# Oxidation, aggregation and immunogenicity of therapeutic proteins

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# Chapter 1

**General introduction**

## Introduction

Recombinant human therapeutic proteins are macromolecules widely used to treat a broad number of diseases for which often no other class of drugs is effective [1].

Administration of highly purified recombinant human therapeutic proteins, however, despite their similarity to their endogenous counterparts, frequently induces the production of anti-drug antibodies (ADAs) [2]. ADAs can be divided into binding antibodies (BABs), which bind to protein regions not relevant for the biological activity, and neutralizing antibodies (NABs) recognizing the active site of the protein. BABs can affect the clearance of the protein and thereby indirectly change its therapeutic efficacy, while NABs directly neutralize the protein's biological activity [3]. These adverse pharmacological consequences resulting from the immunogenicity of therapeutic proteins are a major obstacle during their development.

## Factors influencing immunogenicity

Several factors affect the immunogenicity of therapeutic proteins, including structural aspects (e.g., primary structure, higher-order structures, glycosylation, PEGylation), formulation, treatment regimen, route of administration, co-medication and genetic background of the patient [2].

In addition, the absence of standardized assays for measuring ADAs makes it extremely difficult to compare results between laboratories and between published studies.

Among the product-related factors, structural modifications like aggregation, chemical degradation (particularly oxidation) and combinations thereof are important risk factors for immunogenicity and are therefore discussed in more detail below.

## Aggregation

Aggregation concerns processes by which the smallest naturally occurring monomeric subunit forms larger assemblies, which can be as small as a dimer and as large as a visible precipitate [4]. Such assemblies, hereafter referred to as protein aggregates, can be formed during production, transportation,

storage and administration, and are often accompanied by other physical and chemical degradation processes [4].

Several non-clinical and clinical studies have related therapeutic protein aggregates to immunogenicity [2-3, 5-6]. For instance, the effect of different recombinant human interferon alfa (rhIFNα) aggregates on the immunogenicity has been studied in immune tolerant mouse models. Braun et al. [7] upon i.p. injection of different aggregated rhIFNα-2a formulations, found that all the aggregates tested were highly immunogenic in transgenic immune tolerant Balb/C mice. Similarly, various aggregates of rhIFNα-2b induced ADAs in transgenic immune tolerant mice [8]. Also recombinant human interferon beta (rhIFNβ) aggregation has been correlated with enhanced immunogenicity. Furthermore, it has been demonstrated that the removal of protein aggregates from rhIFNβ formulations reduces the immunogenicity of rhIFNβ [9-11]. In addition to that, rhIFNβ-1b (Betaferon®), which contains more aggregates than rhIFNβ-1a products (Avonex®, Rebif®), has been found to be the most immunogenic product in several preclinical and clinical studies [12].

## Chemical degradation

Oxidation and deamidation are major chemical degradation processes in proteins. One of the impurities found in rhIFNα-2a formulations because of improper storage was an oxidized form [13]. Although the oxidation sites were not determined, in a clinical trial it emerged that the oxidized and aggregated form was more immunogenic when compared with the native counterpart [13].

Particularly interesting are observations that several protein aggregates induced via metal catalyzed oxidation are immunogenic in preclinical models. For instance, Hermeling et al. showed that upon oxidative stress induced by $Cu^{2+}$/ascorbate catalyzed oxidation, rhIFNα-2b formed aggregates that were more immunogenic than other types of rhIFNα-2b aggregates [8, 14]. Similarly, rhIFNβ-1a aggregates [15] and monoclonal antibody aggregates [16] (IgG1), both produced via the same oxidative system, were shown to be highly immunogenic in transgenic immune tolerant mouse models. In addition, immunization of $H^{2k}$ mice with oxidized insulin B chain yielded a higher immune response when compared with the non oxidized form [17].

Deamidation, which mainly occurs at asparagine residues, could elicit immune responses as a result of generation of newly exposed antigenic

determinants [18]. Deamidated serum albumin is immunogenic and potentially could induce autoimmune reactions [19]. Furthermore, deamidation of glutamine residues in gliadin peptides is critical for the creation of epitopes involved in coeliac autoimmune disease [20].

## Mouse models to predict the immunogenicity of protein therapeutics and to investigate underlying immune mechanisms

Nearly all protein therapeutics are immunogenic. Immunogenicity prediction and prevention is an important issue to consider when developing novel therapeutic products.

To this end, animal models are increasingly used to study immunogenicity of therapeutic proteins. The employment of classical non transgenic animal models is not suitable for immunological studies of non conserved proteins, which being foreign for the animal will induce a classical immune reaction [21].

Using transgenic immune tolerant mouse models overcome this problem, since these mice express the human endogenous counterpart of the therapeutic protein under investigation. Such models have been used mainly to investigate product-related factors, especially the presence of various structurally different protein aggregates, involved in immunogenicity [5, 15].

The use of animals rather than in vitro models has the advantage that the immune mechanisms underlying immunogenicity of therapeutic proteins can be studied in an organism with intact immune system and responses.

## Aim and outline of the thesis

The aim of the research described in this thesis is to study the chemical mechanisms responsible for protein aggregation induced by metal catalyzed oxidation and to investigate the relationship between protein oxidation, aggregation and immunogenicity. To this end, recombinant human insulin (further referred to as insulin), rhIFNβ-1a and rhIFNα-2a are used in conjunction with transgenic mice immune tolerant for the respective human endogenous counterparts.

The impact of PEGylation on aggregate formation and excipients to prevent metal catalyzed oxidation are also evaluated.

**Chapter 2** reviews the structural and biological consequences of oxidation of protein and peptide therapeutics.

**Chapter 3** evaluates the susceptibility of PEGylated insulin on aggregation upon chemical and physical stress. Insulin is conjugated on lysine B29 with 5-kDa PEG. Next, insulin and PEG-insulin are subjected to heating, metal-catalyzed oxidation, and glutaraldehyde mediated cross-linking. Finally, the products are characterized physicochemically by complementary analytical methods.

**Chapter 4** investigates the chemical mechanism responsible for insulin aggregation during metal-catalyzed oxidation. Bivalent copper and ascorbate are used as oxidative system and liquid chromatography electrospray ionization tandem mass spectrometry (ESI-LC-MS/MS) is employed for identifying the target of oxidation and cross-links mediating insulin aggregation.

**Chapter 5** describes the antioxidant properties of several excipients, which are evaluated by quantifying aggregate content and chemical degradation in several insulin formulations exposed to bivalent copper and ascorbate.

**Chapter 6** aims to study the chemical changes in aggregated and oxidized rhIFNβ-1a, generated by metal catalyzed oxidation, and the correlation between the structural changes in the aggregated protein and its immunogenicity, previously evaluated in transgenic mice immune tolerant for rhIFNβ.

**Chapter 7** sheds light on the immune mechanism responsible for the immunogenicity of oxidized and aggregated rhIFNα-2a and studies if the presence of aggregated rhIFNβ increases rhIFNα's immunogenicity, using transgenic mice immune tolerant for the human protein. The presence of immunological memory is evaluated after rechallenge with aggregated or native rhIFNα and depletion from CD4+ T-cells is used to test for CD4+ T-cell involvement in immunogenicity.

**Chapter 8** describes the development and the application of a transgenic mouse model for studying the immunogenicity of insulin. The immunogenicity of aggregated and oxidized insulin, non-aggregated oxidized insulin and several commercial insulin formulations is evaluated.

**Chapter 9** summarizes the findings and conclusions of this thesis and discusses the perspectives for further research on strategies to prevent protein degradation and analytical tools for protein characterization.

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