Review

Minimizing immunogenicity of biopharmaceuticals by controlling critical quality attributes of proteins

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Adverse immune responses severely hamper the success of biopharmaceutical therapies. Possible clinical consequences include anaphylaxis, reduced drug half-life and neutralization of the therapeutic protein as well as the endogenous human homologue. Controlling potential triggers of the immune system helps to minimize the immunogenicity of biopharmaceuticals, a crucial consideration in biopharmaceutical manufacturing. This review summarizes the latest advancements that have been made towards insight into the impact of structural characteristics on the immunogenicity of therapeutic proteins. Examples are given to illustrate the role of critical quality attributes, such as protein conformation, glycosylation, chemical modifications and aggregation, in immunogenicity. During the development of biopharmaceutical products, it is important to not just assess the risk for immunogenicity in clinical trials, but to ensure product quality throughout drug design, cell-line selection, upstream and downstream processing, all the way to to the final product.

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1 Introduction

More than 200 pharmaceutical products currently on the market belong to the group of the so-called biopharmaceuticals [1]. Biopharmaceuticals approved for use include hormones, growth factors, cytokines, therapeutic enzymes, and monoclonal antibodies (mAbs). In 2013, biopharmaceuticals are expected to represent four of the five top-selling drugs worldwide [2]. Some of the recently approved biopharmaceuticals are biosimilars and more first-generation biopharmaceuticals are losing patent protection in the next years. Although nucleic acid-based products and stem cell therapies are now underway, the majority of approved biopharmaceuticals in the foreseeable future will continue to be protein-based.

An important feature of biopharmaceuticals is their high structural complexity compared to traditional small-molecule drugs. Whereas small-molecule drugs may face challenges due to intrinsic toxicity, biopharmaceuticals are more likely to induce an immune response in patients [3]. Adverse immune responses severely hamper the successful application of therapeutic proteins. A biopharmaceutical product is immunogenic when it stimulates an immune response followed by the generation of antibodies that specifically react with the product. Repeated administration to patients over an extended period of time generally enhances the risk of raising antibodies against the protein-based drug [4]. Such antibodies can induce anaphylaxis, alter the pharmacokinetic properties of the protein, or inhibit binding of the drug to its target receptor rendering the protein ineffective. Anaphylaxis is caused by an immediate allergic reaction mediated by immunoglobulin E (IgE) antibodies against the product, while an immune response with high titers of neutralizing IgG antibodies strongly decreases the therapeutic activity of the protein. Another possible life-threatening clinical

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Abbreviations: **ADA**, anti-drug antibody; **CHO**, Chinese hamster ovary; **α-Gal**, galactose-α1,3-galactose; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **hGH**, human growth hormone; **IFN**, interferon; **mAb**, monoclonal antibody; **Ig**, immunoglobulin; **Neu5Gc**, N-glycolylneuraminic acid; **rh**, recombinant human

consequence of antibody formation is cross-reactivity with the endogenous protein produced by the patient [5].

When the first therapeutic insulin products were introduced in humans for the treatment of diabetes in the 1920s, anti-drug antibodies (ADAs) were detected after a single or only a few injections, and some fatal anaphylactic responses were reported [6]. Nowadays, the high immunogenicity of the first-generation insulin products is not considered surprising, since these products were of bovine and porcine origin. Proteins purified from animal sources possess a molecular structure that is different from the natural human counterparts, and are thus foreign to the human immune system. Nevertheless, a remarkable decrease in immunogenicity was observed when impurities such as proinsulin, C-peptide, glucagon and somatostatin were removed from porcine insulin preparations [7], indicating that deviation from the structure of the human homologue is not the only determinant of immunogenicity for therapeutic proteins.

Impurities have been held responsible for the immunogenicity of several other therapeutic proteins, such as human growth hormone (hGH) derived from the pituitary glands of cadavers for the treatment of deficiencies in growth hormone production or response. In the 1970s, hGH was reported to induce antibodies in approximately 50% of the treated children despite its human source [8]. The high incidence of antibodies was hypothesized to be related to the presence of 40–70% aggregated hGH in the first "clinical grade" products. Upon improving the purification strategy of the therapeutic protein, aggregation was reduced to less than 5%. This resulted in a slower onset of antibody production, with antibodies that had higher affinities but were significantly less persistent.

Currently, an increasing number of mAbs are in clinical use or in the pipeline for a wide variety of diseases such as immune disorders and different types of cancer [9]. Early therapeutic mAbs were of murine origin and their clinical application was limited due to high rates of ADA generation. Later, advancements in hybridoma and recombinant DNA techniques enabled the production of chimeric (human constant regions), humanized (human, except for complementarity determining regions), and fully human antibodies. Despite major reductions in immunogenicity, ADAs against fully human antibodies such as adalimumab can still be observed [10]. Immunogenicity is also of great concern in the biosimilars industry [3], and in the design of novel protein delivery systems [11].

Various factors that influence the immunogenicity of therapeutic proteins have been identified, which can be divided into product related, patient related and treatment related factors [4]. Since ADAs can cause a decrease in drug response over time and directly affect the efficacy and safety of protein therapeutics, insight into the product related factors which trigger immunogenicity is crucial during the development of biopharmaceuticals. Excellent reviews have been written on this subject, such as those of Hermeling et al. [12], and Singh [13]. Product related factors that influence immunogenicity are part of the critical quality attributes of proteins, which are defined as those characteristics that potentially impact safety and efficacy and should be kept within a certain range to ensure product quality [14, 15]. In this review, we focus on the important quality attributes of proteins (including protein structure, glycosylation, chemical modification and aggregation) that should be controlled during bioprocessing to minimize immunogenicity.

2 Why are therapeutic proteins immunogenic?

Immunogenicity is defined as the ability of a compound to provoke an immune response. A therapeutic protein can be immunogenic because the human immune system categorizes it as non-self. A protein injected into patients will be taken up by antigen presenting cells and processed into smaller peptides. T cells generated in the thymus are able to bind to the peptides presented in the grooves of major histocompatibility complex molecules on the surface of antigen presenting cells. When the T cells recognize these peptides as foreign, they induce B cell proliferation. B and T cells are both part of the adaptive immune system; however, B cells interact directly with the protein owing to the immunoglobulins present on their cell surfaces. After binding to the specific three-dimensional structure of the protein, activated B cells recruit the complement system and macrophages from the innate immune system to destroy and remove the antigen. Such an immune response against a foreign protein is called a classical immune response, which typically leads to the production of high affinity antibodies of different isotypes, as well as memory cells responsible for an enhanced response upon repeated challenge with the antigen (the principle behind vaccination) [16].

On the other hand, humans develop immune tolerance for proteins produced by their own body (self-proteins) in early embryonic life to prevent immune responses to endogenous proteins. Through a mechanism called negative selection, T cells reacting with self-proteins undergo apoptosis during development in the thymus before they can complete maturation [17]. Sometimes autoreactive T cells escape negative selection and are able to reach the periphery. However, these cells are subjected to functional inactivation (anergy) or deletion if adequate levels of costimulators (second signals) are lacking [17]. Similar processes protect autoreactive B cells from proliferating and producing antibodies against self-proteins. In patients with an autoimmune disease, the immune tolerance for self-antigens is impaired, or the immune tolerance for a specific protein may be missing completely in the case of an endogenous protein deficiency. In other patients and healthy individuals, immune tolerance can be

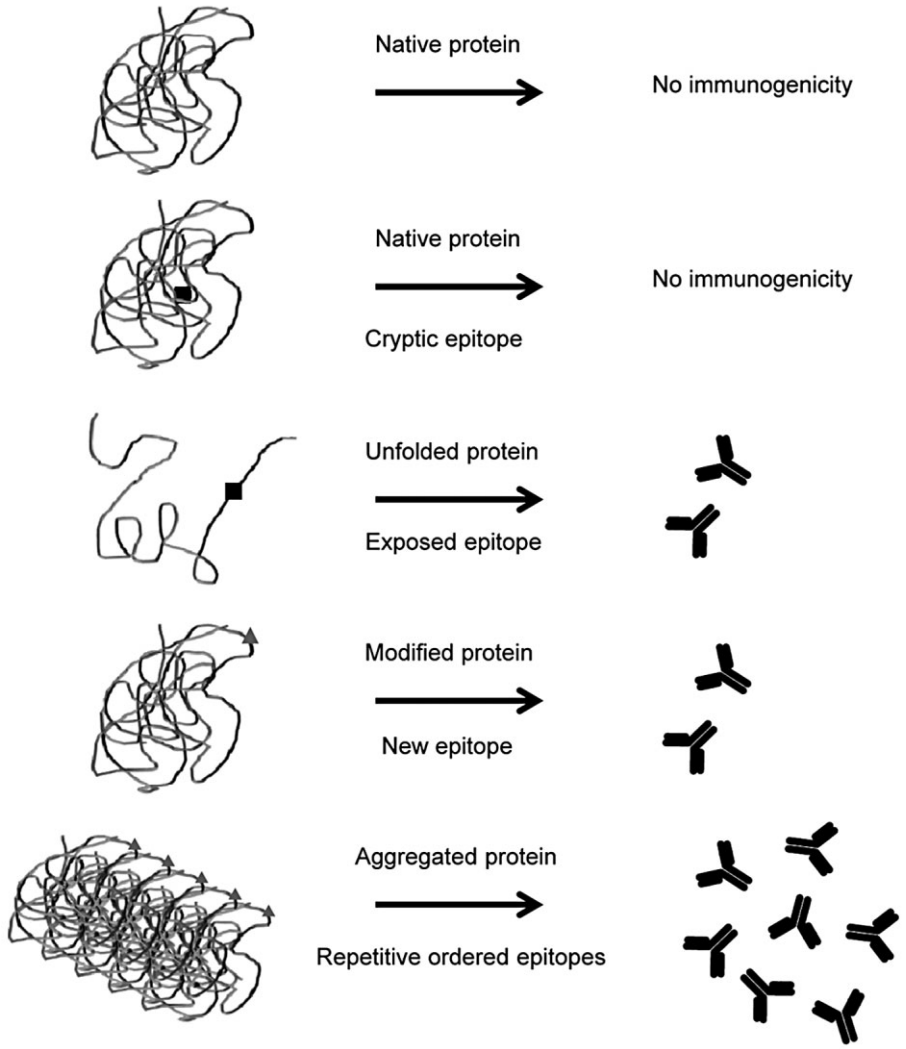

Figure 1. Potential immunogenicity of native, unfolded, modified and aggregated therapeutic proteins. Different structural changes that can occur on proteins and the relative extent of the resulting antibody responses are depicted. Immunogenicity depends on how antigenic epitopes (shown as black squares or grey triangles) are presented to the immune system.

overcome if anergic autoreactive B cells are activated by a self-antigen that is accompanied by a danger signal, such as the presence of protein aggregates displaying repetitive ordered epitopes (Figure 1). Presentation of a multivalent array of the same epitope to a B cell can result in cross-linking of multiple B cell receptors [18]. The surfaces of bacteria and viruses often display highly repetitive ordered antigens, which may explain why the immune system has evolved to react vigorously to these types of structures. Extensive cross-linking of B cell receptors can activate B cells to proliferate and produce antibodies [19–21]. It is by this mechanism that protein aggregates have been proposed to stimulate antibody production against monomeric protein therapeutics; however, the details of this mechanism including the role of T cells are yet to be elucidated.

In addition to the presence of aggregates, the degree of foreignness of the biopharmaceutical protein compared with the natural endogenous protein is another product related factor influencing immunogenicity [12, 22]. Whether the immune system considers the product self or non-self depends on differences in amino acid sequence and glycosylation between the administered protein and its human homologue. Furthermore, not only the sequence but also higher-order protein structure and chemical modifications have an effect on the potential immunogenicity of biopharmaceuticals (Figure 1). In the next sections, we will discuss the influence of product characteristics on immunogenicity and explain how this knowledge can be used during drug design and manufacturing with the aim of minimizing the potential immunogenicity of biopharmaceuticals.

3 Impact of critical quality attributes on immunogenicity

3.1 Protein structure

To preserve immune tolerance for an injected protein, the protein based drug needs to have high similarity to its human homologue. Amino acid sequences of therapeutic and endogenous proteins are preferably identical, which is usually not the case if the therapeutic protein is of animal origin. For this reason, animal insulin was found to be more immunogenic than human insulin [6]. The highly immunogenic bovine insulin has two additional amino acid changes in the exposed A-chain compared with porcine insulin, which is less immunogenic and has only one amino acid mutation in the less exposed B-chain. Antibodies against mutated insulin show cross-reactivity with human insulin.

Neutralizing antibodies against the first murine mAb therapeutics greatly decreased the efficacy of these products [13]. In the 1980s, genetic engineering enabled the production of chimeric antibodies with murine variable regions and human constant regions. Later, humanized antibodies with an amino acid sequence that is human except for the complementarity determining regions and also fully human antibodies were developed [23]. Nevertheless, chimeric antibodies such as infliximab and even fully human antibodies such as adalimumab induced ADAs in humans [10]. In case of chimeric antibodies, one would expect that immune responses are primarily directed towards the murine variable regions resulting in the generation of so-called anti-idiotype antibodies. In humanized and fully human mAbs, immunogenicity can be directed against the complementarity determining regions [24]. However, ADAs may also form against constant domains of human heavy chains due to the polymorphism in the gene encoding these domains [23]. One allelic form of a therapeutic antibody can be immunogenic in patients that are homozygous for the other allotype, thus resulting in anti-allotype antibodies. Lastly, anti-isotype antibodies may bind to certain epitopes in the Fc-portion of an antibody, e.g. epitopes in the hinge region of human IgG [25]. Anti-isotype and anti-allotype antibodies are less likely to neutralize drug activity than anti-idiotype antibodies.

Moreover, epitopes that are identified in a protein do not necessarily cause immunogenicity. A cryptic epitope in domain I of β_2-glycoprotein I, which is believed to cause thrombosis in patients with the antiphospholipid syndrome, only becomes immunogenic after a conformational change in the protein [26]. Conformation was also shown to play a role in the enhanced immunogenicity of recombinant human interferon alpha-2b in a transgenic mouse model, which was caused by misfolding of the therapeutic protein [27]. In fact, immunogenicity depends on how epitopes are presented to the immune system (Figure 1). It is possible that a single amino acid difference in the primary structure of a protein induces a strong immune response, while in another protein multiple mutations have no such effect. Immunogenicity caused by mutations not only depends on the formation of new epitopes, but also on whether the new epitopes are detected by the immune system. New epitopes may be created due to chemical modification of the protein whereby new covalent cross-links between amino acid residues are formed (as discussed later in section 3.3), sometimes accompanied by aggregation (section 3.4). The presence of aggregates that may contain danger signals greatly enhances immunogenicity. Currently, in silico and in vitro methods are being developed to predict T and B cell epitopes based on the sequence of drug candidates. Sequence modification may be applied to reduce the number of T cell epitopes in therapeutic proteins [28]. A potential overlap is described between the location of epitopes and aggregation prone regions [29]. Such studies should lead to the rational design of therapeutic proteins that are less immunogenic and less prone to aggregation, but are beyond the scope of this review.

3.2 Glycosylation

Almost half of the therapeutic proteins that are approved or in clinical trials are glycosylated [1, 13]. Glycosylation is one of the most common and complex post-translational modifications, which leads to the enzymatic addition of glycans on proteins. Glycans can influence the physicochemical (e.g. solubility, electrical charge, mass, size, folding, stability) as well as the biological (e.g. activity, half-life, cell surface receptor function) properties of proteins [12]. The glycosylation profile of a protein is species-specific and depends on the cell-line and culture conditions that are used for production [30]. The presence and structure of carbohydrate moieties can have a direct or indirect impact on the immunogenicity of therapeutic proteins; that is the glycan structure itself can induce an immune response or its presence can affect protein structure in such a way that the protein becomes immunogenic.

3.2.1 Non-human glycan structures

Over the past decade at least four non-human carbohydrate structures that are able to induce an immune response in humans have been identified: galactose-α1,3-galactose (α-Gal), N-glycolylneuraminic acid (Neu5Gc), β1,2-xylose (core-xylose) and α1,3-fucose (core-α1,3-fucose) (Figure 2). The first observations of immune reactions against the glycoepitopes α-Gal and Neu5Gc were described in the context of xenotransplantation of pig organs in humans [31], and the targeting of vaccines to antigen presenting cells in cancer immunotherapy [32]. More recently, the presence of α-Gal and/or Neu5Gc was demonstrated in several therapeutic mAbs [33–36], in-

cluding cetuximab, a chimeric mouse-human IgG1 monoclonal antibody approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck. About 3% of patients develop severe hypersensitivity reactions within minutes after the first exposure to cetuximab, and a higher prevalence (up to 33%) may be seen in certain geographical regions [33]. Most patients with hypersensitivity possess IgE antibodies against cetuximab before the start of the therapy. The antibodies were found to be specific for the α-Gal epitope and related to IgE antibodies involved in anaphylactic reactions to red meat [33, 37]. All humans have IgA, IgM, and IgG antibodies against α-Gal, representing approximately 1% of circulating immunoglobulin. Qian et al. [35] demonstrated that the α-Gal epitopes are located in the Fab regions of the cetuximab antibody. The intravenous injection method and the presence of α-Gal on both Fab regions, which enables efficient cross-linking of IgE on mast cells, may explain the prompt immune response to cetuximab. The murine cell line SP2/0 used to produce cetuximab expresses the gene encoding for α1,3-galactosyltransferase, the enzyme responsible for the synthesis of the α-Gal epitope. Possible options to prevent incorporation of the terminal α-Gal motif in therapeutic mAbs during production include knocking out the gene for α1,3-galactosyltransferase in murine cells or using another expression system. For example, Chinese hamster ovary (CHO) cells may not produce the α-Gal epitope glycoform, although this is still controversial [38, 39].

Humans synthesize the sialic acid N-acetylneuraminic acid (Neu5Ac) but, unlike other mammals, are not able to synthesize Neu5Gc (Figure 2). As a result, the human immune system recognizes Neu5Gc as foreign and humans show high levels of IgA, IgM and IgG antibodies against Neu5Gc (up to 0.1 to 0.2% of circulating immunoglobulin [40]). Injecting products that contain Neu5Gc in individuals with pre-existing antibodies can cause the formation of immune complexes that potentially activate complement or affect half-life of the drug. In fact, it was shown by Gadheri et al. [41] that the clearance of cetuximab increased significantly in mice when anti-Neu5Gc antibodies were pre-injected. In a different study. Maeda et al. [36] detected the presence of the Neu5Gc epitope in three commercial mAb pharmaceuticals produced in murine cell lines (cetuximab, gemtuzumab ozogamicin and infliximab), whereas it was absent in other mAbs (tocilizumab, bevacizumab and adalimumab) produced in CHO cell lines. For this reason, CHO cells are generally preferred over Neu5Gc-producing cell lines for mAb production. Furthermore, it is important to note that CHO cells, like human cells, are able to take up Neu5Gc from cell culture media that contain animal-derived materials and metabolically incorporate the glycoepitope into the secreted protein [42]. Therefore, manufacturers should prevent its uptake by using Neu5Gc-free media to eliminate Neu5Gc contamination in the final product [41, 42].

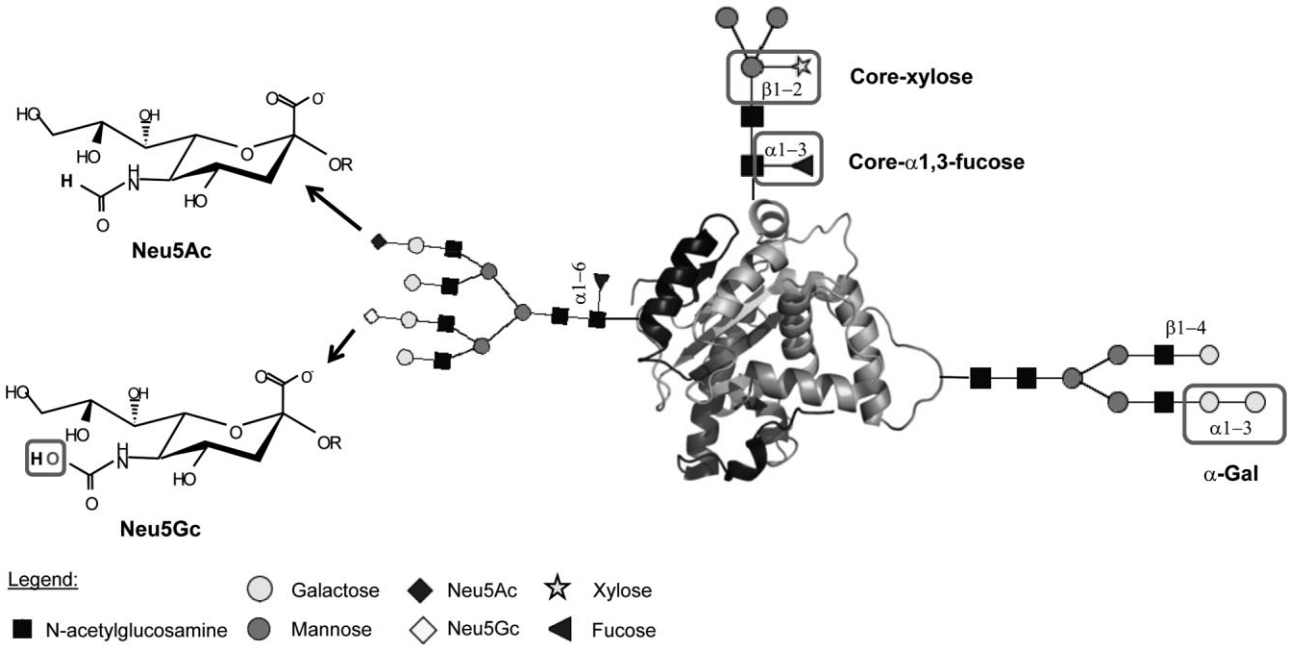

Figure 2. Four glycoepitopes that can be found on biopharmaceuticals and are able to induce an immune response in humans. N-glycolylneuraminic acid (Neu5Gc) is a sialic acid that is synthesized by all mammalian cells except human cells. Neu5Gc has only one oxygen atom difference with its homologue, N-acetylneuraminic acid (Neu5Ac), which is sufficient for Neu5Gc to be immunogenic in humans. The α-Gal epitope is formed by the action of the α1,3-galactosyltransferase in non-human mammalian cells and is a foreign glycoepitope that can induce immune responses and anaphylaxis in humans. Core-xylose and core-α1,3-fucose epitopes can be found in plant-derived biopharmaceuticals causing immunogenicity in humans.

Besides the commonly used protein expression systems of bacteria, yeast, insect and mammalian cells, plant cells are emerging cell factories for the production of biopharmaceutical proteins. In May 2012, the FDA approved the first biopharmaceutical derived from transgenic plants, which is recombinant human glucocerebrosidase for the treatment of Gaucher's disease [43]. Fully functional mAbs also can be efficiently synthesized in transgenic plants [44, 45]. A major drawback of plant-derived glycoproteins is the presence of complex N-glycans with core-xylose and core-α1,3-fucose structures (Figure 2). These two glycoepitopes are foreign to humans due to differences in plant and mammalian glycosyltransferase repertoires [46]. Core-xylose and core-α1,3-fucose specific antibodies (IgM and IgG_1) were detected in 50% and 25% of healthy human blood donors, respectively [47], while specific IgE was found in 25% of allergic patients [48]. Although the clinical impact of such pre-existing antibodies remains to be shown, strategies are investigated to engineer the N-glycosylation pathway of plants to preclude the introduction of glycoepitopes in plant-derived therapeutic proteins [46].

To conclude, non-human glycan structures present on biopharmaceuticals can induce IgE mediated reactions and/or anaphylaxis in allergic patients. Moreover, those glycoepitopes may enhance clearance and decrease therapeutic effect of biopharmaceuticals due to pre-existing IgA, IgM and IgG antibodies in certain patients. Neutralization of the therapeutic protein or cross-reactivity with the endogenous protein resulting from the presence of glycoepitopes is less likely, due to lack of reactivity towards the underlying protein backbone.

3.2.2 *Glycosylation affects protein structure and immunogenicity*

Glycans may impact the immunogenicity of therapeutic proteins in an indirect manner through their influence on folding, solubility and structural stability of proteins. Indeed, glycosylation can improve protein solubility and stability, and thereby decrease the immunogenicity of therapeutic proteins, as in the case of recombinant human interferon beta (rhIFNβ) (Table 1). The non-glycosylated form (rhIFNβ-1b; produced in *E. coli*) is less soluble and more prone to thermal denaturation than the glyco-

Table 1. Impact of product quality on the immunogenicity of the therapeutic protein recombinant human interferon beta (rhIFNβ) as an example.

rhIFNβ type	Quality attribute	Product characteristic	Host	Immunogenicity	Reference
-1b Betaferon®	Protein structure Glycosylation Chemical modification Aggregation	Sequence variation Non-glycosylated AGEs[a)] 60% Aggregates	Patients	Moderate to high	[49, 50, 94]
-1a Rebif®	Protein structure Glycosylation Chemical modification Aggregation	No sequence variation Glycosylated AGEs[a)] Aggregate % unknown	Patients	Moderate	[50, 94]
-1a Avonex®	Protein structure Glycosylation Chemical modification Aggregation	No sequence variation Glycosylated AGEs[a)] <2% Aggregates	Patients	Moderate	[49, 50, 94]
-1b	Chemical modification	PEGylation	Rats	Low	[54]
-1a	Chemical modification Aggregation	Oxidation and aggregation with Cu^{2+}/ ascorbic acid or H_2O_2	TG mice[b)]	High	[64]
-1a	Aggregation	Adsorbed to stainless steel microparticles	TG mice[b)]	High	[87]
-1a	Aggregation	Filtration/reformulation reduces aggregate %	TG mice[b)]	Low	[95]
-1b Betaferon®	Aggregation	Surfactant reduces aggregate %	Mice	Low	[92]
-1b	Aggregation	High hydrostatic pressure reduces aggregate %	TG mice[b)]	Low	[96]

a) AGEs, Advanced glycation end-products
b) TG mice, Transgenic mice immune tolerant for human IFNβ

sylated form (rhIFNβ-1a; produced in CHO cells) [49]. The lack of glycosylation most likely contributes to the high aggregate content of rhIFNβ-1b: it contains about 60% aggregates and elicits neutralizing antibodies in a high percentage of patients, whereas rhIFNβ-1a contains less than 2% aggregates and is less immunogenic [49, 50].

Besides making a protein more soluble, a carbohydrate moiety is sometimes able to cover an antigenic epitope. Antibodies against rhGM-CSF that were generated in patients showed cross-reactivity with rhGM-CSF produced in yeast and *E. coli*, but not with rhGM-CSF produced in CHO cells [12, 51]. It was concluded that the antibodies were directed against a part of the peptide backbone that is protected by O-linked glycosylation in the natural protein and the protein derived from CHO cells. A similar effect was observed upon the introduction of site-specific glycosylation in bovine lactoglobulin, a major milk allergen [52]. The immunogenicity of β-lactoglobulin in mice was drastically reduced by positioning high-mannose chains in the vicinity of B cell epitopes. Antigenic sites were masked while the protein remained functional, allowing the protein to escape from recognition by the immune system. Nevertheless, it should be recognized that the introduction of an altered sequence in the primary structure of a therapeutic protein could enhance immunogenicity, as discussed in section 3.1, or may compromise the chemical or conformational stability of the protein.

3.3 Chemical modification

3.3.1 Intended modifications

Biopharmaceuticals can be chemically modified with the purpose of extending half-life or facilitating uptake by target receptors [53]. An increasingly common type of engineering is the covalent attachment of polyethylene glycol (PEG) polymers to the peptide backbone. PEGylation is intended to lower renal filtration by increasing the molecular weight and to protect the therapeutic protein from proteolytic degradation. Similar to glycosylation, PEGylation may decrease immunogenicity by shielding the immunogenic epitopes, while maintaining the native conformation of the protein. PEG polymers ranging in molecular weight from 12 to 40 kDa were attached to different sites on the hydrophobic and immunogenic therapeutic protein rhIFNβ-1b [54]. The mono-PEGylated rhIFNβ-1b preparations retained 20-70% of the antiviral activity and displayed improved solubility, stability and pharmacokinetic properties compared with unmodified rhIFNβ-1b. Importantly, PEGylation greatly reduced the aggregation propensity of rhIFNβ-1b as well as its immunogenicity in rats (Table 1). The immunogenicity of a PEGylated version of rhIFNβ-1a is currently being evaluated in clinical trials [55]. Yet, it is believed that PEGylation is more likely to reduce the immunogenicity of non-human proteins [13].

3.3.2 Chemical degradation

In addition to intended modifications, a biopharmaceutical may be chemically modified through accidental degradation in one of the many bioprocessing steps: fermentation, virus inactivation, purification, polishing, formulation, filtration, filling, storage, transport and administration. Chemical modifications during bioprocessing may include deamidation, oxidation, isomerisation, hydrolysis, glycation and C/N terminal heterogeneity of the protein, sometimes leading to aggregation [56]. The susceptibility of an individual amino acid residue to chemical modification is dependent on neighbouring residues, the tertiary structure of the protein and solution conditions such as temperature, pH and ionic strength. Chemical modification may give rise to a less favourable charge of the protein, thus leading to structural changes or even the formation of new covalent cross-links [57]. Covalent aggregation is also a form of chemical degradation, which however will be discussed in section 3.4 dedicated to aggregation.

The impact of deamidation on immunogenicity has been described for several products [58-60]. Deamidation of proteins accelerates at high temperature and high pH, and can occur during bioprocessing and storage. Moreover, deamidation can be accompanied by some degree of oxidation, conformational changes, fragmentation and aggregation, posing serious risks for immunogenicity. Oxidation, another major chemical modification, can also reduce conformational stability and may cause the protein to aggregate. Oxidation of human serum albumin with hydroxyl radicals resulted in structural alterations and exposure of hydrophobic patches, causing increased immunogenicity [61]. High ADA titers were observed after injection of a metal-catalyzed oxidized and aggregated IgG1 sample in non-transgenic and transgenic mice [62]. Therapeutic interferons oxidized and aggregated via the same metal-catalysis method were able to overcome the immune tolerance of transgenic mice that were immune tolerant for the administered human proteins (Table 1) [63, 64]. The transgenic mice also developed antibodies against oxidized and aggregated rhIFNβ-1a treated with H_2O_2 [64], but not against oxidized rhIFNα-2b treated with H_2O_2 [63], probably due to the absence of aggregation.

Chemical stresses during manufacturing and storage can be caused by exposure to light or elevated temperatures and the presence of oxygen, metal ions or peroxide impurities from excipients in the formulation. Trace amounts of iron, chromium and nickel were found to leach into the formulation buffer via contact with stainless steel surfaces typically used during bioprocessing [65]. Despite limited data on the extent to which actual chemical modifications occur during biopharmaceutical manufacturing, it is clear that they form a serious risk of inducing an immune response in patients. Preventative measures should include the careful evaluation of buffers, surface

materials and conditions used during manufacturing, transport and storage.

3.4 Aggregation

The immunogenicity of therapeutic proteins is frequently associated with the presence of aggregates [4, 12, 18, 20]. Aggregation can occur through different mechanisms, and the causes are highly variable [56, 66]. In the previous sections, we described that certain amino acid sequences are more prone to aggregation, and that glycosylation, PEGylation and chemical degradation can affect aggregation. Since aggregation is often accompanied by other structural changes on the protein, it is difficult to distinguish the individual impact of each factor on immunogenicity. Nevertheless, it is well known that aggregation may induce an immune response, and fifty years ago it was already described that the removal of aggregates from bovine IgG eliminated immunogenicity in mice [67]. In 1970, Weksler et al. [68] reported that aggregate-free equine IgG did not induce any immune response in organ transplant patients, whereas the aggregated product did [68]. A very recent example of the link between aggregation and immunogenicity [69] showed that contaminating soluble tungsten from syringes caused aggregation of a biosimilar recombinant human erythropoietin, probably explaining the presence of neutralizing antibodies after administration of the product to chronic renal failure patients.

Since information on the nature of immunogenic aggregates from clinical studies is generally limited, animal models are used to provide insight into the link between aggregation and immunogenicity [70]. Studies employing such models have elucidated that not all aggregates appear to be immunogenic, as demonstrated for example for rhGH [71]. Transgenic mice immune tolerant for human IFNα have been used to study the immunogenicity of aggregated rhIFNa products [63, 72]. rhIFNα-2b aggregates composed of native-like proteins were able to overcome the immune tolerance of transgenic mice, resulting in antibodies cross-reacting with the monomeric protein [63]. IgG titers were found to be dependent on the level of aggregation [73]. Others [74] have shown that native-like aggregates of factor VIII were also more immunogenic than non-native aggregates or native protein.

Non-native aggregation can trigger structural changes in the protein leading to the creation of new epitopes or the exposure of existing epitopes (Figure 1). Native-like aggregates, however, are more likely to elicit ADAs that cross-react with the native protein and thus pose a greater risk for the patient. Native-like aggregates may resemble haptens on the surface of pathogens that form organized and repetitive structures that can cross-link B cell receptors in a multivalent manner [75]. Experimental data from Dintzis et al. [76] indicated that such highly ordered structures need to have a molecular weight over 100 kDa featuring 10 to 20 haptens with a spacing of 10 nm for direct activation of B cells [76]. Likewise, self-proteins presented in a highly ordered, repetitive manner through conjugation to virus-like particles were able to induce high antibody levels in mice [77, 78]. For details of the immunological effect of protein aggregates and the relationship between protein aggregation and immunogenicity, we refer to reviews by Rosenberg [18], Sauerborn et al. [20] and Wang et al. [79].

In an ideal world, we would exactly know the type of aggregates, amount of aggregates, aggregate percentage and number of injections that cause immunogenicity. This remains largely unknown due to the heterogeneity and complexity of aggregate structures. Aggregates can be classified according to size, reversibility, secondary or tertiary structure, covalent modification and morphology [80]. One class of aggregates that are of particular concern include subvisible particles or, more specifically, aggregates ranging from 0.1 to 10 µm in size [81]. Despite case studies demonstrating the presence of particles smaller than 10 µm in biopharmaceutical products and their potential high risk for immunogenicity, subvisible particles have not been given the attention they deserve. The current USP light obscuration test for drug approval requires that the number of particulates over 10 µm is ≤6000 per container, while the number of particulates over 25 µm is ≤ 600 per container. Industrial researchers agree that additional analysis of subvisible particles smaller than 10 µm would support product characterization and development [82]. However, our understanding of the nature of particulate matter in protein products is limited. Likewise, we currently lack the means to monitor this class of aggregates using small-volume, high-throughput techniques. Subvisible particles are too large to be analyzed by standard quality control methods such as SEC and SDS-PAGE, but too small to be visually detected. Therefore, use of additional, less routine methods such as asymmetrical flow field flow fractionation and micro-flow imaging has been recommended for extended characterization following a risk-based approach [83].

Moreover, product characterization should not be limited to monitoring protein particles, but also focus on non-protein particles [84]. Foreign micro- and nanoparticles, for example shed from filling pumps or product containers, are able to induce protein aggregation or nucleate the formation of heterogeneous aggregates. Several therapeutic proteins were shown to adsorb readily to glass and stainless steel microparticles, subsequently generating ADAs in non-transgenic and transgenic mice immune tolerant for the protein [85-87]. In addition to material surfaces, air-liquid interfaces, shear forces, pump cavitation and local thermal effects may cause unfolding and induce protein aggregation [88].

In the early stages of drug development, computational methods can be applied to evaluate the protein aggregation propensity for candidate selection [89, 90]. During subsequent stages of cell line development, a screen

may help to identify high-producing clones that generate minimal amounts of aggregated protein [91]. Furthermore, aggregation can take place during upstream and downstream processing, fill-finish operations, transport and storage [66]. Surface materials and conditions such as light, pH and temperature, should be chosen to minimize the physical and chemical stresses during the entire production process. Aggregation occurring in the later stages of biopharmaceutical manufacturing can be minimized by the addition of an excipient [92], although care should be taken that the excipient itself does not cause immunogenicity. Nowadays, several protein products make use of an in-line low protein-binding filter, an option that can be considered to prevent administration of particulates [79]; however, filters can also shed particulate matter. Furthermore, the quaternary, tertiary and secondary structure of the protein can be stabilized by optimizing the formulation buffer, dosage form (lyophilized or ready-to-use syringe), and container material of the final product.

4 Implications for biopharmaceutical manufacturing

There is a wide range of factors that influence the immunogenicity of therapeutic proteins [4], including product related factors that have important implications for bioprocessing. Our understanding of the relationship between the critical quality attributes of proteins and immunogenicity still shows gaps, despite advances that have been made since Hermeling et al. [12], who described the influence of protein structure on immunogenicity. In this review, we summarize the current literature on the relationship between product quality and immunogenicity, and place these findings in the context of biopharmaceutical manufacturing. The protein structure of the biopharmaceutical, as well as its glycosylation, chemical modifications and aggregation properties, should be considered for their potential impact on immunogenicity. If an impact is proven or suspected, the quality attribute will need to be monitored and controlled during product development and biopharmaceutical manufacturing [14]. This has implications throughout the different development and manufacturing steps from drug design, cell-line selection, upstream and downstream processing to the final drug product. All biopharmaceutical proteins carry combinations and variations of the critical quality attributes described in this review, each contributing to the potential risk for immunogenicity. Together with non-product related factors such as route and frequency of administration, concomitant medication, patient features and disease state, they determine the immune response seen in patients.

Risk analysis is part of the quality by design approach currently advocated in biopharmaceutical manufacturing [15]. The influence of specific structural aspects of the recombinant protein such as sequence mutations and the presence of aggregates should be assessed *in silico*, in vitro and/or in vivo for their impact on immunogenicity. Nevertheless, computational methods, cell-based assays and animal models only provide information on the probability of raising an immune response against the therapeutic protein but not on the clinical consequences. Therefore, a risk determination for immunogenicity needs to include the probability that the protein will induce antibody formation as well as the severity of the consequences [93]. Potential consequences include anaphylaxis, increased clearance rate, neutralization of the drug, loss of biological function and cross-reactivity with the endogenous protein, and is dependent on the nature of the disease and the possibility to switch to another product. Defining the critical quality attributes that impact the immunogenicity of a biopharmaceutical provides a better understanding of the product, helps to control immunogenicity and thus improves safety and efficacy in patients.

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5 References

[1] Walsh, G., Biopharmaceutical benchmarks 2010. *Nat. Biotech.* 2010, *28*, 917–924.
[2] Goodman, M., Market watch: sales of biologics to show robust growth through to 2013. *Nat. Rev. Drug Discov.* 2009, *8*, 837–837.
[3] Schellekens, H., Biosimilar therapeutics – what do we need to consider? *NDT Plus* 2009, *2*, i27–i36.
[4] Schellekens, H., Bioequivalence and the immunogenicity of biopharmaceuticals. *Nat. Rev. Drug Discov.* 2002, *1*, 457–462.
[5] Casadevall, N., Nataf, J., Viron, B., Kolta, A. et al., Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N. Engl. J. Med.* 2002, *346*, 469–475.
[6] Schernthaner, G., Immunogenicity and allergenic potential of animal and human insulins. *Diabetes Care* 1993, *16*, 155–165.
[7] Heding, L. G., Larsson, Y., Ludvigsson, J., The immunogenicity of insulin preparation. Antibody levels before and after transfer to highly purified porcine insulin. *Diabetologia* 1980, *19*, 511–515.
[8] Moore, W. V., Leppert, P., Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J. Clin. Endocrinol. Metab.* 1980, *51*, 691–697.
[9] Baker, M., Reynolds, H. M., Lumicisi, B., Bryson, C. J., Immunogenicity of protein therapeutics: the key causes, consequences and challenges. *Self Nonself* 2010, *1*, 314–322.
[10] Ordas, I., Mould, D. R., Feagan, B. G., Sandborn, W. J., Anti-TNF monoclonal antibodies in inflammatory bowel disease: pharmacokinetics-based dosing paradigms. *Clin. Pharmacol. Ther.* 2012, *91*, 635–646.

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Dr **Muriel Bardor's** PhD thesis focused on the humanization of the N-glycosylation of recombinant mAb produced in transgenic plants. After her graduation in 2001, she joined Prof Ajit Varki's group at the Glycobiology Research and Training Center, University of California San Diego, USA, where she demonstrated the uptake mechanism of the non-human sialic acid N-glycolylneuraminic acid into human and mammalian cells. At the end of 2003, she obtained an associate professor position at the University of Rouen, France. There, she initiated and led a project regarding the characterization of the N-glycosylation processing in microalgae. She is currently doing an overseas long-period stay in Singapore as a group leader of the Analytics department at Bioprocessing Technology Institute, A*STAR, Singapore. Her group is developing analytical tools for the glycoanalysis of recombinant proteins produced in mammalian cells and for the characterization of the glycosylation of the host producer cells.

[11] Jiskoot, W., Randolph, T. W., Volkin, D. B., Middaugh, C. R. et al., Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. *J. Pharm. Sci.* 2012, *101*, 946–954.
[12] Hermeling, S., Crommelin, D., Schellekens, H., Jiskoot, W., Structure-immunogenicity relationships of therapeutic proteins. *Pharm. Res.* 2004, *21*, 897–903.
[13] Singh, S. K., Impact of product-related factors on immunogenicity of biotherapeutics. *J. Pharm. Sci.* 2011, *100*, 354–387.
[14] Eon-Duval, A., Broly, H., Gleixner, R., Quality attributes of recombinant therapeutic proteins: an assessment of impact on safety and efficacy as part of a quality by design development approach. *Biotechnol. Prog.* 2012, *28*, 608–622.
[15] Rathore, A. S., Winkle, H., Quality by design for biopharmaceuticals. *Nat. Biotechnol.* 2009, *27*, 26–34.
[16] Marini, J. C., Chapter 8: Cell cooperation in the antibody response, in: Male, D., Brostoff, J., Roth, D. B., Roitt, I. (Eds.), *Immunology*, Elsevier Limited, Philadelphia 2006, pp. 163–180.
[17] Abbas, A. K., Lichtman, A. H., Chapter 9: Immunologic tolerance and autoimmunity, in: Malley, J. (Ed.), *Basic Immunology: Functions and Disorders of the Immune System*, Elsevier Saunders, Philadelphia 2006, pp. 161–176.
[18] Rosenberg, A. S., Effects of protein aggregates: an immunologic perspective. *AAPS J.* 2006, *8*, E501–E507.
[19] Bachmann, M. F., Zinkernagel, R. M., Neutralizing antiviral B cell responses. *Annu. Rev. Immunol.* 1997, *15*, 235–270.
[20] Sauerborn, M., Brinks, V., Jiskoot, W., Schellekens, H., Immunological mechanism underlying the immune response to recombinant human protein therapeutics. *Trends Pharmacol. Sci.* 2010, *31*, 53–59.
[21] Defrance, T., Taillardet, M., Genestier, L., T cell-independent B cell memory. *Curr. Opin. Immunol.* 2011, *23*, 330–336.
[22] Schellekens, H., Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin. Ther.* 2002, *24*, 1720–1740.
[23] Jefferis, R., Human immunoglobulin allotypes: possible implications for immunogenicity. *mAbs* 2009, *1*, 332–338.
[24] Harding, F. A., Stickler, M. M., Razo, J., DuBridge, R. B., The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions. *MAbs* 2010, *2*, 256–265.
[25] Rispens, T., de Vrieze, H., de Groot, E., Wouters, D. et al., Antibodies to constant domains of therapeutic monoclonal antibodies: anti-hinge antibodies in immunogenicity testing. *J. Immunol. Methods* 2012, *375*, 93–99.
[26] de Laat, B., van Berkel, M., Urbanus, R. T., Siregar, B. et al., Immune responses against domain I of β2-glycoprotein I are driven by conformational changes: domain I of β2-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis Rheum.* 2011, *63*, 3960–3968.
[27] Maas, C., Hermeling, S., Bouma, B., Jiskoot, W., Gebbink, M. F. B. G., A role for protein misfolding in immunogenicity of biopharmaceuticals. *J. Biol. Chem.* 2007, *282*, 2229–2236.
[28] De Groot, A. S., Scott, D. W., Immunogenicity of protein therapeutics. *Trends in Immunology* 2007, *28*, 482–490.
[29] Kumar, S., Singh, S., Wang, X., Rup, B., Gill, D., Coupling of aggregation and immunogenicity in biotherapeutics: T- and B-cell immune epitopes may contain aggregation-prone regions. *Pharm. Res.* 2011, *28*, 949–961.
[30] Brooks, S. A., Chapter 4: Species differences in protein glycosylation and their implications for biotechnology, in: Brooks, S., Rudd, P., Appelmelk, B. (Eds.), *Glycosylation in Diverse Cell Systems: Challenges and New Frontiers in Experimental Biology*, Society for Experimental Biology, London 2011, pp. 74–92.
[31] Ezzelarab, M., Ayares, D., Cooper, D. K. C., Carbohydrates in xenotransplantation. *Immunol. Cell. Biol.* 2005, *83*, 396–404.
[32] Galili, U., The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. *Immunol. Cell. Biol.* 2005, *83*, 674–686.
[33] Chung, C. H., Mirakhur, B., Chan, E., Le, Q.-T. et al., Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3-galactose. *N. Engl. J. Med.* 2008, *358*, 1109–1117.
[34] Chung, C. H., Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. *Oncologist* 2008, *13*, 725–732.
[35] Qian, J., Liu, T., Yang, L., Daus, A. et al., Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole–quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion. *Anal. Biochem.* 2007, *364*, 8–18.

[36] Maeda, E., Kita, S., Kinoshita, M., Urakami, K. et al., Analysis of non-human N-glycans as the minor constituents in recombinant monoclonal antibody pharmaceuticals. *Anal. Chem.* 2012, *84*, 2373–2379.

[37] Saleh, H., Embry, S., Nauli, A., Atyia, S., Krishnaswamy, G., Anaphylactic reactions to oligosaccharides in red meat: a syndrome in evolution. *Clin. Mol. Allergy* 2012, *10*, 5.

[38] Jefferis, R., Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends Pharmacol. Sci.* 2009, *30*, 356–362.

[39] Bosques, C. J., Collins, B. E., Meador, J. W., Sarvaiya, H. et al., Chinese hamster ovary cells can produce galactose-α-1,3-galactose antigens on proteins. *Nat. Biotechnol.* 2010, *28*, 1153–1156.

[40] Padler-Karavani, V., Varki, A., Potential impact of the non-human sialic acid N-glycolylneuraminic acid on transplant rejection risk. *Xenotransplantation* 2011, *18*, 1–5.

[41] Ghaderi, D., Taylor, R. E., Padler-Karavani, V., Diaz, S., Varki, A., Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nat. Biotechnol.* 2010, *28*, 863–867.

[42] Bardor, M., Nguyen, D. H., Diaz, S., Varki, A., Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells. *J. Biol. Chem.* 2005, *280*, 4228–4237.

[43] Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S. et al., A plant-derived recombinant human glucocerebrosidase enzyme - A preclinical and phase I investigation. *PLoS One* 2009, *4*, e4792.

[44] Hiatt, A., Caffferkey, R., Bowdish, K., Production of antibodies in transgenic plants. *Nature* 1989, *342*, 76–78.

[45] Bardor, M., Loutelier-Bourhis, C., Paccalet, T., Cosette, P. et al., Monoclonal C5-1 antibody produced in transgenic alfalfa plants exhibits a N-glycosylation that is homogenous and suitable for glyco-engineering into human-compatible structures. *Plant Biotechnol. J.* 2003, *1*, 451–462.

[46] Bardor, M., Burel, C., Villajero, A., Cadoret, J. P. et al., Chapter 5: Plant N-glycosylation: an engineered pathway for the production of therapeutical plant-derived glycoproteins, in: Brooks, S., Rudd, P., Appelmelk, B. (Eds.), *Glycosylation in Diverse Cell Systems: Challenges and New Frontiers in Experimental Biology*, Society for Experimental Biology, London 2011, pp. 93–118.

[47] Bardor, M., Faveeuw, C., Fitchette, A.-C., Gilbert, D. et al., Immunoreactivity in mammals of two typical plant glyco-epitopes, core α(1,3)-fucose and core xylose. *Glycobiology* 2003, *13*, 427–434.

[48] Manduzio, H., Fitchette, A.-C., Hrabina, M., Chabre, H. et al., Glycoproteins are species-specific markers and major IgE reactants in grass pollens. *Plant Biotechnol. J.* 2012, *10*, 184–194.

[49] Runkel, L., Meier, W., Pepinsky, R. B., Karpusas, M. et al., Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). *Pharm. Res.* 1998, *15*, 641–649.

[50] van Beers, M. M. C., Jiskoot, W., Schellekens, H., On the role of aggregates in the immunogenicity of recombinant human interferon beta in patients with multiple sclerosis. *J. Interferon Cytokine Res.* 2010, *30*, 767–775.

[51] Gribben, J. G., Devereux, S., Thomas, N. S. B., Keim, M. et al., Development of antibodies to unprotected glycosylation sites on recombinant human GM-CSF. *Lancet* 1990, *335*, 434–437.

[52] Tatsumi, Y., Sasahara, Y., Kohyama, N., Ayano, S. et al., Introducing site-specific glycosylation using protein engineering techniques reduces the immunogenicity of β-lactoglobulin. *Biosci. Biotechnol. Biochem.* 2012, *76*, 478–485.

[53] Walsh, G., Jefferis, R., Post-translational modifications in the context of therapeutic proteins. *Nat. Biotechnol.* 2006, *24*, 1241–1252.

[54] Basu, A., Yang, K., Wang, M., Liu, S. et al., Structure-function engineering of interferon-β-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjug. Chem.* 2006, *17*, 618–630.

[55] Baker, D. P., Lin, E. Y., Lin, K., Pellegrini, M. et al., N-terminally PE-Gylated human interferon-β-1a with improved pharmacokinetic properties and in vivo efficacy in a melanoma angiogenesis model. *Bioconjug. Chem.* 2006, *17*, 179–188.

[56] Manning, M., Chou, D., Murphy, B., Payne, R., Katayama, D., Stability of protein pharmaceuticals: an update. *Pharm. Res.* 2010, *27*, 544–575.

[57] Torosantucci, R., Mozziconacci, O., Sharov, V., Schöneich, C., Jiskoot, W., Chemical modifications in aggregates of recombinant human insulin induced by metal-catalyzed oxidation: covalent cross-linking via Michael addition to tyrosine oxidation products. *Pharm. Res.* 2012, *29*, 2276–2293.

[58] Cleland, J., Powell, M., Shire, S., The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Ther. Drug Carrier Syst.* 1993, *10*, 307–377.

[59] Chen, W., Ede, N. J., Jackson, D. C., McCluskey, J., Purcell, A. W., CTL recognition of an altered peptide associated with asparagine bond rearrangement. Implications for immunity and vaccine design. *J. Immunol.* 1996, *157*, 1000–1005.

[60] Doyle, H. A., Zhou, J., Wolff, M. J., Harvey, B. P. et al., Isoaspartyl post-translational modification triggers anti-tumor T and B lymphocyte immunity. *J. Biol. Chem.* 2006, *281*, 32676–32683.

[61] Rasheed, Z., Ali, R., Reactive oxygen species damaged human serum albumin in patients with type 1 diabetes mellitus: biochemical and immunological studies. *Life Sci.* 2006, *79*, 2320–2328.

[62] Filipe, V. L. S., Chapter 9: Immunogenicity of different stressed monoclonal IgG formulations in immune tolerant transgenic mice, in: *Monoclonal antibody aggregates: physicochemical characteristics, stability in biological fluids and immunogenicity*, PhD thesis, Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands 2012, pp.171–200.

[63] Hermeling, S., Aranha, L., Damen, J. M. A., Slijper, M. et al., Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b. *Pharm. Res.* 2005, *22*, 1997–2006.

[64] van Beers, M. M. C., Sauerborn, M., Gilli, F., Brinks, V. et al., Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. *Pharm. Res.* 2011, *28*, 2393–2402.

[65] Zhou, S., Schöneich, C., Singh, S., Biologics formulation factors affecting metal leachables from stainless steel. *AAPS PharmSciTech.* 2011, *12*, 411–421.

[66] Cromwell, M. E. M., Hilario, E., Jacobson, F., Protein aggregation and bioprocessing. *AAPS J.* 2006, *8*, E572–E579.

[67] Dresser, D. W., Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunology* 1962, *5*, 378–388.

[68] Weksler, M. E., Bull, G., Schwartz, G. H., Stenzel, K. H., Rubin, A. L., Immunologic responses of graft recipients to antilymphocyte globulin: effect of prior treatment with aggregate-free gamma globulin. *J. Clin. Invest.* 1970, *49*, 1589–1595.

[69] Seidl, A., Hainzl, O., Richter, M., Fischer, R. et al., Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity. *Pharm. Res.* 2012, 1–14.

[70] Brinks, V., Jiskoot, W., Schellekens, H., Immunogenicity of therapeutic proteins: the use of animal models. *Pharm. Res.* 2011, *28*, 2379–2385.

[71] Fradkin, A. H., Carpenter, J. F., Randolph, T. W., Immunogenicity of aggregates of recombinant human growth hormone in mouse models. *J. Pharm. Sci.* 2009, *98*, 3247–3264.

[72] Braun, A., Kwee, L., Labow, M. A., Alsenz, J., Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm. Res.* 1997, *14*, 1472–1478.

[73] Hermeling, S., Schellekens, H., Maas, C., Gebbink, M. F. B. G. et al., Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. *J. Pharm. Sci.* 2006, *95*, 1084–1096.

[74] Pisal, D. S., Kosloski, M. P., Middaugh, C. R., Bankert, R. B., Balu-iyer, S. V., Native-like aggregates of factor VIII are immunogenic in von Willebrand factor deficient and hemophilia a mice. *J. Pharm. Sci.* 2012, *101*, 2055–2065.

[75] Vos, Q., Lees, A., Wu, Z. Q., Snapper, C. M., Mond, J. J., B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol. Rev.* 2000, *176*, 154–170.

[76] Dintzis, H. M., Dintzis, R. Z., Vogelstein, B., Molecular determinants of immunogenicity: the immunon model of immune response. *Proc. Natl. Acad. Sci. USA* 1976, *73*, 3671–3675.

[77] Chackerian, B., Lenz, P., Lowy, D. R., Schiller, J. T., Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J. Immunol.* 2002, *169*, 6120–6126.

[78] Jegerlehner, A., Tissot, A., Lechner, F., Sebbel, P. et al., A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 2002, *20*, 3104–3112.

[79] Wang, W., Singh, S. K., Li, N., Toler, M. R. et al., Immunogenicity of protein aggregates - Concerns and realities. *Int. J. Pharm.* 2012, *431*, 1–11.

[80] Narhi, L. O., Schmit, J., Bechtold-Peters, K., Sharma, D., Classification of protein aggregates. *J. Pharm. Sci.* 2012, *101*, 493–498.

[81] Carpenter, J. F., Randolph, T. W., Jiskoot, W., Crommelin, D. J. A. et al., Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. *J. Pharm. Sci.* 2009, *98*, 1201–1205.

[82] Singh, S. K., Afonina, N., Awwad, M., Bechtold-Peters, K. et al., An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. *J. Pharm. Sci.* 2010, *99*, 3302–3321.

[83] den Engelsman, J., Garidel, P., Smulders, R., Koll, H. et al., Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. *Pharm. Res.* 2011, *28*, 920–933.

[84] Zölls, S., Tantipolphan, R., Wiggenhorn, M., Winter, G. et al., Particles in therapeutic protein formulations, Part 1: overview of analytical methods. *J. Pharm. Sci.* 2012, *101*, 914–935.

[85] Bee, J. S., Davis, M., Freund, E., Carpenter, J. F., Randolph, T. W., Aggregation of a monoclonal antibody induced by adsorption to stainless steel. *Biotechnol. Bioeng.* 2010, *105*, 121–129.

[86] Fradkin, A. H., Carpenter, J. F., Randolph, T. W., Glass particles as an adjuvant: a model for adverse immunogenicity of therapeutic proteins. *J. Pharm. Sci.* 2011, *100*, 4953–4964.

[87] van Beers, M. M. C., Gilli, F., Schellekens, H., Randolph, T. W., Jiskoot, W., Immunogenicity of recombinant human interferon beta interacting with particles of glass, metal, and polystyrene. *J. Pharm. Sci.* 2012, *101*, 187–199.

[88] Luo, Q., Joubert, M. K., Stevenson, R., Ketchem, R. R. et al., Chemical modifications in therapeutic protein aggregates generated under different stress conditions. *J. Biol. Chem.* 2011, *286*, 25134–25144.

[89] Castillo, V., Graña-Montes, R., Sabate, R., Ventura, S., Prediction of the aggregation propensity of proteins from the primary sequence: aggregation properties of proteomes. *Biotechnol. J.* 2011, *6*, 674–685.

[90] Fernandez-Escamilla, A.-M., Rousseau, F., Schymkowitz, J., Serrano, L., Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins. *Nat. Biotechnol.* 2004, *22*, 1302–1306.

[91] Lee, C. J., Seth, G., Tsukuda, J., Hamilton, R. W., A clone screening method using mRNA levels to determine specific productivity and product quality for monoclonal antibodies. *Biotechnol. Bioeng.* 2009, *102*, 1107–1118.

[92] Rifkin, R., Maggio, E., Dike, S., Kerr, D., Levy, M., n-Dodecyl-β-D-maltoside inhibits aggregation of human interferon-β-1b and reduces its immunogenicity. *J. Neuroimmune Pharmacol.* 2011, *6*, 158–162.

[93] Buettel, I. C., Chamberlain, P., Chowers, Y., Ehmann, F. et al., Taking immunogenicity assessment of therapeutic proteins to the next level. *Biologicals* 2011, *39*, 100–109.

[94] Bozhinov, A., Handzhiyski, Y., Genov, K., Daskalovska, V. et al., Advanced glycation end products contribute to the immunogenicity of IFN-β pharmaceuticals. *J. Allergy Clin. Immunol.* 2012, *129*, 855–858.

[95] van Beers, M. M. C., Sauerborn, M., Gilli, F., Brinks, V. et al., Aggregated recombinant human interferon beta induces antibodies but no memory in immune-tolerant transgenic mice. *Pharm. Res.* 2010, *27*, 1812–1824.

[96] Seefeldt, M. B., Rosendahl, M. S., Cleland, J. L., Hesterberg, L. K., Application of high hydrostatic pressure to dissociate aggregates and refold proteins. *Curr. Pharm. Biotechnol.* 2009, *10*, 447–455.