG in a 250 mL Hospira IV bag at a ratio of 1:160 labeled to unlabeled protein concentration wherein the unlabeled protein concentration was 0.4 mg/mL. This solution was then pumped through the infusion system with a 0.2 μm in-line filter in place. After 250 mL of IVIG solution was pumped through the system, the tube downstream from the filter membrane was cut off, and the IV tube, the filter membrane and the tube downstream from the filter membrane were rinsed individually with 5 mL of saline solution. Images of particles desorbed from these infusion system components were captured using a Nikon Eclipse microscope model TE 300 (Nikon Inc, Melville, NY) equipped with a super high pressure Hg source and a 488 nm green excitation fluorescent filter.
Results

Infusion setup in the lab

Figure 13: Setup in the lab replicating a typical IV infusion system used at the Outpatient Infusion Center at the University of Colorado Hospital
Figure 14: Particle concentrations in saline from an IV bag, and after the saline solution was processed through the infusion set, with or without an in-line filter connected to the IV tube. (Left panel) - Particles ≥ 1 µm from MFI analysis for three individual 250 mL IV saline bags manufactured by Hospira and (right panel) - particle distribution between 60-500 nm for one representative IV bag from NTA analysis. Results are shown for: initial sample collected from the injection port of the IV bag (a and b); sample processed through the infusion set connected without a filter (c and d); sample processed with a 1.2 µm Baxter in-line filter (e and f); and sample processed with a 0.2 µm Baxter in-line filter (g and h). Error bars indicate SD for 5 independent measurements of particles in the same sample.
Saline from IV bags manufactured by Hospira and Baxter (250 mL and 100 mL volumes) were characterized for micro- and nanoparticle levels. Fig.14 shows detailed results obtained from three bags of 250 mL IV saline manufactured by Hospira. In the initial saline sample from these IV bags there were several thousand microparticles ≥ 1µm in size (Fig.14a). The microparticle counts increased slightly when saline was processed through the IV tube without an in-line filter (Fig.14c). As expected there were substantial decreases in microparticle concentrations when in-line filters were employed (Fig.14e, g). Nanoparticle concentrations presented in the right-hand panels of Fig.14 were representative particle distributions between 60-500 nm from one bag of 250 mL Hospira saline. Interestingly, there was no significant difference in the nanoparticle size distribution between the initial sample and the saline sample pumped through the IV system without an in-line filter (Fig.14b, d). There was only a slight decrease in the nanoparticle concentration when the 1.2 µm Baxter filter was used (Fig.14f). Use of a 0.2 µm Baxter filter also resulted in a decrease in the number of nanoparticles, with the majority of particles remaining having sizes smaller than 200 nm (Fig.14h).

The same assessments were conducted for particle concentrations in 100 mL IV saline bags manufactured by Hospira, and in 250 mL and 100 mL IV saline bags manufactured by Baxter (Fig.15). For each of these IV bags, the initial saline sample contained hundreds to several thousand microparticles per mL, as measured by MFI (Fig.15a) and by FC (Fig.15b). When the IV solution was pumped through the IV system without an in-line filter, microparticle concentrations were similar to those in the initial saline solutions. The use of the three different in-line filters resulted in substantial reductions in the microparticle concentrations (Fig.15a, b).
Figure 15: Particle concentrations in IV saline bags and after the saline solution was processed through the infusion set, with or without an in-line filter connected to the IV tube. Three IV bags manufactured by Hospira and Baxter in 250 mL and 100 mL volume each were tested. Total particle concentrations are shown for: ≥ 1 µm from MFI analysis (a); ≥ 2 µm from FC analysis (b); and ≥ 60 nm from NTA analysis (c). Three different in-line filters were tested with the IV tubes; 1.2 µm pore size manufactured by CareFusion (CF); 1.2 µm pore size manufactured by Baxter (BX); and a 0.2 µm pore size manufactured by Baxter.
Each datum point shows mean of particle counts obtained for a sample processed from one IV bag. Error bars indicate SD for 5 independent measurements of particles in the same sample.

Nanoparticle concentrations of about 4-75×10^6 particles per mL were observed in the initial saline samples from the three different IV bags (Fig.15c). The particle concentrations were comparable between the initial saline sample and the saline sample processed with the IV system with no filter, but decreased to 1-21×10^6 particles per mL when any of the three in-line filters were employed (Fig.15c). Overall, the micro- and nanoparticle concentrations were comparable in IV saline from different bags from the two manufacturers and also between the 100 and 250 mL bag volumes.

Particle concentrations in solutions of IVIG in IV saline

For characterizing the micro- and nanoparticle concentrations, solutions of IVIG were prepared by dilution of the formulation into saline IV bags (250 mL and 100 mL) manufactured by Hospira or Baxter. Detailed results are presented in Fig.16 for particle contents in IVIG diluted into saline in IV bags (250 mL) manufactured by Hospira. There was bag-to-bag variability in the initial IVIG sample particle counts, with 8000 to 16,000 particles per mL ≥ 1 µm measured by MFI (Fig.16a). Processing the IVIG solutions through the IV system without an in-line filter produced particle counts that were slightly higher compared to the initial IVIG-saline sample (Fig.16c), and with in-line filters in place, the microparticle counts were greatly reduced (Fig 16e, g). Nanoparticle concentrations in the solution of IVIG in IV saline were not altered by processing through the IV system without a filter (Fig.16b, d), and also did not change considerably when the in-line filters were employed during pumping through IV system (Fig.16f and 16h).
Micro- and nanoparticle concentrations were also analyzed for IVIG diluted into saline in 100 mL IV saline bags manufactured by Hospira and in 250 mL and 100 mL IV saline bags manufactured by Baxter (Fig. 17).

Figure 16: Particle concentrations in IVIG diluted in IV saline bag, and after it was processed through the infusion set, with or without an in-line filter connected to the IV tube. (Left
(right panel) - particle distribution between 60-500 nm for one representative IV bag from NTA analysis. Results are shown for: initial sample collected from the injection port of the IV bag (a and b); sample processed through the infusion set connected without a filter (c and d); sample processed with a 1.2 µm Baxter in-line filter (e and f); and sample processed with a 0.2 µm Baxter in-line filter (g and h). Error bars indicate SD for 5 independent measurements of particles in the same sample.

Figure 17: Particle concentrations in IVIG diluted in IV saline bag and after it was processed through the infusion set, with or without an in-line filter connected to the IV tube. IVIG diluted into three IV bags manufactured by Hospira and Baxter in 250 mL and 100 mL.
volume each were tested. Total particle concentrations are shown for: ≥ 1 µm from MFI analysis (a); ≥ 2 µm from FC analysis (b); and ≥ 60 nm from NTA analysis (c). Three different in-line filters were tested with the IV tubes: 1.2 µm pore size manufactured by CareFusion (CF); 1.2 µm pore size manufactured by Baxter (BX); and a 0.2 µm pore size manufactured by Baxter (BX). Each datum point shows mean of particle counts obtained for a sample processed from one IV bag. Error bars indicate SD for 5 independent measurements of particles in the same sample.

There were 3,700-23,000 particles per mL ≥ 1 µm analyzed by MFI (Fig.17a) and 1,130-12,500 particles per mL ≥ 2 µm analyzed by FC (Fig.17b). Although the microparticle concentrations in the initial IVIG samples from these IV bags varied considerably, they were generally higher than those observed in saline alone (Fig.15a and 15b). In the IVIG solutions processed through the IV system without the in-line filters, the total microparticle concentrations ranged from 3,600-15,000 particles per mL ≥ 1 µm (Fig.17a) and from 2,660-18,900 particles ≥ 2 µm (Fig.17b). Each of the three in-line filters connected to the IV tube reduced the microparticles to 200-4000 particles per mL (Fig.17a and 17b). The nanoparticle concentrations in IVIG solution prepared in saline varied considerably between the different bags, with 18-240×10^6 particles per mL measured in the initial samples (Fig.17c), and levels remained high and variable in samples pumped through the IV system without a filter (Fig.17c). However unlike the reduction in microparticle concentrations with the use of in-line filters, the nanoparticle concentrations in the IVIG-saline solution remained relatively high (7-83×10^6 per mL) in the samples processed through the IV system with in-line filters in place (Fig.17c). Thus, even the smaller pore size filter tested (0.2 µm) was not effective at reducing nanoparticle concentrations in solutions of IVIG in IV saline that were pumped through the IV system.
Particle concentration as a function of sample volume processed through the IV system.

To investigate whether the particle levels in IVIG processed through the IV system with in-line filters would vary during the time course of infusion of an entire bag of 250 mL, we determined particle concentrations as a function of volume processed. Microparticle concentrations were analyzed by MFI for 5 aliquots of 50 mL each. These aliquots were obtained sequentially as the 250 mL IVIG saline solution was pumped. Overall, with all three filters tested there were no trends in the levels of microparticles as a function of volume of solution processed through the IV system. (Fig.18). Interestingly, however, there were a considerable number of particles ≥ 10 µm (Fig.18b) and ≥ 15 µm (Fig.18c) in the IVIG-saline solutions. Representative images for these particles ≥ 20 µm and as large as 60 µm obtained with FC analysis are presented in Fig.19.

Identification of particles in the IV infusion system using the Morphologi G3-ID

To identify the chemical characteristic and potential sources for microparticles, Raman microscopy was used to acquire images and Raman spectra for selected particles. Particles in the IV solution of IVIG processed through the IV infusion set connected to the 0.2 µm in-line filter were collected onto a quartz filter membrane for identification by a Morphologi G3-ID automated Raman microscope. This technique enabled the imaging and identification of proteinaceous particles (Fig.20a, b), based on the characteristic bands in the Raman spectrum for phenylalanine, tyrosine and beta-sheet structure. Also detected were non-proteinaceous particles composed of polycarbonate (Fig.20c, d), which were identified by a Raman spectrum that match that of polycarbonate in a reference spectral library. The polycarbonate particles were probably shed from the IV line connectors and from the housing...
for the in-line filter. Also, particles composed of PVC were identified (data not shown), which most likely were shed from the IV line downstream from the filter.

**Protein adsorption to the infusion system and desorption during saline flush**

We hypothesized that one source of protein particles could be protein that had adsorbed to the liquid-solid interfaces (e.g., downstream side of the filter membrane, filter housing and tubing walls) that formed a film, which was then sloughed off. In particular, formation of particles by this mechanism in areas downstream from the upper surface of the filter membrane could account for the substantial number of microparticles observed; even when an in-line filter with a 0.2 µm pore size was used. In initial studies, we incubated fluorescently labeled IVIG with the entire infusion system and observed protein adsorption to the walls of the IV bag and the IV tubing. Representative images from the latter are shown in Fig.21a. We next spiked fluorescently labeled IVIG into a solution of the protein in a 250 mL bag of IV saline, and the entire bag volume was processed through the IV system with a 0.2 µm in-line filter in place. After the infusion procedure, the tube downstream from the filter membrane was cut off and the filter membrane, the tube downstream from the filter membrane and the IV tube were flushed with protein-free IV saline. It should be noted that a similar saline flush is often used in the clinic to prevent wastage of the drug product that may still be in the IV tubes. In our study, after saline flush, we observed numerous particles with detectable fluorescent signal in the size range of ca. > 10 µm that sloughed off from the IV tubes (Fig.21b), filter membrane (Fig.21c); and the tube downstream from the filter membrane (Fig.21d). These results support our hypothesis and show that even with an in-line filter in place; relatively large protein microparticles can form downstream from the filter.
membrane and be delivered to patients, in addition to the high level of nanoparticles that are not removed by the filter.
Figure 18: Particle concentrations as a function of volume of IVIG-saline solution processed through the IV tube with an in-line filter connected to the IV tube. Total particle concentrations ≥ 1 µm (a); ≥ 10 µm (b); and ≥ 15 µm (c) from MFI analysis. Three different in-line filters were tested: a 0.2 µm pore size manufactured by Baxter (BX); a 1.2 µm pore size manufactured by Baxter (BX); and a 1.2 µm pore size manufactured by CareFusion (CF). Error bars indicate SD for 3 independent measurements of particles in the same sample.
Figure 19: Particle images captured during FlowCAM analysis of IVIG-saline solution processed through the IV tubes with in-line filters from Baxter (BX) or Care Fusion (CF).

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<thead>
<tr>
<th>In-line filters</th>
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<tr>
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<td><img src="image1" alt="Particle images" /></td>
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<td>1.2 μm BX</td>
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<td>0.2 μm BX</td>
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Figure 20: Representative images and Raman spectrum obtained by Morphologi G3-ID upon analysis of IVIG processed through the IV tube connected to a 0.2μm Baxter in-line filter. Image of a protein particle (a); Raman spectrum for the protein particle (b); image for polycarbonate particle (c); and Raman spectrum for polycarbonate (d). Black spectrum is for the sample particle and orange is the reference spectrum from the Raman spectral library.
Figure 21: Representative images of: fluorescently labeled IVIG adsorbing to the surface of an IV tube (a); particle images from saline used to flush the IV tube (b); filter membrane (c); and tube downstream from the filter membrane (d). An IVIG-saline solution spiked with fluorescently labeled IVIG molecules was processed through the IV system with a 0.2μm Baxter in-line filter.

Figure 22: Representative images of coomassie staining of IV bag containing saline alone (top panel) and IVIG-saline solution (bottom panel).
In addition, protein adsorption to the IV bags was also investigated without altering the protein by fluorescence labeling. IV bag that previously contained IVIG-saline solution was incubated with coomassie dye for 24 hours. The staining for this bag was compared to an IV bag that held saline solution alone without any protein. The results presented in Fig. 22 show higher commassie staining for bags incubated with protein than saline alone.

**Discussion**

We observed high numbers of subvisible particles -- particularly in the submicron size range -- in both IV saline and solutions of IVIG in IV saline, which were processed through an IV infusion system. Importantly, even when in-line filters were employed during the IV infusion procedure there were high levels of particles in the processed solution. Therefore, with IV administration of therapeutic proteins, high levels of subvisible particles are delivered to patients. Presently regulatory authorities, such as the US Food and Drug Administration (FDA), recognize such particles as critical product quality attributes that may pose a risk to patient safety. Numerous studies -- including human clinical investigations, in animal models and with *in vitro* assays with human cells -- have shown a link between subvisible particles and unwanted immunogenicity of therapeutic protein products.\(^{15,24,34,54-56,88}\)

A debate in the literature centers on whether there is absolute proof that particles at this concentration cause adverse immunogenicity in patients.\(^{36,53}\) However, regulatory agencies advocate precaution when dealing with factors that could pose a risk to patient health, but where this link has not been definitively and generally established. For example, a Commentary authored by FDA regulators states that “a strong scientific basis for a product attribute having a potential outcome of harm should warrant that the attribute be presumed to
be critical, even if a completely defined link to clinical performance has not been
demonstrated”. Subvisible particles are presently in this regulatory domain. Hence their
formation and presence in therapeutic protein products needs be monitored rigorously at all
stages of a product’s life history, from product manufacture through delivery to the patient.
The Commentary further states, in reference to subvisible particles, “assessment of such an
attribute needs to evolve with increased scientific knowledge and technical capabilities and
thereafter actions should be undertaken to lessen the probability of occurrences and severity
of harm pertaining to that product quality attribute”.64

This statement translates to the expectation that methods used for quantifying and
characterizing subvisible particles should be based on the technical advances in the field.
Further, all sources of subvisible particles should be identified and control strategies should
be developed to minimize their presence. Given these expectations and the results of the
present study, it is imperative that the quality of IV delivered therapeutic protein products be
assessed quantitatively with state-of-the art instrumentation. Furthermore, such studies
should include the complete clinical infusion protocol and systems employed in clinical
practice. Such work is needed for each product on the market and should be executed with
recommended IV solutions, lines, pumps and in-line filters.

Recently, however, quality assessments based on particles in IV solutions has focused
mostly on visible particles. Observations of visible particles have resulted in recalls of
several lots of IV saline in the last few years and therefore a shortage in supply of IV saline
manufactured by both Hospira and Baxter.97 Our study suggests that IV saline is also a major
contributor of subvisible particles. Development of successful therapeutic products requires
minimizing aggregates or particles. Because of the high levels of subvisible particles in IV
saline, even if the protein formulation itself in the product vial was particle-free to begin with, a high level of particles might be delivered into patients during an IV infusion.

This problem is especially of concern for drug products that are delivered via IV infusion without an in-line filter. Based on our data for the particle concentrations in IVIG in IV saline solution processed though the infusion system without an in-line filter, in a 250 mL infusion there would be billions of nano- and millions of microparticles delivered to the patient. For more than 50% of the mAb products currently marketed in the U.S., package inserts do not recommend the use of an in-line filter.82 Similarly, a recent review by Werner et al. noted that more than 80% of the approved therapeutics mentioned in the Rote List ® (list of medicinal products marketed in Germany) have no recommendations for filtration during preparation or administration.98

However, we observed that even when an in-line filter was employed during the IV infusion procedure, there were high numbers of nanoparticles present in the processed solutions of IV saline and IVIG. As expected, the total counts of microparticles were greatly decreased by the in-line filters, but there were still substantial levels of microparticles ≥10 µm. We hypothesized that these larger sized particles may be contributed by the components downstream from the filter membrane. The framework on which the membrane for the in-line filters rests and the filter housing both are made of polycarbonate. We found evidence with Raman microscopy that some of the particles recovered distal to the filter were polycarbonate, which most likely represent washed off components downstream from the membrane surface. Also, the polycarbonate components and the polyvinyl IV tubing are relatively hydrophobic surfaces, and protein molecules readily adsorb onto such surfaces.99 95 Microparticles, therefore, shed from these surfaces would most likely be coated with protein...
molecules. Furthermore, our studies with fluorescently labeled IVIG molecules demonstrated that the protein readily adsorbed to the tubing and other parts of the IV system, resulting in visible films of adsorbed protein. As solution is run through the system, parts of this film may slough off resulting in relatively large numbers of micron-sized particles. Because this phenomenon could also occur downstream from the filter membrane, particles generated herein would be present in the IV solution administered to patients. Overall, current in-line filter systems are not effective at preventing infusion of large quantities of particles into patients during IV administration.

Approaches to mitigate delivery of particles to patients during IV infusion should include development of manufacturing methods to lower the particle levels in IV solutions. More importantly, it is critical that there is also development of in-line filters that effectively reduce the levels of both nano- and microparticles. As part of these efforts, it is also important that problems caused by the filters themselves are also addressed. As found in the current study, some earlier investigators have also reported undesirable effects of in-line IV filters such as protein adsorption to the filter components and accelerated particle formation due to foreign particles shedding from the filter membranes. Thus, extensive work is needed in developing/designing in-line filters that are compatible with protein products and are effective in removing particles, while not contributing to the generation of particles themselves.
CHAPTER IV
SUBVISIBLE PARTICLES IN SOLUTIONS OF REMICADE® IN INTRAVENOUS SALINE ACTIVATE IMMUNE SYSTEM PATHWAYS IN IN VITRO HUMAN CELL SYSTEMS

Introduction

Immunogenicity to therapeutic proteins has been a concern since the introduction of the first biologic into the clinic in early 1920s. Initially the cause of the immune responses in human patients was the non-human (e.g., porcine and bovine) origin of the biologics. Development of recombinant protein products that closely matched the endogenous human proteins decreased the occurrence of immune responses. Intriguingly, neutralizing antibodies are still observed in patients receiving humanized and fully human recombinant proteins. For example, for some therapeutic monoclonal antibodies (mAbs) currently on the market, studies have reported neutralizing antibodies in 5-70% of the patients against adalimumab, abciximab, daclizumab, and infliximab (i.e., Remicade®). Side-to-side comparison of Remsima and Remicade® also revealed similar rates of immunogenicity between the two products.

Clinical manifestations of neutralizing antibodies can range in severity from a decreased biological activity of the drug product to complete loss of product efficacy. Dosing escalation can usually remedy reduced product efficacy. Patients who stop responding to the original product are usually treated with a different protein drug product prescribed for the same indication. For example, with multiple antagonists of TNF-α on the market, non-responding patients have the benefit of switching between Remicade®, Enbrel® and Humira®. It is important to note that this is not an option for numerous medical
conditions addressed by only one therapeutic product; for example in case of enzyme replacement therapies. Moreover, these neutralizing antibodies may also cross-react and neutralize the endogenous protein. In some cases, no immediate clinical effects have also been documented, but anti-drug antibody generation in patients could potentially compromise the therapy in the long-term.

Currently, most mAb therapies are administered via intravenous (IV) infusion, which, in addition to potentially provoking an immune response against the drug, may also result in infusion related hypersensitivity reactions in patients. Symptoms for infusion reactions range in severity from mild headaches, flushing and itching, to tightness in throat, dizziness and even anaphylactic shock. The mechanisms for infusion reactions are not clear. It is speculated that therapeutic proteins upon interaction with their molecular targets may stimulate the release of inflammatory cytokines. Infusion reactions can also be facilitated by antigen mediated IgE activation leading to mast cell sensitization which may further release cytokines amplifying the symptoms for infusion reactions. For mAbs, mild to moderate reactions are commonly observed with trastuzumab eliciting a reaction in 40% of the patients, rituximab in 77-85% of the patients, cetuximab in 16-19% of the patients and panitumumab in about 5% of the patients. Immunogenic responses due to the presence of neutralizing antibodies may also provoke infusion reactions as observed in a study in patients with Crohn’s disease being treated with Remicade®. The study also noted the use of immunosuppressive agents such as anti-histamines, acetaminophen or corticosteroids in reducing incidences of infusion reactions.

Contribution of subvisible particles in protein solutions in eliciting adverse immunogenicity to therapeutic proteins has been reported in numerous studies.
Levels and sizes of these particles are also considered important product quality attributes, and FDA recommends a rigorous analysis to characterize subvisible particles in therapeutic protein solutions.\(^{64}\) A recent article authored by FDA researchers further highlights the importance of proper particle analyses. There were 49 cases of anaphylaxis including 7 fatalities among patients receiving peginesatide, a commercial pegylated erythropoietin peptide mimetic. With the use of more sensitive nanoparticle tracking analysis and flow imaging methods, much higher concentration of nano- and microparticles were observed in the commercial product compared to the product used in the clinic. In contrast, when characterized with the light obscuration lot release assay, there were no differences in the subvisible particle levels between the commercial and the clinical lots. This study clearly demonstrates the risk to patient safety from particles in solution when appropriate particle characterization methods that can detect potentially lethal differences in product quality are not employed.\(^{52}\)

For decades preclinical studies conducted in murine models have provided important comparative immunogenicity data for products during different stages of production, after changes in manufacturing processes and/or to assess the effects of different forced degradation procedures.\(^{24,31,33,56,116}\) More recently the value and efficiency of \textit{in vitro} human cell immunogenicity assays have been demonstrated for small amounts of aggregates generated by forced degradation.\(^{34,53,54}\) For example, Joubert \textit{et al} have reported a strong innate immune response that progressed to adaptive immune response against protein particles generated via exposure to freeze-thawing, stirring or shear stress. They observed the release of various inflammatory cytokines in peripheral blood mononuclear cell (PBMC) cultures and a partial to complete inhibition of the innate immune response in the presence of
inhibitors for cell surface receptors like FcγRs, TLR-2 and TLR-4. They further report an enhanced T cell proliferation in PBMC cultures against protein particle containing samples compared to the monomeric control sample. Rombach et al have documented that proteinaceous subvisible particles provoked an enhanced antigen presentation capacity, co-stimulatory activity and cytokine secretion profiles in dendritic cells (DCs). These are the DC driven activities associated with subsequent T cell dependent immune responses. They also report a change in the antigenic peptide numbers and clusters presented on the DCs in the presence of protein particles. These two studies were conducted on model mAbs that had been purposely degraded, but a study by Ahmadi et al compared particle concentrations and immune responses to formulations in commercial vials of Rituxin® and Herceptin®. They further investigated activation of immune response against these products after exposure to stirring, heat stress or freeze-thawing stresses, which greatly increased the levels of proteinaceous subvisible particles. DC activation resulting in T cell proliferation was observed for both degraded and non-degraded Rituxin® and Herceptin®. It is important to note that the known mechanism of action of Herceptin is via binding to FcγRs and it does not have CD4+ T cell epitopes. But subvisible particles in this protein solution resulted in a strong activation of the immune system including T cell proliferation.

Relatively high rates of both infusion-related hypersensitivity reactions and immunogenic responses in patients have been reported during IV administration of Remicade®, the model protein for the current study. More than 20% of the patients develop infusion reactions and more than 50% develop neutralizing antibodies against Remicade®. It is a chimeric monoclonal antibody (mAb) used to treat numerous indications including Crohn’s disease, ulcerative colitis, rheumatoid arthritis, ankylosing
spondylitis and psoriatic arthritis.\textsuperscript{119} It blocks and neutralizes the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-\(\alpha\)), a cytokine that causes inflammation and induces the secretion of other pro-inflammatory cytokines.\textsuperscript{120} Remicade\textsuperscript{®} is administered through intravenous infusion (IV) by diluting in 0.9\% saline, using an in-line filter with a pore size of 1.2 \(\mu\text{m}\) or less; at 0, 2 and 6 weeks following which maintenance dose is administered every 6-8 weeks thereafter.\textsuperscript{119}

Most of the studies that tested the role of subvisible particles on immunogenicity were conducted with particles generated by accelerated degradation conditions. Such particle concentrations may not directly represent particle levels present during IV administration of therapeutic proteins. To date, there has not been a published study that investigated the levels of particles in IV administered monoclonal antibodies and the effects of these clinically-relevant particle levels on immunogenicity or infusion reactions in a model system. Therefore in the current study, we characterized particle concentrations in the micron and sub-micron ranges in IV solution of Remicade\textsuperscript{®} that was processed through an infusion pump system that is routinely used in the clinic for IV administration of therapeutic proteins. This infusion setup is similar the setup used in the clinic and demonstrated previously (unpublished data Pardeshi \textit{et. al}). In this earlier study with intravenous immunoglobulin, we found the presence of high levels of nano- and microparticles in IV saline that contributed greatly to the total particle concentration initially present in the bag of IV solution containing protein. Furthermore, even with the use of in-line IV filters, we observed high levels of nanoparticles in the antibody solution processed through the infusion system. Therefore, we hypothesized that the particles in solutions of Remicade\textsuperscript{®} that are administered to patients during IV infusion play an important role in eliciting infusion related reactions and
immunogenic responses. To test this hypothesis, particles levels were measured in IV solutions of Remicade® processed through the IV infusion system, either with or without in-line filters. The biological effects of the solutions were then tested -- with human primary cells -- for cytokine release in whole blood and in PBMC cultures, stimulation of toll like receptors (TLRs), upregulation of surface receptors on DCs and T cell proliferation. To assess specifically the roles of particles, samples of the processed Remicade® solutions were centrifuged to prepare particle enriched and particle free fractions, which were also tested in the in vitro human cell systems.

**Materials and methods**

**Materials**

Remicade® (100 mg/mL infliximab) manufactured by Janssen Biotech Inc was used as the model protein for this study. Clinical IV administration was replicated in the lab using an Alaris 8100 pump module and an Alaris 8015 pump controller. IV tubes (CareFusion, serial # 2426-0500), in-line filters (Baxter: 1.2 µm pore size, serial # 2C1103, and 0.2 µm pore size, serial # 2C8671), and IV saline bags (Hospira, Lake Forest, IL, 250 mL serial # NDC 0409-7983-02) were purchased from various medical equipment suppliers and distributors.

For cytokine release in whole blood assay, whole blood samples from 14 donors were obtained from Bioscience Cambridge (CTLS). The non-sterile 96 U well microplates (polypropylene) used for the assay were purchased from BA Greiner. (Cat. No.: 650201). The CBA kits for IL-1b flex set (Bead B4-558279), IFN-g flex set (Bead E7-558269), IL-6 flex set (Bead A7-558276) and IL-10 flex set (Bead B7-558274) were purchased from BD Biosciences.
For the cytokine release in PBMCs and T cell proliferation assays, AIMV culture media from Invitrogen (via Fisher VX12055083) was used. Tritiated thymidine for pulsing PBMC cultures was purchased from Perkin Elmer (NET027Z). The positive controls for the assays testing for cytokine release in PBMCs, upregulation of surface receptors on DCs and for T cell proliferation were KLH protein purchased from Sigma (#H7017) and A33 generated in house (batch 5-7.68mg/ml).

For isolating immature DCs, Human PBMCs isolated from blood were obtained by National Blood Transfusion Service, Addenbrookes Hospital, Cambridge. AIM V® Culture Medium from Gibco via Life technologies (12055-083) was used. Human IL-4 (Peprotech 200-04), Human GM-CSF (Peprotech 300-03), TNF-α (Peprotech 300-01A), PBS (DPBS/Modified, Hyclone (Thermo Scientific)–BBT stores), EDTA (Fluka Analytical, 03690 – 100ml) (0.5M EDTA in PBS), Human Serum (Sigma, H4522 - 100ml), (heat-inactivated at 56°C for 30 minutes), MACS Buffer (2mM EDTA, 0.5% Heat Inactivated Human Serum in PBS). MACS CD4+ T cell isolation kit (Miltenyi Biotec 130-096-533). MACS Pan monocyte Isolation kit (Miltenyi Biotec 130-096-537), MACS LS separation columns (Miltenyi Biotec 130-042-401) and the compensation beads from BD Biosciences (Anti-Mouse Ig, κ/Negative Control, 552843) were used for the assay.

Methods

*Processing the IV solution of Remicade® through the infusion system*

We replicated the infusion setup typically used for administration of therapeutic proteins by the Outpatient Infusion Center at the University of Colorado Hospital for Remicade®. Accordingly, the commercial lyophilized Remicade® product was reconstituted in 10 mL of water for injection. This solution was then introduced back into the IV bag. For
analysis of particle concentrations in the ‘initial sample’, using a non-siliconized syringe, 10 mL of the Remicade®-saline solution was withdrawn from the injection port of the IV bag. The IV tube was then connected to the IV bag, and 250 mL of the solution was processed through the infusion system at a rate of 140 mL/hr, without an in-line filter in place (no filter sample) to collect 5 aliquots of 12 mL each. Then, a 1.2 µm Baxter in-line filter was attached to the IV tube and similar aliquots were collected. This filter unit was removed, a 0.2 µm Baxter filter was attached to the IV tube and 5 aliquots of 12 mL of the solution were collected. Particle concentrations were determined five times on the single aliquot of the initial sample and one time each for the 5 aliquots for each of the filtered samples.

For in vitro cell assays, solution of Remicade®-saline from two IV bags processed in the manner described above were pooled together. Then to prepare particle free and particle enriched fractions, each sample of pooled aliquots were centrifuged. Ultracentrifugation was conducted using the Beckman XL-80 ultracentrifuge with a Beckman fixed angle Ti 50 rotor. A sample of 25 mL of each of the IV solution of Remicade®-saline aliquots was centrifuged. The 20 mL supernatant on the top of the solution in the centrifuge tube was collected as the particle-free fraction and the 4 mL of the solution was collected as the particle-enriched fraction.

**Particle characterization**

Particles ≥ 1 µm in size were characterized using a Protein Simple (Ottawa, ON, Canada) 4200 MFI system equipped with a 100 µm flow cell. Sample volume of 0.5 mL was analyzed, of which 0.1 mL was used as purge volume, 0.05 mL was used for background optimization and 0.35 mL was used for particle analysis.
A Nanosight Model NS 300 (Malvern Instrument Ltd., Amesbury, UK) with a laser (488 nm), CMOS camera and an integrated syringe pump was used for characterizing submicron particles (≥ 60 nm). The instrument was controlled with NTA 2.3 software build 033. Using a 1 mL silicone oil-free plastic syringe (National Scientific Company, TN, USA) the sample chamber was manually primed with the sample, and then the syringe was connected to the syringe pump for analysis using a flow rate of 10 and a camera setting of 12. Video analysis was done at a detection threshold of 12.

**Cytokine release in whole blood**

The assay was conducted according to the protocol by Wolf *et al.* An aliquot of 12.5 µL from each of the Remicade® samples was incubated with 237.5 µL of whole blood sample from each donor in a 96 well U-bottom plate overnight at 37° C, 5% CO₂. After 24 hours of incubation, the 96 well plates were centrifuged at 1800 g for 10 mins at 4° C. Post centrifugation, 100 µL of the supernatant from each well was transferred to appropriate wells of another 96 well plate and frozen at -80° C until further analysis. Concentration of IL-6, IL-10, TNF-α and IFN-γ was analyzed in these supernatants using cytokine bead array (CBA) kits obtained from BD. Donors with response higher than the 95 percentile for PBS (negative control) are considered positive.

**Isolation of Peripheral blood mononuclear cells (PBMCs)**

PBMCs isolated from healthy donor buffy coats from blood drawn within 24 hours obtained from the UK National Blood Transfusion Service (Addenbrooke’s Hospital, Cambridge, UK) and according to approval granted by Cambridgeshire Local Research Ethics Committee was used for this study. Lymphoprep (Axis-shield, Dundee, UK) was used
to isolate PBMCs from buffy coats by density gradient, depleting the CD8$^+$ T cells using CD8$^+$ RosetteSep™ (StemCell Technologies, Inc., London, UK). After isolation, PBMCs were frozen and stored in liquid nitrogen until use. The assay protocol as mentioned by Ahmadi et al.$^{54}$

**Cytokine release in PBMCs**

The Remicade® samples (500 µL) were incubated with AIMV serum free media (500 µL) and 1 mL of PBMCs (4x10$^6$ cells/mL) from 11 healthy donors in a 24 well flat bottom plate at 37° C, 5% CO$_2$. On day 2, 200 µL of the cell suspension from each well was transferred to a V-bottom 96 well plate for centrifugation for 7 mins at 1200 RPM. 90 µL of the supernatant was then transferred to each of the two 96 well U-bottom plates and frozen at -80° C until further analysis. Concentration of IL-6, IFN-$\gamma$, IL-10 and IL-1β was analyzed in the supernatants using the CBA kits obtained from BD.

**TLR activation**

HEK-293 cells co-transfected with human toll-like receptor (TLRs) gene (genes for TLR 2, 3, 4, 5, 8, 9), the MD-2/CD-14 co-receptor gene and a secreted embryonic alkaline phosphatase (SEAP) reporter gene were obtained. The SEAP reporter gene is fused to NF-$\kappa$B and AP-1 binding sites. Stimulation with a ligand specific to the TLR activates the NF-$\kappa$B and AP-1 resulting in the production of SEAP which can be detected by HEK-Blue detection media. The color change can be detected by a UV spectrophotometer at 620-650 nm.

Initial cell culture suspension generated as per the recommendations by Invivogen. For stimulation of TLRs, suspension of approximately 2.8x10$^5$ HEK-TLR cells per mL was prepared in HEK-Blue detection media, and 180 µL of the cell suspension was added per
well (~50,000 cells per well). 20 µL of the Remicade® samples were incubated with the cells at 37° C in 5% CO₂ for 22 hours before reading the plate at 620-655 nm using a spectrophotometer. The procedure was according to the recommendations by Invivogen specific for each TLRs. Recommendations for HEK-Blue™ hTLR4 cells as per Invivogen.¹²²

**Dendritic cell activation**

Monocytes were isolated from PBMCs from 9 healthy donors using the Pan Monocyte kit (procedure as mentioned in the manufacturer’s protocol; to a final concentration of 1x10⁶ cells per mL).¹²³ The cell suspension (2 mL) was plated in a 24 well flat bottom plate, and the monocytes were allowed to mature into immature dendritic cells with GM-CSF (1000 U/mL) and IL-4 (1000 U/mL) in AIMV media for 4 days. Media change was done on day 2, 4 and 7. On day 4, TNF-α was added to the well containing positive control, and Remicade® samples were added to the respective wells for each plate. The plates were then incubated at 37° C, 5% CO₂. The dendritic cells were harvested on day 8, wherein the supernatant was collected in a 15 mL falcon tube and the wells were washed twice with 2 mL of PBS. Remaining adherent cells were removed by incubating the wells with 1 mL of 2 mM EDTA in PBS for 5-10 mins at 37° C. This solution was pooled together in 15 mL tubes; the tubes were then filled with PBS and centrifuged for 10 minutes at 1200 RPM. The supernatant was discarded, and the dendritic cells in the pellet were resuspended in 900 µL of PBS and counted using trypan blue stain. Cells were then resuspended in PBS and centrifuged for 10 minutes at 1200 RPM after which they were resuspended at a concentration of 50,000 cells per 100 µL PBS. Of this cell suspension, 100 µL was then transferred to each well in a 96 well U-bottom plate. For staining, the cells were centrifuged at 4° C for 20 minutes at 2200 RPM, the supernatant was discarded, and 9 µL of diluted
FcγR blocker was added to each well. The contents of the wells were mixed by placing the plate on a digital shaker for 2 mins at 500 RPM after which the plates were incubated for 10 minutes at room temperature. 38 µL of antibody mix specific to CD11c, CD14, CD40, CD80, CD83, CD86 and HLA-DR was added to each well, and the plates were shaken for 2 mins on the digital shaker at RPM followed by incubation in the dark at 4°C for 20 minutes. Cold MACS buffer (150 µL) was added to each well after incubation and the plates were centrifuged for 3 mins at 2200 RPM at 4°C. The supernatant was discarded and this wash step was repeated one more time after which the wells were resuspended in 50 µL of fix and 50 µL of MACS buffer and the plates were stored at 4°C in the dark until FACS analysis.

Fluorescence compensation settings for multicolor flow cytometry analyses were optimized using compensation beads purchased from BD. For compensation beads, a 96 well plate was prepared for 7 colors specific to each antibody (CD11c, CD14, CD40, CD80, CD83, CD86 and HLA-DR). The beads were vortexed and positive beads (anti-mouse Ig, κ particles) and negative beads (no binding capacity) were added to each well. Single antibody (CD11c, CD14, CD40, CD80, CD83, CD86 and HLA-DR) was added to each well, and the plates were incubated for 20 minutes at room temperature. Cold MACS buffer (150 µL) was added to each well following incubation after which the plates were centrifuged for 3 minutes at 4°C at 2200 RPM. The supernatant was discarded and the wash step was repeated one more time. Each well was then resuspended in 200 µL of MACS buffer and the plates were stored at 4°C in the dark until FACS analysis.

T cell proliferation

T cell proliferation assay was conducted as mentioned by Ahmadi et al. Remicade® samples (0.5 mL) with AIMV serum free media (0.5 mL) and 4x10^6 PBMCs/mL (1 mL)
isolated from 22 donors were incubated in a 24 well flat bottom plate for 5 days at 37° C, 5% CO₂. At day 5 the culture in each well was gently resuspended and three 100 µL aliquots were transferred to a 96 well U bottom plate. These cultures were then pulsed with AIMV media that was spiked with 0.75 µCi of tritium and the cells were allowed to proliferate for 24 hours at 37° C, 5% CO₂. Following 24 hour incubation the plates were harvested and the tritium incorporated into the cells was measured using liquid scintillation counting. Donors with 2 fold higher response compared to media are considered positive.

**Statistical analysis**

Statistical analysis was conducted using GraphPad Prism software version 5 (GraphPad Software Inc, California, USA). Repeated measures Friedman test was conducted for analyses of data set for cytokine release in whole blood and in PBMC cultures, paired student t-test for stimulation of TLRs, and repeated measures one-way ANOVA test for T cell proliferation.

**Results**

**Particle characterization in IV solution of Infliximab**

Particle concentrations were characterized in intravenous solution of Remicade® prepared by dilution (1 to 25) of the reconstituted commercial formulation into saline IV bags (250 mL) manufactured by Hospira. There was bag-to-bag variability in the microparticle counts in the initial Remicade® solution collected from the injection port of each of the three IV bags with 4300-10,700 particles per mL ≥ 1 µm (Fig.23a). Previously, we have determined that IV saline itself contains thousands of microparticles per mL and that there is a high degree of variation in the microparticle concentrations between IV bags (4 -73×10⁶ nanoparticles/mL) (Unpublished data, Pardeshi, *et.al*).
When the Remicade®-saline solution from the bag was processed through the IV system without an in-line filter (no filter samples), particle concentrations at 4700-10,400 particles/mL ≥ 1 µm didn’t change significantly (Fig.23c). However, with either in-line filter in place microparticle concentrations were greatly reduced to 200-800 particles/mL ≥ 1 µm (Fig.23e, g).

Representative nanoparticle counts and size distributions from one bag of Remicade® diluted in IV solution manufactured by Hospira are presented in Fig.23 (right panel). Two bags of Remicade®–saline solution had a high concentration of particles < 100 nm resulting in a highly varied total nanoparticle concentrations in the initial samples. However, the nanoparticle concentrations or size distributions did not change when the Remicade®-saline from the bag was processed through the infusion system without a filter (Fig.23b, d) or when in-line filters were employed (Fig.23f, h).

**Effects of Remicade® solutions on cytokine release in whole blood samples**

To monitor the secretion of inflammatory cytokines such as IL-6, IFN-γ, TNF-α and IL-10, whole blood samples from 14 healthy donors were incubated with solutions of Remicade®-saline processed through the IV system. Interestingly, only IL-6 secretion was observed (Fig.24). The particle enriched samples of Remicade®-saline processed through the infusion system without an in-line filter in place (no filter samples) resulted in a high concentration of IL-6 secretion. The frequency of donor responses (10/14 positive donors) for this sample were also high. In support for the contribution of particles in eliciting IL-6 secretion, cells from 4 donors secreted > 1000 pg/mL IL-6 in the no filter solution of Remicade®-saline in the particle enriched sample compared to 1 and 0 donors for supernatant and no centrifugation samples, respectively (Fig.24).
The secretion of IL-6 was generally lower for Remicade®-saline processed through the IV system with in-line filters (Fig.24). However even for these samples, cells from some of the donors had high IL-6 secretion in response to Remicade®-saline. For example, cells from some donors incubated with the particle enriched samples for Remicade®-saline processed through either in-line filter (1.2 and 0.2 µm pore size) or those incubated with the supernatant sample for Remicade® solution filtered through the 0.2 µm in-line filter.

Furthermore, comparing between the particle-enriched samples of Remicade®-saline solution (no filter, filtered through the 1.2µm or the 0.2µm in-line filter) the frequency of donor responses increased as a function of microparticle concentration in solution. No filter solution with 10/14 positive donors had the highest donor frequency followed by 1.2 µm filtered solution (6/14) and 0.2 µm filtered solution (3/14). Speculatively, the high IL-6 secretion induced by the supernatant of Remicade®-saline processed through the 0.2 µm in-line filter may be due to either the inherent immunogenicity of Remicade® or to extrinsic factors (e.g., leachates) contributed by the in-line filter.
Figure 23: Particle concentrations in infliximab diluted in IV saline. (Left panel) particles greater than 1 µm from 3 individual 250 mL IV saline bags and (right panel) particle distribution between 60-500 nm for a representative IV bag. (a and b) Initial saline sample; (c and d) No filter sample; (e and f) samples filtered through 1.2 µm Baxter filter; (g and h) samples filtered through the 0.2 µm Baxter filter. Error bars represent 5 independent measurements of the same sample.
Figure 24: IL-6 secretion in whole blood samples from 14 donors in response to various infliximab solutions. Infliximab solution processed through the infusion system either without an in-line filter (no filter) or with an in-line filter connected to the infusion system (1.2 µm or 0.2 µm pore size) were further subjected to a centrifugation step to generate the supernatant (Sup) and particle enriched sample (P) along with the no centrifugation (NC) sample. The number of positive donors compared to PBS is noted above each of the data set. Statistical significance was determined by Friedman test by comparison to negative control (PBS) *** p < 0.001.

Effects of Remicade® solutions on cytokine secretion from PBMCs

PBMC cultures from 11 donors were monitored for the secretion of IFN-γ, IL-6, IL-1β and IL-10 in response to IV solution of Remicade® (Fig.25). There was no significant secretion of any of the cytokines in PBMC cultures in response to Remicade®-saline samples when compared to the negative control (media).
Effects of Remicade® solutions on stimulation of Toll like Receptors (TLRs)

Figure 25: Cytokine secretion in PBMCs from 11 donors in response to various infliximab solutions. Infliximab solution processed through the infusion system either without an in-line filter (no filter) or with an in-line filter connected to the infusion system (1.2 µm or 0.2 µm pore size) were further subjected to a centrifugation step to generate the supernatant (Sup) and particle enriched sample (P) along with the no centrifugation (NC) sample. The number of positive donors compared to media is noted above each of the data set.

TLR reporter cell lines expressing various TLRs were responsive only to Remicade® solution processed through the infusion system and filtered through the 0.2 µm in-line filter. The response was observed in all three samples of the solution filtered through the 0.2 µm in-line filter independent of the centrifugation processing. Samples of Remicade®-saline
processed either without an in-line filter or with the 1.2 µm in-line filter did not induce stimulation of the TLRs (Fig.26).

![Figure 26: TLR activation in response to various infliximab solutions. Infliximab solution processed through the infusion system either without an in-line filter (NF) or with an in-line filter connected to the infusion system (1.2 µm or 0.2 µm pore size) were further subjected to a centrifugation step to generate the supernatant (Sup) and particle enriched sample (P) along with the no centrifugation (NC) sample. Statistical significance was determined by one way ANOVA test by comparison to media. *** p < 0.001, ** p < 0.01 and * p < 0.05](image)

**Effects of Remicade® solutions on upregulation of surface receptors on dendritic cells (DCs)**

Surface receptors on DCs are highly expressed in mature cells. Therefore, immature DCs that generally express low levels of differentiation markers were used in this assay to investigate the upregulation of surface receptors in response to Remicade®-saline solutions (Fig.27). We did not observe upregulation of any of the DC surface receptors in response to any of the Remicade®-saline samples.
Figure 27: Upregulation of DC surface receptors in response to various infliximab solutions: (a) CD 83, (b) CD 40, (c) CD80, and (d) CD 86. Infliximab solution processed through the infusion system either without an in-line filter (NF) or with a 0.2 µm in-line filter (0.2) connected to the infusion system were further subjected to a centrifugation step to generate the supernatant (Sup) and particle enriched sample (P) along with the no centrifugation (NC) sample.

Effects of Remicade® solutions on T cell proliferation

The particle-enriched fraction of Remicade®-saline processed through the infusion system without a filter stimulated significant T cell proliferation compared to that observed with the particle-free and no centrifugation samples (Fig.28). For Remicade®-saline processed with a 0.2 µm filter, we also observe a high level of T cell proliferation for the
particle-enriched sample, but it was not statistically significant compared to the effects observed with particle-free and no centrifugation samples. Importantly, T cell proliferation responses varied across cells obtained from different donors, but a higher frequency of response was noted for the particle enriched fractions for Remicade® solution processed through the infusion system without an in-line filter (5/22) and with the 0.2 µm in-line filter (4/22) compared to the respective supernatant and no centrifugation samples.

Importantly, the donor response for T cell proliferation was varied but higher donor frequency was noted for the particle enriched fractions for infliximab solution processed through the infusion system without an in-line filter (5/22) and with the 0.2 µm in-line filter (4/22) compared to the respective supernatant and no centrifugation samples.

![Figure 28: T cell proliferation in response to various infliximab solutions.](image)

Figure 28: T cell proliferation in response to various infliximab solutions. Infliximab solution processed through the infusion system either without an in-line filter (no filter) or with a 0.2 µm in-line filter connected to the infusion system were further subjected to a centrifugation step to generate the supernatant (Sup) and particle enriched sample (P) along with the no centrifugation (NC) sample. The number of positive donors compared to media is noted.
above each of the data set. Statistical significance was determined by repeated measures one-way ANOVA test by comparison within each solution group. ** p < 0.01.

Discussion

Although there are only a few published studies that have tested for immunogenicity due to subvisible particles in therapeutic protein solutions using *in vitro* human cell assays, the method has shown great value. It has been particularly useful for comparing relative effects of samples subjected to different accelerated degradation stresses. And in one study, particles, even at the relative low levels in unstressed samples of Rituxin were shown to be immunogenic.\textsuperscript{54} In the current study, we found that the *in vitro* human cell assays clearly documented immunogenicity of Remicade\textsuperscript{®} diluted into IV saline and processed through a clinical IV infusion system.

Even though the particulate levels in samples tested in these earlier studies comprised a relatively small amount of the protein (~3% of the total protein), the microparticle concentrations in these samples were approximately 100,000 per mL.\textsuperscript{34,53,54} In contrast, in the Remicade\textsuperscript{®}-saline samples used in the current study, levels of particles ≥ 1 μm were around few thousand particles/mL (Fig. 1). But nanoparticle levels were much higher with more than 30×10\textsuperscript{6} particles per mL. Importantly, these levels (less than 0.1% of the total protein mass in solution) reflect the particle loads that are essentially delivered to patients during IV infusion. At even this relatively low mass percent of protein in particles, the Remicade\textsuperscript{®}-saline samples could induce responses in the *in vitro* cell models that were indicative of infusion reactions and immunogenicity; including significant IL-6 secretion in whole blood samples, stimulation of TLRs and T cell proliferation. The frequency of positive donors for these responses was lower for the essentially particle-free Remicade\textsuperscript{®}-saline samples, relative to
particle-enriched or non-centrifuged samples. In addition, the dilution factor of 1:20 for Remicade® samples in whole blood in this assay is similar to the dilution that may occur as 250 mL of Remicade®-saline solution is administered to patients with approximately 5 L of blood. Overall, the results indicate that even with in-line filters in place the relative miniscule mass of Remicade® protein infused into patients as nanoparticles is sufficient to cause adverse responses such as infusion reactions and immunogenicity.

IV infusion of mAbs usually provokes infusion reactions characterized by the release of various inflammatory cytokines such as TNF-α, IFN-γ, IL-10 and IL-6.\textsuperscript{20} We did not observe significant concentrations of TNF-α, IFN-γ or IL-10 in this study. Remicade® is an antagonist of TNF-α, and it suppresses the secretion of IFN-γ.\textsuperscript{125} Moreover, IL-10 is an anti-inflammatory cytokine that works to inhibit the secretion of various pro-inflammatory cytokines, and it is likely that the immune response against particles in Remicade®-saline solution is not immunosuppressive as noted for responses against aggregates of some mAbs.\textsuperscript{53,126}

For IL-6, secretion of 1000-100,000 pg/mL were previously reported in response to Remicade® samples that were greatly aggregated (0.1-100 µg/mL).\textsuperscript{127} In current study, subvisible particles that constituted < 0.1% of the total Remicade® concentration in solution (0.2 mg/mL) induced the secretion of 400-10,000 pg/mL of IL-6 in whole blood cultures. Thus, a potent effect of even trace levels of particles in IV administered Remicade® is to stimulate secretion of relatively high levels of a cytokine implicated in infusion reactions in patients. Surprisingly, we did not see a similar IL-6 response in PBMC cultures. Since cells from different donors were tested in these two assays, the donor-to-donor variation is likely to have contributed to the differences. Also, whole blood samples consist of cells other than
monocytes and lymphocytes that can amplify the IL-6 secretion resulting in the high concentration of IL-6 observed in whole blood samples compared to the PBMC cultures.

As part of the activation of the innate immune system, we also monitored the stimulation of TLRs in response to particles in IV solution of Remicade®. TLRs are pattern recognition receptors. They may also recognize certain epitopes presented by protein aggregates. We observed activation of TLR’s 2, 4 and 8. Involvement of TLR’s 2 and 4 in response to protein aggregates has been reported by Joubert et al., but the likely epitopes in our samples for activating TLR 8 still need to be determined. Importantly the activation of TLRs in this study was observed only for samples filtered through the 0.2 µm in-line filter, and was observed even with the particle-free supernatant sample. The cause of this activation by particle-free samples is not known, but could be due to some leachates shed from this specific in-line filter into the protein solution processed through the infusion system. Importantly, however, the results show that even the in-line filter may play a role in activating parts of the immune system during IV infusions.

In this study we did not observe a significant upregulation of various receptors on the surface of DCs. This is in contrast to the observations documented by Rombach et al. They report enhanced activation of DCs and an increased antigen presentation capacity in response to protein particles generated by exposure of model antibody solutions to stress conditions. Perhaps the levels of subvisible particles in samples tested in the current study were not high enough to stimulate activation of DCs. Another explanation could be the donor-to-donor variations. The HLA-class II molecules that are unique to each donor are important for peptide presentation. The monocytes from which the DCs were derived represented a cohort of healthy donors, and testing only 9 donors may not be a large enough sample set to
have tested cells from donors that are sensitive enough to cause upregulation of DC surface receptors for such low level of particles in solution. Moreover, DC maturation by mAb aggregates did not account for complement-related mechanisms. The heat treated serum used as a supplement in the culture media has very low levels of complement proteins to prevent interference with the response elicited specifically due to DC activation. Therefore in comparable *in vivo* conditions, the particles in solution may induce higher DC maturation.

In contrast to results with DC receptor upregulation, the levels of particles in the samples of Remicade®-saline tested were sufficient to induce T cell proliferation. Importantly, the magnitude of T cell proliferation was in a similar range to the observations by Ahmadi *et al* for subvisible particles in solutions of both Rituxan and Herceptin, samples that had much higher particle content than the samples tested in this study.\(^5^4\) Again, overall the results indicate that even trace levels of micro- and/or nanoparticles in infused Remicade® can stimulate activation of an important component of the immune response.

Intravenous administration results in the accumulation of nanoparticles in the spleen, liver and the lungs mostly by the action of the mononuclear phagocytic system also known as the reticuloendothelial system.\(^1^2^9\)-\(^1^3^1\) Of these organs, spleen is the main organ filtering out blood-borne pathogen and antigens.\(^1^3^2\) Researchers have also shown selective accumulation of protein aggregates in the spleen compared to the respective monomers.\(^1^3^1\) Spleen consists of numerous immune cells such as macrophages, dendritic cells, monocytes, T cells and B cells.\(^1^3^2\) In this study we did not test for involvement of other APCs and it is likely that the response in our system was driven by macrophages. Macrophages are as capable as the DCs in driving the Th17 inflammatory response.\(^1^3^3\) This ties in with the high IL-6 secretion observed in whole blood samples since IL-6 is also secreted by macrophages.\(^1^3^4\) Moreover,
both IL-6 and activated macrophages are involved in differentiating naïve T cells into Th17 cells and in localizing T cells at the inflammation site suggesting a staging of Th17 response against particles in Remicade®-saline solution.\textsuperscript{133,134} In line with this mechanism, TLRs are also expressed on T cells\textsuperscript{135} and both TLR-2 and TLR-4 promote Th17 responses.\textsuperscript{136,137} Therefore patients receiving infusion with the 0.2 µm in-line filter may have the stimulated TLRs also contributing to the immunogenic responses in addition to other mechanisms for activating the immune system.

Finally, it is important to consider the clinical implications of our observations of the effects of particles and infusion system on cellular response to Remicade®-saline processed through the IV system. Although Remicade® is prescribed for administration using an in-line filter, the majority of mAb products currently on the market have no recommendations for using an in-line filter during administration.\textsuperscript{82} Thus, the full particle load in the IV solution will be delivered to patients with a high potential of inducing immune responses and/or infusion reactions. Even though inline-filters could reduce the particulate load present in the infused solution, they may also introduce leachates and particles (e.g., polycarbonate, glass) into protein solutions. These introduced components, in turn, could have an immunogenic effect and/or could act as seeds to generate more protein particles in solution.\textsuperscript{138,139}

Much more research is needed to understand and reduce the risk of particle-induced adverse reactions in patients receiving IV infusion of therapeutic proteins. Overall, for each therapeutic protein the underlying causes of infusion reactions and immunogenicity should be investigated thoroughly. As part of this work, it is important that appropriate methods be used to understand sources of subvisible particles in IV solution, the protein formulation and the infusion systems. Importantly, better in-line filter systems are needed that can effectively
reduce concentrations of even relatively small sub-micron particles, without themselves causing problems in patients due to leachates or particle shedding. These research and engineering efforts could ultimately lead to improved outcomes in patients and reduced adverse events due to IV infusion.
The field of therapeutic proteins has tremendous potential to grow considering the unmet need for effective treatment options for numerous chronic medical conditions. An important step in this direction is a better understanding of factors causing immunogenicity against therapeutic proteins and in turn reducing the incidences of such responses so that fewer patients turn non-responsive to therapy. Protein aggregates that may form from physicochemical instability of the protein product represent important product quality attributes that can provoke immunogenicity. Current thesis work focused on aggregates in the subvisible size range (60 nm – 100 µm) and attempted to understand their role as critical attributes in protein stability and immunogenicity.

This thesis work demonstrated that subvisible particles can result from exposure to most pharmaceutically relevant stress conditions. We have shown that nanoparticles are the pre-cursors to microparticle formation in IVIG solutions particularly for the interface induced stress conditions like freeze-thawing and agitation. Other studies have also documented protein aggregation at the interface during surface-induced stresses but our study shows that protein molecules in the bulk solution also contribute to particle formation. Importantly, the nanoparticles already present in the bulk solution accelerated microparticle formation upon exposure to interface induced stresses. Our observation – the mechanism of microparticle formation is via both agglomeration of nanoparticles and step-wise addition of protein monomers to nanoparticles -- is an important conclusion towards understanding the mechanism of particle formation. While developing therapeutic proteins, this observation can help in designing formulation strategies to counteract the underlying mechanisms for particle
formation. This study also shows the impact of multiple stress conditions such as freeze-thawing and agitation on particle formation. This directly translates to production processes where a bulk drug product may be exposed to multiple freeze-thaw cycles and attention must be given to the resultant particle formation that can seed further particle formation upon exposure to downstream processes such as filling and transport of the product. Moreover, this work has shown further potential for flow cytometry as a particle counting tool. Few studies have used this instrument for counting particles\textsuperscript{140-142} but we have combined counting and fluorescence capability of the instrument to investigate the mechanism for particle formation.

Particle formation can be a complex process that can involve numerous mechanisms of formation.\textsuperscript{47,48,61} In our study, using IVIG which is IgG molecules from pooled plasma from human donors added further complexity due to the heterogeneous nature of the protein solution. In addition to the lot-to-lot variability, we also observed variability within the same vial with protein molecules that had different propensities to aggregate/particulate. This made investigating the mechanism of particle formation extremely challenging. We had to introduce additional step of agitation to consume the aggregate prone species in IVIG solution and then process the solution through an ultracentrifugation step to remove these species before conducting our experiments.

Also since we were investigating the transition of protein particles from nanometer size range to micron size range, it would have been extremely helpful if the analytical techniques were available that covered the full spectrum of the size range. Analytical techniques with overlapping size ranges are available, but the results generated from each of these instruments could not be corroborated either due to different detection principles or
different limit of detections. With this work, we hope to further the interest of the field in developing robust analytical techniques that can support analysis through the entire size range that is critical for stability of protein formulations.

Furthermore, we investigated particle formation during delivery to patients via IV infusion. For the first time, particulate levels in IV saline and in therapeutic proteins in saline solutions were characterized replicating the IV infusion procedure followed in the clinic. An important observation during this study was that IV saline is one of the major contributors of subvisible particles in protein-saline solutions. With this work we would like to impress upon the field the imminent need to generate cleaner saline solutions.

In addition, we have shown that various components of the infusion system are potential surfaces for the protein to adsorb onto and aggregate. Further, a saline flush after processing the protein-saline solution through infusion system resulted in desorption of proteinaceous particles into solutions that will be delivered to patients. During conversations with the nurses at the University of Colorado Outpatient Infusion Center, we found that such a saline flush is routinely used in the clinic to prevent wastage of the product remaining in the IV lines. Furthermore, practices like using a repetitor pump to aliquot smaller volumes of IV fluids or protein solutions and the pneumatic tubing used to deliver the prepared solutions of therapeutic products to the patients are detrimental to the stability of the drug product. We hope that this work brings to attention that certain procedures conducted in the clinic may not be best practices and wish that collaborations such as this study are a step towards improved communication between professionals involved through all stages of a product’s life cycle that can help bring better therapeutic products to the patients.
During background research for this study we found that a large number of monoclonal antibody products currently on the market do not have recommendations for using an in-line filter during administration. This means that the entire particulate load observed in one bag of protein-saline solution is delivered to patients. These package inserts are the only source of information for professionals preparing and administering the protein product and hence care must be taken to include all important instructions in the package inserts. Furthermore, our results indicate that on occasions when in-line filters are used during IV infusion, they may contribute to larger sized particles since the tube downstream from the filter membrane is a potential surface for the protein to adsorb onto. We also observed particles of polycarbonate that probably sloughed off from the housing of the filter membranes. These in addition to the high concentration of nanoparticles that were too small for the membrane pore size to filter out highlight the need for developing better in-line filters that can remove particles without contributing more into protein-saline solutions.

Lastly we investigated the activation of various pathways of the innate and the adaptive immune system due to subvisible particles in therapeutic protein solutions. Recent studies using human cells in *in vitro* assays have shown their sensitivity to detect immune responses even against a small amount (~3% of the total mass of the protein in solution) of particle content in protein solutions. In the current work, we have shown the contribution of even lower concentrations (clinically relevant concentrations) of subvisible particles in activating various stages of the immune system. Along with the protein particles we have reported potential contribution by extrinsic factors such as leachates from in-line in eliciting the activation of both innate and adaptive immune response.
Remicade®, is a chimeric monoclonal antibody and hence has an inherent sequence related immunogenicity. Studies such as current work help in our understanding of the factors that can contribute to the current rate of incidences of immunogenicity. But for a robust determination of the contribution of particles in immunogenicity it would be useful to conduct such studies with a therapeutic protein that is fully human and hence has a relatively low incidence of immunogenicity.

Overall this thesis work is able to show that subvisible particles are indeed an important product quality attribute affecting not only the stability of protein formulations but also contributing to immunogenic responses. They can form at any step of the product manufacturing process up until delivery to patients and hence every processing stage needs to be considered rigorously for its role in contributing to subvisible particles in protein solutions. From a formulation perspective, this study suggests taking into consideration the mechanism of particle formation at the molecular level, protein’s sensitivity to particular stress conditions, various surfaces the protein may interact with and extrinsic sources of particles such as IV saline and in-line filters when designing robust formulations. These would have implications not only on product quality but also on patient safety.

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