



Review

Immunogenicity of protein aggregates—Concerns and realities

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ABSTRACT

Protein aggregation is one of the key challenges in the development of protein biotherapeutics. It is a critical product quality issue as well as a potential safety concern due to the increased immunogenicity potential of these aggregates. The overwhelming safety concern has led to an increased development effort and regulatory scrutiny in recent years. The main purposes of this review are to examine the literature data on the relationship between protein aggregates and immunogenicity, to highlight the linkage and existing inconsistencies/uncertainties, and to propose directions for future investigations/development.

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1. Introduction

Advances in biotechnology have brought to the market more than 174 biotherapeutic drug products in the US and EU (Giezen et al., 2008). This achievement is the result of years of painstaking research and development addressing myriad discovery, quality, preclinical, and clinical challenges. One of the key development challenges that has received increasing scrutiny of late, is the aggregation propensity of proteins, not typically seen for small-molecule drugs. Protein aggregation has been under extensive investigation addressing a variety of aspects and issues such as aggregation mechanisms, influencing factors, formulation/process control, and related analytical methodologies (Chi et al., 2003; Roberts, 2003;

Mahler et al., 2009; Philo and Arakawa, 2009; Wang et al., 2010; den Engelsman et al., 2011; Zolls et al., 2012).

In addition to the quality-related consequences of protein aggregation such as possible loss of protein activity and undesirable aesthetics of drug product, soluble protein aggregates have been shown to have significant cytotoxic effects for a variety of proteins (Curatolo et al., 1997; Kaye et al., 2003; Poon et al., 2009). More importantly, protein aggregation was considered “the most important”, recognized structural change known to increase the immune response in protein products (Hermeling et al., 2004). Reduction of protein aggregation has been claimed to “reduce immunogenicity” (Sauerborn et al., 2010). Immunogenicity here refers to formation of serum anti-drug antibodies (ADAs) specific for the protein of interest. Unwanted immunogenicity of a protein product potentially makes the product less or in-effective. In worst-case scenarios, formation of ADAs may pose serious safety concerns, and can even be life-threatening as in the cases of pure red cell aplasia (PRCA) after administration of Eprex. This justifies the requirement of

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evaluating protein product-related immunogenicity as an integral part of product clinical trials (Shankar et al., 2006; Kaliyaperumal and Jing, 2009). Immunogenicity of protein aggregates has now become a topic of intensive research (Hermeling et al., 2004; Rosenberg, 2006; Buttel et al., 2011).

In 2009, a group of academic scientists and FDA representatives published a commentary, raising concern about the potential role of protein subvisible particles in product immunogenicity, specifically those in the size range of 2–10 μm and the lack of regulatory guidelines on these particles (Carpenter et al., 2009). This article elevated the general concern for protein aggregation/particulate formation among regulatory agencies, healthcare professionals, and the pharmaceutical industry as well. The following year saw a responding commentary on these issues by a group of “industrial” scientists (Singh et al., 2010). They generally agree to (1) the need for additional work to understand the immunogenicity impact of these particles and (2) the need to develop methods/instruments for monitoring particles in the range of 0.1–10 μm . They also pointed out (1) the immunogenicity potential of protein aggregates is a debatable subject and (2) monitoring particles of <10 μm is not a suitable test for release, stability and comparability at this time, although monitoring these particles could facilitate product development.

Notwithstanding the ongoing debate, the US FDA has been acting on these concerns and significantly increased their scrutiny and requirement on analysis of proteinaceous particles. This is exemplified by the following recent FDA correspondence in connection with both Investigational New Drug and Biological Licensing Applications.

“... USP <788> testing results are critical to mitigate the risk associated with occlusion of small blood vessels and small sub-visible particles may pose an immunogenicity risk. Provide USP <788> particulate testing data for in-use stability studies and an analysis of particulates between 2 and 10 microns.”

“...in addition to measuring particulates that are $\geq 10 \mu\text{m}$ in size, subvisible particulates in the 2–10 μm range should also be characterized and quantified using technique(s) that can accurately estimate the amount of subvisible protein particulates present. Sub-visible particulates in the 0.1–1 μm range should be qualitatively assessed.”

“...additionally characterize the types and amounts of sub-visible particles (2–10 μm) in the drug product under stress conditions, at release, and throughout the shelf-life, and also propose an appropriate control strategy based on the risk to product quality.”

It is clear that significant efforts are urgently needed to address the increasing concern about the potential immunogenicity enhancement of protein aggregates. The main purposes of this review is to examine the literature data on the relationship between protein aggregates and their immunogenicity, to highlight the uncertainties, and to propose future directions for further investigations/development.

2. Immunogenicity of protein aggregates

Protein aggregates can be defined as any physically-associated or chemically-linked non-native species of two or more protein monomers. They can generally be classified into two major categories—soluble (usually measurable by SEC) and insoluble (particulates). Soluble aggregates cover the size range of roughly 1 to 100 nm, and protein particulates cover subvisible (~ 0.1 –100 μm) and visible ($>100 \mu\text{m}$) ranges. A more detailed classification of protein aggregates has been proposed (Narhi et al., 2011).

Do soluble protein aggregates enhance the immunogenicity potential of a protein product? It is generally believed that a potential linkage exists between protein aggregation and enhanced product immunogenicity (Porter, 2001; Hermeling et al., 2004; Rosenberg, 2006; Baker et al., 2010; Richard and Prang, 2010). Early evidence for enhanced immunogenicity of protein aggregates was reported in 1960s, when aggregated human γ -globulin was found to be more immunogenic than the aggregate-free form in mice in a dose-dependent manner (Gamble, 1966). Around the same time, clinical evidence was reported based on simple skin test of aggregated vs non-aggregated human γ -globulin and human serum albumin (Christian, 1960; Ring et al., 1979). Removal of aggregates in human γ -globulin clearly reduced its clinical immunogenicity (Weksler et al., 1970). Over the years, many other proteins have been reported to have enhanced immunogenicity upon aggregation under a variety of storage or stressed conditions. Table 1 lists these proteins, including vaccine examples. Dose-dependent immunogenicity of protein aggregates has also been reported for a few proteins. Based on these examples, it appears undeniable that a linkage exists between protein aggregation and enhanced product immunogenicity.

A widely-accepted theory for immunogenicity enhancement by protein aggregates is the additional T cell-independent activation of B-cells through cross-linking mechanisms by repetitive and ordered structures (conformational) in protein aggregates in native forms (Baker and Carr, 2010; Sauerborn et al., 2010; Defrance et al., 2011). These aggregates may resemble “immunons”, spatially continuous clusters of haptens on a linear polymer ($>100 \text{ kD}$ in size with >10 –20 haptens spaced in 100 Angstroms) for direct B-cell activation (receptor clustering) proposed by Dintzis (Dintzis et al., 1976). Other studies further solidified the immunon concept and demonstrated a minimum number of receptor sites (~ 10), that needs to be bound to a single stimulatory ligand for activation of a B-cell (Sulzer and Perelson, 1997).

Another obvious mechanism for the enhanced immunogenicity of protein aggregates is aggregation-induced structural changes toward more foreignness, induced simply by physical intermolecular interactions, chemical linkages, or other chemical modifications. Many chemical modifications such as methionine oxidation or deamidation have been found in protein aggregates formed under simple physical stress conditions (Luo et al., 2011). Thus, when chemical degradations lead to protein aggregation and enhanced immunogenicity, the enhancement can be attributable to several possibilities - degradation-induced structural change, aggregation-induced structural change, increased structural repetitiveness, or a combination of these. Understanding the true cause of the immunogenicity enhancement may facilitate design or stabilization of protein products for less immunogenicity. For example, administration of heavily aggregated recombinant human interferon $\alpha 2\text{b}$ (rhIFN- $\alpha 2\text{b}$) (aged and oxidized) induced significant formation of antibodies relative to fresh samples in both wild-type and transgenic mice (Hermeling et al., 2006). Samples containing progressively greater fractions of aggregated (oxidized) protein generated proportionally more antibodies in transgenic mice. Since administration of H_2O_2 -induced oxidation products of recombinant human interferon $\alpha 2\text{b}$ (rhIFN- $\alpha 2\text{b}$) induced higher IgG titers than native forms in wild-type mice (Hermeling et al., 2005), could the apparent high immunogenicity of aggregated rhIFN- $\alpha 2\text{b}$ samples be attributable partially to chemical degradation-induced structural changes? In the evaluation of a closely related protein, it was observed that a higher proportion of multiple sclerosis (MS) patients developed antibodies after receiving recombinant human IFN- $\beta 1\text{b}$ than IFN- $\beta 1\text{a}$ (Kivisakk et al., 2000). It is reasonable that the difference in antibody response was attributed to the apparent difference - higher amount of aggregates in IFN- $\beta 1\text{b}$ samples and the structural difference (van Beers et al., 2010a). However, recent

Table 1
Examples of protein aggregates linked to enhanced immunogenicity.

Protein	Aggregate level and type	Dose and administration	Test model	References
Soluble aggregates				
Epoetin α	Low amount of dimers and other aggregates induced by tungsten	25 IU/kg 3 times per week or 75 IU/kg weekly; SC	Anemic patients	(Seidl et al., 2011)
Human γ -globulin	Variable levels of aggregates by heating at 63 °C for 15'	5 mg; IV	Rabbits	(Biro and Garcia, 1965)
Human γ -globulin	Variable levels of aggregates by heating at 63 °C for 30'	100 μ g; IP	A/J mice; IP	(Gamble, 1966)
Human growth hormone	Variable levels of aggregates	0.1–2U 3 times per week; SC(?)	Children	(Moore and Leppert, 1980)
Human interferon- α 2	50% dimer and very small amount of oligomers	0.3 μ g weekly for 5 weeks; IP	Balb/C and transgenic mice	(Braun et al., 1997)
Human interferon- α 2b	Variable levels of oxidized aggregates	10 μ g repeated dose; SC	Wild-type and transgenic mice	(Hermeling et al., 2006)
Human interferon- β 1a	~80% oligomeric or large aggregates formed by metal catalysis	5 μ g repeated dose; IP	Transgenic mice	(van Beers et al., 2011)
Human interferon- β 1a	Small amount of mainly non-covalent aggregates	5 μ g repeated dose; IP	Transgenic mice	(van Beers et al., 2010a)
IFN- α 2b-HSA fusion protein	20% to 42% dimers and aggregates by shaking	100 μ g twice per week for 4 weeks; SC	Mice	(Zhao et al., 2009)
Insoluble aggregates or mixtures				
Bovine γ -globulin	Insoluble aggregates by centrifugation	2 mg adsorbed on Bentonite; IP	CBA mice	(Claman, 1963)
Murine growth hormone	1.2% subvisible particles (based MFI) vs control	2 μ g repeated dose; SC	Mice	(Fradkin et al., 2011a)
Human growth hormone (product A & B)	5% soluble and 72% insoluble (A) and 31% soluble through F/T	10 μ g weekly for 2 weeks; SC	Naive and primed mice	(Fradkin et al., 2009)
Human interferon- α 2b	17% large aggregates and 10% insoluble formed by metal catalysis	10 μ g repeated dose; IP	Wild-type & transgenic mice	(Hermeling et al., 2005)
Human interferon- β 1a	More aggregates as monitored by light scattering	0.25 μ g (SC) or 0.5 μ g (IN); 3 days/week for 4 or 5 weeks	C57BL/6 mice	(Rifkin et al., 2011)
Protein aggregates as vaccines				
Bacterial needle protein MxiH ⁴⁵	Multimers, induced with a full length	10 μ g on days 0, 14, 28; IM	Balb/c mice	(Barrett et al., 2010)
Hemagglutinin	Trimers, induced via trimeric motif	3 μ g on days 0 and 14; SC	Balb/c mice	(Weldon et al., 2010)
Hepatitis B peptide antigen	Multimers, derivatized with dipalmityl-lysine	200 μ g twice with Freund's adjuvant; SC	Rabbits	(Hopp, 1984)
HIV Tat101 protein derivative	Disulfide bonded dimers	5 μ g twice; SC	Balb/c mice	(Kittiworakarn et al., 2006)
HIV envelope protein GP120	Trimers, induced via a trimeric motif	7–9 μ g 3 times with 1X Ribi adjuvant; SC	Balb/c mice	(Yang et al., 2001)
Horse heart cytochrome c	Aggregates induced by glutaraldehyde	5 mg per animal with Freund's adjuvant; IV	Rabbits	(Reichlin et al., 1970)
Human muscle creatine kinase	Aggregates induced by glutaraldehyde	150 μ g per animal with Freund's adjuvant; IP	Balb/c mice	(Man et al., 1989)
γ KEI polypeptide	Oligomerized by PEGylation	100 μ g with a 50 μ g boost at week 4; SC	C57BL/6 mice	(Rudra et al., 2010)

analysis of serum samples from 2,711 MS patients for formation of neutralizing antibodies revealed that the formation frequency was greatest (35%) for Rebif (SC IFN- β 1a; 44 μ g thrice weekly), least (7.5%) for Avonex (IM IFN- β 1a; 30 μ g once weekly), and in between for Betaseron (SC IFN- β 1b; 250 μ g every other day) and Rebif (22 μ g thrice weekly) (Grossberg et al., 2011). With variation in dose and route of administration, these results make it difficult to attribute the difference in immunogenicity to the difference in aggregate level and/or the structural differences (lack of the first Met, Cys-17 mutated to Ser, and lack of glycosylation in IFN- β 1b).

Many protein product-related factors could influence the immunogenicity of a protein product, including impurities, contaminants, and formulation excipients (Singh, 2011). Presence of any potential immunogenic or tolerogenic components in testing samples would complicate interpretation of the enhanced immunogenicity of protein aggregates. A recent study showed a positive correlation of immunogenicity with the amount of aggregates in IFN- β in mice (Rifkin et al., 2011). The complication of this study was the use of dodecylmaltoside, an alkylsaccharide surfactant. Was the reduction in immunogenicity due to a lower

degree of aggregation or any possible “protective” effect of the alkylsaccharide surfactant? It has been known that surfactants can bind to proteins (Randolph and Jones, 2002; Hermeling et al., 2003; Chou et al., 2005; Villalobos et al., 2005). If this surfactant binds to the protein, could the binding partially shield any antigenic epitopes on the protein, leading to less immunogenicity, somewhat like the concept of immunogenicity reduction through protein glycosylation (Fagnani et al., 1990) or pegylation (Basu et al., 2006)? Another relevant clinical observation is the high rate of antibody formation associated with administration of aggregate-containing recombinant human IL-2 (Prummer, 1997). In addition to the structural modifications (C125S mutation and lack of Ala1 and glycosylation), the product (Proleukin) contains a significant amount (0.18 mg per vial) of sodium dodecyl sulfate, an ionic surfactant to curb protein aggregation. Could this ionic surfactant contribute to the immunogenicity response, as documented for other similar compounds (Katz et al., 2000; Evans et al., 2004; Mueller et al., 2009)?

Metal ions are common process-related impurities and their presence in protein products can alter the aggregation and

immunogenicity behavior (Zhou et al., 2011). It has been shown that metal-catalysis-induced aggregation of rhIFN- α 2b (Hermeling et al., 2005) and rhIFN- β 1a (van Beers et al., 2011) generate significant immunogenicity in transgenic mice. Tungsten pin extract was shown to induce formation of low level of disulfide-bonded protein dimers and aggregate, which were proposed to be responsible for the increased immunogenicity of epoetin α in clinical trials (Seidl et al., 2011). Since the reference product Eprex, with almost 50% batches containing comparable or even higher level of dimers and aggregates than epoetin α , did not generate any neutralizing antibodies in all the subjects, the type rather than level of aggregates appears responsible for the breaking of immune tolerance. Tungsten-induced aggregates may possess new epitopes, as tungsten alters the conformational structure of the protein (Seidl et al., 2011).

Protein aggregates, generated through different storage or stressed conditions, may induce different degrees of immunogenicity. While aggregated IFN- β 1a sample (~10% more dimers or oligomers) through H₂O₂-induced oxidation was more immunogenic than the native protein, GdnHCl treatment-induced aggregates (70% dimers or oligomers) were not in transgenic mice (van Beers et al., 2011). Administration of aggregated rhIFN- α 2b samples through glutaraldehyde or heat (boiling) treatment actually reduced significantly the IgG titers relative to the native forms in wild-type mice (Hermeling et al., 2005). Similarly, aggregated rFVIII (with a size equivalent to at least 6 rFVIII molecules), induced by heat treatment (80 °C for 2 min) is less immunogenic than native rFVIII in hemophilia A mice, albeit the aggregated rFVIII appeared to act as a distinct antigen (Purohit et al., 2006).

The different immunogenic behaviors of protein aggregates suggest one or more of the following explanations—(1) enhanced immunogenicity by oxidation-induced structural change; (2) enhanced immunogenicity by formation of repetitive structures in oxidation-derived aggregates; and (3) reduced immunogenicity by alteration or even loss of immunogenic epitopes in protein aggregates after different treatments. Similarly, in the evaluation of immunogenicity of stressed growth hormone (GH) in mice, stressed samples of one GH product containing either 31% soluble aggregates (induced by freeze/thaw) or 69% soluble aggregates (induced by agitation) did not enhance the immunogenicity of the original product (containing non-detectable level of aggregates) in terms of anti-GH titers in both neonatally primed and naive mice (Fradkin et al., 2009). The failure of GH aggregates to initiate an enhanced immunogenicity could also possibly be due to non-optimum spacing of epitopes in GH aggregates as immunons, or an anergic behavior of GH aggregates, as immunon-like polymers could actually inhibit the B-cell activation, if binding to B-cell receptors is non-stimulatory (Sulzer and Perelson, 1997).

All the above examples demonstrate a reasonable correlation of enhanced immunogenicity with protein aggregation but not with structural features in protein aggregates, specifically responsible for the enhanced immunogenicity. Such a correlation is difficult to establish given the complicated mechanisms of an immunogenic event and its many influencing factors. Protein aggregates generated under stressed conditions may or may not represent those seen in a product after long-term storage, and may or may not be more (or less) immunogenic. Therefore, immunogenicity data on stressed protein samples, presumably strongly protein dependent, need to be interpreted with caution.

It would be ideal to identify the structural features in protein and/or protein aggregates, that are responsible for initiation of immunogenicity. This would allow possible reduction of immunogenicity (deimmunization) of protein products through mutation of the key immunogenic site and/or insertion of tolerogenic epitopes (Scott and De Groot, 2010; Kumar et al., 2011). It, however, has been difficult to link clearly the potential immunogenicity of a

pure protein to a particular type of sequence/structure with *in-silico* methods (Porter, 2001; Flower, 2009; Brinks et al., 2011; Singh, 2011). An unusual epitope on one protein may not be recognizable on another (Porter, 2001). Nevertheless, presence of T cell epitopes in proteins has been considered a pre-requisite for immunogenicity and these potential immunogenic sites can be screened and modified (Bryson et al., 2010; Scott and De Groot, 2010). The measured immunogenicity potential by *ex vivo* EpiScreen™ showed a reasonable correlation with the clinical immunogenicity of 16 commercial protein products (Barker, 2010). Measurement of the binding affinity of peptides/proteins to HLA has been used to predict approximately their relative immunogenicity (Cohen et al., 2010). Recently, scientists have attempted to examine the coincidence between the aggregation-prone regions and the T-cell immune epitopes and proposed a potential linkage between the repetitiveness of cross β motif structure in protein aggregates and direct B-cell activation (Kumar et al., 2011). Much more analysis and experimental work are still needed to establish a clear relationship.

3. Immunogenicity of protein particulates

Would insoluble protein aggregates, i.e. proteinaceous particulates, have higher immunogenicity potential than native proteins or smaller aggregates? Some believe that particulate aggregates are more immunogenic than soluble aggregates, and smaller particulates are more immunogenic than larger ones (Singh, 2011). As shown in Table 1, protein particulates or mixtures of protein particulates and soluble aggregates can be more immunogenic than monomeric proteins. Particulates can enhance immunogenicity through several mechanisms, including enhancement of antigen uptake by dendritic cells, stimulation of both B and T lymphocytes, facilitation of maturation, activation, or proliferation of dendritic cells, and prolonged release of protein antigens (Seferian and Martinez, 2000; Wang et al., 2008; Aline et al., 2009; Prego et al., 2010; Jones et al., 2011).

As early as 1963, Claman clearly demonstrated a higher degree of antibody formation to sedimented bovine γ -globulin than soluble protein with Bentonite in mice (Claman, 1963). Latest studies demonstrated that particulates of recombinant murine growth hormone (mGH) even at levels below the detection limits of SEC-HPLC (<1%), induced immune responses in mice (Fradkin et al., 2011a). On the other hand, some results from a few studies on the immunogenicity of protein particulates have not been consistent and data interpretation seems difficult. For example, it was shown that a freeze-thawed commercial GH product (5% soluble + 72% particulates of <9 μ m) induced significantly higher anti-GH titers than the unprocessed GH product containing only 2% aggregates in naive adult mice, but interestingly, not in neonatally primed mice (Fradkin et al., 2009). In contrast, the agitated commercial GH (12% soluble + 42% particulates of <9 μ m) did not change the immunogenicity of unprocessed samples in naive mice, and in fact, decreased the immunogenicity of the unprocessed GH product in neonatally primed mice. In addition, none of the processed and unprocessed GH induced any detectable immunogenicity in GH transgenic mice. The negative results challenges the capacity of protein particulates in breaking the immune tolerance (Fradkin et al., 2009). The variety of responses has been explained based on the structure of epitope exposed in the aggregates, the amount of insoluble aggregates, the need for adjuvanticity in neonatal mice as well as in transgenic mice. In a recent study, agitated mGH sample containing about 50% of protein particulates significantly increased the IgG2a level, but the high-pressure treated mGH (containing only 0.08% of protein particles) generated a similar level of IgG2a. Another observation in this study is the uncertainty of the amount of soluble aggregates, hence their potential contribution

to immunogenicity, as the SEC method could not detect any in all samples, including those containing a substantial level of protein particulates. It is not uncommon that dissociation or loss of protein aggregates takes place due to sample dilution in mobile phase and/or column binding during SEC analysis (Roberts et al., 2003; Ejima et al., 2005).

Transgenic animal models are more relevant than wild-type animals in testing the immunogenicity of therapeutic proteins (Hermeling et al., 2004; Brinks et al., 2011). With this model, antibody formation was compared upon administration of oxidized and aged rhIFN- α 2b samples at different pH's, and the antibody titer inversely correlated roughly with the unrecoverable amount of protein, presumably particulates, in the samples (Hermeling et al., 2006). In another example, administration of stressed IFN- α 2b containing a large amount (>50% visually) of mainly covalent aggregates in transgenic mice is no more immunogenic than the sample containing a smaller amount of mainly non-covalent aggregates (van Beers et al., 2010b).

Although a couple of mechanisms have been proposed for promoting immunogenicity of particulates, the immunogenicity of protein particulates is not well understood at this time. While protein particulates may possess repetitive structures and structural changes, most native antigenic sites might be altered or completely covered in protein particulates. These particulates need to be processed/digested for subsequent presentation by MHC II molecules on the surfaces of APCs. Since more conformationally stable proteins are less immunogenic due to slow unfolding or digestion (Maas et al., 2007; Ohkuri et al., 2010), protein particulates would be even less likely digested intracellularly due to requirement of particulate dissolution. Dissolution of particulates takes time and the measured immunogenicity within the experimental time scale may not be long enough to pick up an immune signal, which could explain some of the negative results (Naim and van Oss, 1992). The immunogenicity of particulates vs soluble aggregates or monomeric proteins needs further investigations.

Overall, investigations have been very limited on the immunogenicity of proteinaceous particulates. Lack of such studies is partly due to the challenges in generating, separating, and quantifying various kinds of proteinaceous particulates. The mechanisms of protein particulate formation and the associated amount, size, type, morphology, and reversibility can be significantly different depending on the experimental conditions (Joubert et al., 2011). Controlled formation of protein particulates for a fixed amount and in a specific size range is not easy to achieve simply based on the protein's known aggregation behavior (He et al., 2010). The amount of proteins in particulates may have to be estimated roughly based on the sizing results with certain assumptions on the shape and density of particulates (Fradkin et al., 2011b).

4. Lessons from vaccine development

Many protein vaccines have been developed and commercialized. An effective vaccine has to be able to generate sufficient immunogenicity, which is measured in two aspects—humoral immunity (antibody formation) and cellular immunity. The lessons learned during vaccine development in linking protein antigen aggregation and humoral immunity can help understand the role of protein aggregates in immunogenicity of protein products and vice versa. Vaccines are purposely designed to induce an immune response, and are often dosed along with adjuvants once or a few times, while protein therapeutics are generally dosed many times or chronically with immunogenicity being a great concern.

Structural modifications in a protein antigen can have a strong influence on immunogenicity. For example, K58I mutation in the

hemagglutinin (HA) induced a superior systemic and local antibody response after intranasal immunization due to improved virus uptake by the nasal epithelial cells in mice (Krenn et al., 2011). Addition of aldehyde groups into several monomeric model proteins enhanced by several orders of magnitude the immunogenicity in mice (Allison and Fearon, 2000). This result may explain why presence of 0.2% formaldehyde in recombinant protective antigen (rPA) vaccine induced significantly higher anti-rPA IgG titers than the control vaccine (Little et al., 2007). These examples confirm the potential enhancement of immunogenicity through chemical degradations in protein products, which would contribute to the enhanced immunogenicity of chemically-modified protein aggregates.

As with protein products, formulation components, impurities or contaminants can strongly influence the immunogenicity of a vaccine product. For example, several studies have demonstrated significant enhancement of vaccine immunogenicity in the presence of Cu^{2+} (Mustafaev and Norimov, 1990; Mustafaev et al., 1996; Basalp et al., 2002). Metal ions are able to facilitate complex formation among the same or different protein antigens with increased stability by acting as “fasteners” between macromolecules (Mustafaev et al., 1996; Basalp et al., 2002). This binding and stabilizing activity could make a decapeptide or even heptapeptide immunogenic (Yang et al., 1993). As discussed before, metal ions have been or proposed to be a source of aggregate-induced immunogenicity in protein products (Hermeling et al., 2005; Seidl et al., 2011). An obvious interpretation for the enhanced immunogenicity is the facilitation of metal in forming a metal-protein complex and/or stabilizing conformational epitopes in the protein aggregates.

Does a protein antigen in a vaccine product have an enhanced immunogenicity (antibody formation) upon oligomerization or aggregation? As shown in Table 1, many oligomerized protein antigens are more immunogenic than their monomeric forms with or without adjuvants. Even disulfide-bonded dimers of HIV Tat101 derivatives induced antibody response higher than the monomer without an adjuvant in mice (Kittiworakarn et al., 2006). Some of these results are easily understandable as oligomerized antigens are structurally more like the surfaces of real foreign microorganisms—trimeric structures on viruses (Lu et al., 1995; Rao et al., 1995; Weldon et al., 2010), and polymeric “needle” on bacteria (Barrett et al., 2010). Some of these protein antigens were oligomerized or polymerized by using a cross-linking agent or a polymer-inducing chain/motif. It is very possible that these added components in the antigen would have some positive influence. It has been shown that antigen immunogenicity can be significantly impacted by attachment of a fatty acid (Oda et al., 2004), a his tag, an often-used affinity tag for facilitating protein purification (Khan et al., 2011), or a specific linker (Buskas et al., 2004; Lawatscheck et al., 2007; Chia et al., 2010). The altered immunogenicity could be due to the attachment-induced alteration in the structure, stability, flexibility, or aggregation tendency of the original antigen. An obvious explanation for the enhanced immunogenicity of antigen or protein aggregates is their enhanced repetitive structures, like immunons. Antigens or haptens (10–20) on the surface of pathogens (or aggregates in this case) in an organized and repetitive form can activate B cells by cross-linking B-cell receptors in a multivalent fashion (Vos et al., 2000). For example, the M2-specific antibody levels were found to be proportional to the epitope density (1, 2, 4, 8 and 16 copies) on the influenza virus both in mice and rabbits (Liu et al., 2004) and higher antibody titers were observed for tandem multi-peptide antigen, derived from human papillomavirus (HPV), relative to the mono-peptide form in mice (Rubio et al., 2009).

In other cases, aggregation of a protein antigen may not enhance, or even reduce the immunogenicity. Ovalbumin is a common

model protein antigen for screening adjuvants. Presence of aggregated ovalbumin failed to increase the level of anti-OVA IgG relative to non-aggregated protein in mice (Allison and Fearon, 2000). Administration of soluble non-covalent and disulfide-linked ovalbumin aggregates (adjuvanted with $\text{Al}(\text{OH})_3$), induced by heat denaturation, was less immunogenic than the native protein with the threshold immunogenic dose 100 times higher in mice (Koch et al., 1996). The reduction in immunogenicity was attributed to a lower epitope density in denatured ovalbumin. This is a reasonable interpretation, as antigen aggregation can cover some antigenic sites (Jain and Roy, 2011). Similarly, Valiente-Gabioud (Valiente-Gabioud et al., 2011) examined the number of repeats (repetitiveness) of Flagellar Repetitive Antigen (FRA) on the immunogenicity of the antigen and increasing the number of repeats (1, 2, 3, and 4) did not increase the antibody response in mice. As mentioned above, the negative effect could be due to non-optimized spacing of epitopes, and/or potentially non-stimulatory binding to B-cell receptors (Sulzer and Perelson, 1997).

Do protein antigens have an enhanced immunogenicity upon particulate formation? Clearly, if protein antigens could self assemble into virus-like particles, significant immunogenicity can occur without an additional adjuvant (Jegerlehner et al., 2002) (Warfield et al., 2007; Zhang et al., 2011). Multimeric virus-like particles (VLPs) of the virus surface proteins have been shown to induce a higher antibody titer than the monomeric proteins in mice (Schirmbeck et al., 1994; Denis et al., 2007), although exceptions do exist (Valdes et al., 2009). Further aggregation of VLPs has resulted in either no change (Babiuk et al., 2004; Caparros-Wanderley et al., 2004; Jones et al., 2011), or a reduction in antibody formation (Schirmbeck et al., 1995). Again, one of the possible reasons is the partial coverage of the antigenic sites.

What happens if protein form particulates other than VLPs? A study, claimed to be the first to evaluate immunogenicity of single-component protein particulates, demonstrated superior immunogenicity (high anti-OVA titers) of particulate ovalbumin (prepared by dense carbon dioxide) relative to solubilized ovalbumin at a dose of $10 \mu\text{g}$ in mice (White et al., 2008). It was noted that the soluble ovalbumin was prepared by dissolving the particulate ovalbumin in NaOH solutions, followed by pH neutralization. It is known that proteins can be rapidly degraded in acidic or basic solutions, and the soluble ovalbumin could have been degraded in the preparation process, resulting in a loss of epitopes for reduced immunogenicity, as the integrity of the soluble ovalbumin was never confirmed.

Another argument for enhancing immunogenicity by protein particulates is their non-specific adjuvantation effect (Fradkin et al., 2009). The role of non-specific polymeric or lipid-based particulates in enhancing vaccine immunogenicity has been well documented with mechanisms of action mimicking those of aluminum salt adjuvants (Wang and Singh, 2011). Particulates of a variety of polymers have been shown to be effective adjuvants (Wang and Singh, 2011). Their relative effects depend not only on the size and type of polymers (Fifis et al., 2004; Moschos et al., 2005; Wendorf et al., 2008; Oyewumi et al., 2010), but also on many other factors, such as antigen adsorption capacity, strength, stability, or efficiency of particulate uptake by competent immune cells (Morefield et al., 2005; Thomas et al., 2010). In most cases, antigens need to be encapsulated in or attached to these polymers either physically or chemically for immunogenicity enhancement (Slutter et al., 2010).

Base on the information, one would think intuitively that protein particulates, formed through physical or chemical linkages, could act as an adjuvant in a similar manner, and enhance the immunogenicity of a protein product (Xiang et al., 2006). One could also argue that protein particulates are covered with protein molecules, and the surface protein molecules still have native or close to native

structures for enhancing immunogenicity. Two apparent differences, however, are noted here. The first one is the rigidity of these particulates. While the polymeric particulates as vaccine adjuvants are generally rigid in solution, most protein particulates are likely to be amorphous, more flexible and pliant except fibrils. The second difference is the quantity of these particulates. While the amount of polymeric particulates, used as vaccine adjuvants, is relatively large, up to mg for aluminum salts, protein products would have, if any, an insignificant amount of protein particulates except for a mAb product, where a non-detectable fraction of protein particulates, 0.022% by routine SEC, would be equivalent to about $20 \mu\text{g}/\text{mL}$ at a high protein concentration of $90 \text{ mg}/\text{mL}$ (Wuchner et al., 2010). It is obvious that additional data are still needed to confirm whether a small amount of loose protein particulates or protein fibrils would be able to make a significant difference in immunogenicity for a protein product.

In relation to protein particulates, other contaminating particulates in a protein product may enhance immunogenicity, such as glass (Fradkin et al., 2011a) or stainless steel (Van Beers et al., 2012) particulates. It is noted that a large quantity of such particulates may be needed for immunogenicity enhancement. For example, generation of an equivalent level of immunogenicity (IgG1), induced after administration of recombinant murine growth hormone (mGH) adsorbed on alum, requires the same dose of mGH adsorbed on glass particles exceeding the amount of alum by 76 times (Fradkin et al., 2011b). Administration of rhIFN- β 1a adsorbed on stainless steel microparticles at $2270 \text{ mg}/\text{mg}$ protein induced more immunogenicity (IgG titer) than free protein fractions or protein mixtures with glass or polystyrene particles in transgenic mice (Van Beers et al., 2012). It is unlikely that such a large amount of foreign particles would be present or contaminated in a protein product. Nonetheless, these results indicate the importance to monitor all the particulates and to determine their composition in protein products.

5. Protein aggregation in a biological milieu

Pharmaceutical scientists and regulatory agencies have long recognized the necessity of monitoring the behavior and fate of administered protein products. This necessity was more from a pharmacokinetic and/or pharmacodynamic point of view rather than from an immunological point of view. This is understandable, as the clearance of a product from the injection site or bloodstream cannot be predicted accurately simply from the type or the size of the product (Koide et al., 2010). Recent recognition of the potential immunological significance after administration of a protein product has not been translated into any significant efforts in experimental investigations in this area, partly due to the complication of a biological system and the challenges associated with analytical methodologies. Novel analytical methods are still being developed to characterize protein aggregates in a biological milieu (Filipe et al., 2011; Mach and Arvinte, 2011).

An obvious question would be the short-term and long-term fate of a protein as well as its aggregates after administration. Many proteins are not stable at a body temperature of 37°C and aggregate easily, such as granulocyte-colony stimulating factor (G-CSF) (Krishnan et al., 2002; Raso et al., 2005), recombinant human keratinocyte growth factor (rhKGF) (Chen et al., 1994), recombinant human platelet-activating factor acetylhydrolase (rhPAF-AH) (Chi et al., 2005a), rhIL-1ra (Chi et al., 2005b; Roy et al., 2006), and even IgG molecules (Jiskoot et al., 1990; Chen et al., 2003; Van Buren et al., 2008). Similarly, many proteins are usually stable in a narrow pH range and any pH outside this narrow range causes rapid protein aggregation, such as IL-1 β (Gu et al., 1991). Several proteins have been shown to aggregate readily at neutral to weakly

basic conditions at a moderate temperature, such as, rhGCSF at pH 7, (Thirumangalathu et al., 2006), rhKGF at pH 7 (Chen et al., 1994) and mutated apomyoglobin at pH 7.0 (Vilasi et al., 2008) and botulinum neurotoxin at pH 8.0 (Roy et al., 2008). Therefore, native proteins could rapidly aggregate in a biological milieu due to the temperature and/or pH changes after administration. Understanding the aggregation potential of a protein product after administration would be valuable through *in vitro* as well as *in vivo* evaluations.

Likewise, protein aggregates/particulates present in a protein product at 2–8 °C or room temperature could reverse back to monomers or solubilized due to the temperature increase and/or pH change after administration. High-temperature-induced protein disaggregation has been reported for serum cryoglobulins (Ferri et al., 2002; Ramsland and Farrugia, 2002) and IgG1 (Sukumar et al., 2004). The pH-induced reversibility of aggregation has been observed for some proteins, such as human muscle acylphosphatase (Calamai et al., 2005), and human interferon- γ (Mulkerrin and Wetzell, 1989). If monomerization of soluble aggregates or dissolution of insoluble aggregates occurs due to the temperature and/or pH change upon administration, would immunogenicity of protein aggregates be less of a concern? If yes, could other alternative physiological mechanisms be utilized to process the excessive amount of protein aggregates after administration for the same purpose (Gebbinck et al., 2009)?

Microparticles, consisting mainly of phospholipids and proteins and ranging in sizes from 0.1 to 2 μm , are circulating in the blood, performing many functions related to coagulation, vascular function, inflammation, and stimulating cytokine release, etc. (Puddu et al., 2010). Such components can potentially interact with a protein product and/or its aggregates after administration and change their physiological and/or biochemical behavior. For example, fibrillogenesis of islet amyloid polypeptide (IAPP) is enhanced by >10-fold with human tissue-derived phospholipids (Knight and Miranker, 2004). Membranes containing phosphatidylserine (PS), a negatively charged phospholipid, induce a rapid formation of fibers for a variety of proteins, including lysozyme, insulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, transthyretin, cytochrome c, histone H1, and alpha-lactalbumin (Zhao et al., 2004). A reverse event could be possible, too. It has been demonstrated that dioleoyl phosphatidylcholine (DOPC) could interact with mature A β amyloid fibrils and revert the inert fibrils to neurotoxic protofibrils (not monomers) (Martins et al., 2008). Thus, proteins, that do not have a strong aggregation tendency, can form protein aggregates due to interactions with these tissue and/or cellular components. Will this type of interactions change the immunogenicity potential of a protein product? Lipids and liposomes have been recognized as effective vaccine adjuvants to initiate an enhanced antibody response (Calderon et al., 2006; Hartikka et al., 2009; Shlapobersky et al., 2009). It is conceivable that protein interactions with lipids could lead to a significant change in immunogenicity of a protein product after administration. No literature reports, however, have been found on the evaluation of such effects *in vivo*. In comparison, use of dicaproyl phosphatidylserine, a short-chain water-soluble phospholipid, reduced the immunogenicity of rFVIII in hemophilia A mice (Purohit and Balasubramanian, 2006). Dicaproyl phosphatidylserine was shown to interact with rFVIII, causing subtle changes in the tertiary and secondary structure of the light chain. Similarly, phosphatidylserine (PS)-containing liposomes can also bind to the rFVIII light chain for a lower total- and inhibitory antibody titers in hemophilia A mice (Ramani et al., 2008). The bindings may cause a reduction in the rate of antigen processing by proteolytic enzymes (Ramani et al., 2008) and/or block the antigenic site found in the light chain (Peerlinck et al., 1997).

These results possibly explain why aggregated FVIII showed less immunogenicity than monomeric FVIII, as the lipid-binding sites and/or antigenic sites on the protein may be hidden in the aggregated protein.

Certain protein interactions with tissue components potentially lead to chemical modifications in protein or its aggregates for enhanced immunogenicity. Since disulfide-bonded dimers of HIV Tat101 derivatives induced stronger antibody response than the monomer without an adjuvant in mice (Kittiworakarn et al., 2006), *in vivo* disulfide formation of such compounds in an oxidative environment could lead to enhanced immunogenicity. Polyvinylpyrrolidone, not polyvinylphenol (a structurally similar polymer), has been shown to be immunogenic in rabbits, which was attributed to possible conjugation with host proteins via the carbonyl group of the lactam ring for enhanced immunogenicity (Naim and van Oss, 1992).

The relative number and density of immune cells are different in different tissues and biological fluids to initiate a different degree of immune response. Partly because of this, the route of product administration strongly influences the outcome of immunogenicity. It is generally believed that intravenous (IV) injection would lead to the least immune response relative to other common administration routes, such as intradermal (ID), subcutaneous (SC) and intramuscular (IM) injections (Schellekens, 2002, 2003). Among the non-IV routes, ID injection seems to initiate equal or greater immune response than SC or IM injections, presumably because the dermis contains more dendritic cells (Bonnotte et al., 2003; Chiu et al., 2009; Kunzi et al., 2009; Nicholson et al., 2009). SC administration generally leads to an equal or higher immunogenicity than IM administration in both mice and human subjects (Braun et al., 1997; Prummer, 1997; Perini et al., 2001; Stertman et al., 2004; Martin et al., 2010). Exceptions do exist. For example, IV administration of human recombinant FVIII in hemophilia A mice has generated equal amount of neutralizing antibodies, even though the total antibody titer is lower relative to SC administration (Peng et al., 2009). The frequency for the formation of IL-2-binding antibodies are similar after IV or SC administration, although the formation of neutralizing antibodies is associated with SC administration in patients (Prummer, 1997). The time of administration in a day could also influence the immunogenicity outcome (Cernysiov et al., 2010).

6. Regulatory considerations

It is generally understood that the level of soluble aggregates in a protein product should be no more than 5–10%. The limits on the number of insoluble proteinaceous particulates in all size ranges have not been widely agreed upon. As mentioned above, the FDA has increased their scrutiny on the level of subvisible particulates of <10 μm in size. Should a specification limit be set on particulates in this size range for lot release or stability evaluation? The answer is probably no, as the available data appear not consistent enough to support such a limit. On the other hand, these small subvisible particulates have the potential for enhanced immunogenicity and their size and number can be an indicator of consistency for a manufacturing process. Therefore, these particulates should be monitored routinely. On the other hand, all the currently available methods for particle analysis have certain limitations (Narhi et al., 2009; den Engelsman et al., 2011; Zolls et al., 2011). A clear need is to develop suitable analytical methods for simultaneous determination of particulates' quantity, size, shape, and composition in this and other size ranges.

For subvisible particulates of larger size, pharmaceutical companies generally use USP <788> as a release and stability specification. Traditionally, these limits were set for small-molecule drugs based

on a safety concern that particles of larger than 10 μm lead to occlusion of capillary blood vessels. Would protein particulates in the same size range (>10 μm) present a similar level of risk? An obvious regulatory gap is the lack of a similar guideline designed specifically for protein products. For severely aggregation-prone proteins, USP <788> is difficult to meet. A potential option would be to establish two regulatory limits, one for foreign particulates, and one for proteinaceous particulates. Even if such a distinction is allowed, what data would be needed to justify a limit on proteinaceous particulates? Would batch analysis plus immunogenicity data be adequate? The answer is probably no. In vivo safety data will likely be needed, or the in vivo behavior of protein particulates needs to be evaluated.

Another apparent gap is the lack of a similar pharmacopeial guidance on the particulate limits in the diluted infusion solutions prepared before administration. Many protein products meet the particulate limits easily in the original product container either as a liquid product or after reconstitution for a lyophilized product. The behavior of a protein product could be significantly different upon further dilution into commonly-used infusion solutions such as saline, half normal saline, D5W, etc. and result in a significant difference in particulate counts. Protein aggregation could take place right after dilution (Agarkhed, 2011). In addition, a number of product vials may be needed for pooling into a single infusion bag/container. This additional sample handling and transfer can introduce additional intrinsic and extrinsic particulate matters. Combination of all these factors can easily lead to particulate counts exceeding the limit for large volume injections, if the total infusion volume is >100 mL. An obvious option is to use an in-line low protein-binding filter before infusion and a single filtration step has been proven to be effective in reducing the counts of small particles (Narhi et al., 2009). If the diluent is not compatible with the product, however, particulates can re-form even after filtration (Agarkhed, 2011).

The current USP requires that all injections be “essentially free from visible particles”. Originally regulated for small-molecule drugs, particulate matters in injections mean extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions. The high aggregation tendency of proteins makes it difficult to develop most protein products essentially free of particulates and presence of a small amount of subvisible and/or visible proteinaceous particulates are often unavoidable. Should the description “essentially free from visible particles” be strictly applied to protein products? The answer is probably no, because protein particulates can be formed during manufacturing, inspection, and/or storage, and based on the definition, are not unintentionally present in the solutions. Therefore, some protein products are described as ‘may contain fiber-like particles’, with the understanding that these particles are mainly proteinaceous in nature. A number of approved protein products contain such languages in their package inserts (e.g. Campath, Vectibix, Stellara, etc.). Could these proteinaceous particulates, if not easily dissolvable in the circulation, pose similar level of safety concern as the undissolvable foreign particles, aside from their immunogenicity concern? What are the possible options for developing a commercial product for severely aggregation-prone proteins? One obvious option is to demonstrate safety of these particulates in human subjects and set a specification limit accordingly. This would be difficult to pursue, because there are no easy methods currently for characterization of protein particulates in terms of quantity, shape, rigidity, composition, and surface properties, all of which could contribute to product impact in humans. The other option is to remove physically all protein particulates by using an in-line low protein-binding filter immediately before administration. The latter option is relatively easy and has been adopted for many protein products.

7. Summary

In view of the current literature, the effect of protein aggregates on the immunogenicity of protein products has been evaluated in a limited number of studies. Results from these studies, albeit lack of complete consistency, generally point to a linkage between protein aggregation and enhanced immunogenicity. Limited clinical observations appear to support such a claim (Weksler et al., 1970; Moore and Leppert, 1980; Giovannoni et al., 2007). Yet, the structural features in protein aggregates truly responsible for the enhanced immunogenicity is far from being clear and need further investigations (Joubert et al., 2011).

The increased attention of regulatory agencies to the immunogenicity potential of protein aggregates/particulates is understandable because of the safety and efficacy concerns. On the other hand, the type and level of protein aggregates/particulates that lead to any enhancement of clinically relevant immunogenicity are unknown. Since protein aggregates are associated with product quality, toxicity, and potentially enhanced immunogenicity, the level of protein aggregates in protein products should be considered a critical quality attribute (CQA) and be controlled and monitored during product development and manufacturing processes.

The current gaps and proposed future directions are summarized as follows:

- The mechanisms of protein-induced immune response (either as a product or vaccine antigen) need further elucidation, along with factors that dominate or control the immune response and tolerance induction.
- Additional efforts are needed to understand the structural features in proteins and protein aggregates truly responsible for immunogenicity, and to develop methods for identifying the immunogenic sites/epitopes.
- The mechanisms for the non-specific contribution of proteinaceous particulates as adjuvants to the immunogenicity of proteins need further confirmation.
- The in vivo behavior and fate of protein products and their aggregates after administration need to be examined during product development.
- Development of robust and high-throughput analytical instrumentation/methods is needed for quantitation of proteinaceous and non-proteinaceous particulates with simultaneous analysis of shape, density, and composition.

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