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Oxidation, aggregation and immunogenicity of therapeutic proteins

Riccardo Torosantucci

Oxidation, aggregation and immunogenicity of therapeutic proteins

Oxidatie, aggregatie en immunogeniciteit van therapeutische eiwitten
(met een samenvatting in het Nederlands)

Proefschrift

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Riccardo Torosantucci

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      Prof. dr. ir. W.E. Hennink, Universiteit Utrecht

"Memento audere semper"

Gabriele D'Annunzio

Ad Alberto, Antonella, Gaetana ed Olga Ekin

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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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Chapter 2

Oxidation of therapeutic proteins and peptides: structural and biological consequences

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Abstract

Oxidation is a common degradation pathway that affects therapeutic proteins and peptides during production, purification, formulation, transportation, storage and handling of solid and liquid preparations. In the present work we review the scientific literature about structural and biological consequences of protein/peptide oxidation. Representative examples are discussed of specific products whose oxidation has been recently studied, including monoclonal antibodies, calcitonin, granulocyte colony-stimulating factor, growth hormone, insulin, interferon alpha and beta, oxytocin and parathyroid hormone. These examples illustrate that oxidation often leads to modifications of higher-order structures, including aggregate induction, and can generate products that are pharmacokinetically different, biologically less active and/or potentially more immunogenic than their native counterpart. It is therefore crucially important during the pharmaceutical development of therapeutic proteins and peptides to comprehensively characterize oxidation products and evaluate the impact of oxidation-induced structural modifications on the biological properties of the drug.

Introduction

In the last thirty years proteins and peptides have gained importance in the treatment of a broad number of diseases for which no other therapy is available [1]. Instability, however, represents a serious problem in the development of therapeutic proteins and peptides [2].

In particular oxidation, which has been reported to occur during production [3], purification [4], formulation [5] and storage [6], is a major concern [7], as it can extensively modify the primary structure of proteins and peptides, by which changes in secondary, tertiary and quaternary structure may arise [8-10]. Whereas there are several excellent reviews describing oxidation mechanisms [11], products of amino acid oxidation [12-16], the biochemical basis of protein oxidation [17-18] and strategies to prevent oxidation [2, 11-12, 17], to the best of our knowledge, only one review, published twenty years ago, described the pharmaceutical consequences of protein oxidation [19]. At that time, however, experimental data about biological consequences of oxidation were scarce.

Here we aim to give an update on the current knowledge about the consequences of oxidative modification for amino acid residues (i.e. primary structure), higher-order structures (i.e. secondary, tertiary and quaternary structure), biological activity, half-life and immunogenicity of several protein and peptide therapeutics.

After briefly introducing the potential causes of oxidation during production, purification, formulation and storage, we will discuss the consequences of oxidation for: monoclonal antibodies (mAbs), calcitonin (CT), granulocyte colony-stimulating factor (G-CSF), growth hormone (GH), insulin, recombinant human interferon alpha-2a (IFNα-2a) interferon alpha-2b (IFNα-2b) and interferon beta-1a (IFNβ-1a), oxytocin and parathyroid hormone (PTH).

Oxidation of proteins and peptides

Most biopharmaceuticals are produced by recombinant DNA technologies, usually by employing microbial hosts like E. coli [20] or mammalian cells like Chinese hamster ovary (CHO) cells [21]. Already during the production steps, the concentration of dissolved oxygen (DO) can influence the oxidative state of therapeutic proteins, as demonstrated for the production in E. coli of recombinant human IFNγ, where an increase in carbonyl groups [18] (a general

marker of oxidative modification) correlated with a relatively high DO concentration (i.e. 60% DO), suggesting that the aerobic environment should be scrupulously monitored [3]. However, also low oxygen concentration (a condition known as hypoxia) may induce oxidative stress through the production of reactive oxygen species in mammalian host cells, likely generated by electrons leaking from the mitochondrial electron transport chain [22-25]. In support of this, oxidation-induced fragmentation of recombinant human IgG1 produced in CHO cells was observed in the purified material and, interestingly, the same degradation was reproduced by *in vitro* incubation of the protein with hydrogen peroxide [26].

Besides oxidation that may arise during the production in cell culture, oxidation can occur in the subsequent downstream processes. For instance, purification of lactate dehydrogenase, using metal affinity chromatography, yielded an oxidized product [4].

During formulation and storage several excipients and impurities can directly or indirectly favor oxidation. For instance, hydrogen peroxide has been encountered as an impurity in polymeric excipients such as polyethylene glycol (PEG) or polysorbate [5, 27]. Additionally, these polymeric excipients can spontaneously oxidize in aerobic environment, without the aid of a catalyst (auto-oxidation) [28-29], generating several peroxides. Among the impurities which might favor oxidation, transition metals represent a common threat, as they can catalyze oxidation reactions [14] already at submicromolar concentration [30]. Furthermore, transition metals being air pollutants [31-32] may contaminate buffers [33] and excipients such as sugars, surfactants and amino acids [34]. Also, they can be released from containers [11], making it difficult to fully avoid their presence in formulations.

Chemical modifications in amino acids induced by oxidation

Potentially all 20 natural amino acids can be oxidized [16], however, cysteine (Cys), histidine (His), methionine (Met), phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) are generally most prone to oxidation, due to the high reactivity of sulfur atoms and aromatic rings towards various reactive oxygen species [12]. Table I provides a comprehensive summary of reported cases of protein/peptide oxidation, including the chemical changes in primary and higher-order structures, as well as observed biological consequences.

Table I. Summary of reported structural and biological consequences of oxidation of protein and peptide therapeutics.

Protein [a]	Oxidative stress [b]	Chemical changes in primary structure [c]	Changes in higher-order structure	Biological consequences	Ref.
IgG1 (humanized monoclonal)	H_2O_2	M252 (HC [d]), M428 (HC), M358 (HC)	Reduced melting temperature (Tm) of CH2 domain	Decreased binding to FcRn	49
	H_2O_2	M252 (HC), M428 (HC)	Subtle conformational changes	Reduction of serum half-life	50, 52
IgG1 Fc (human, E. coli derived)	H_2O_2	M33 (HC), M209 (HC)	Altered secondary and tertiary structure; Reduced melting temperature of CH2 domain	n.i.; n.i.	51
IgG1 (not specified)	H_2O_2	M34 (HC), M83 (HC), M253 (HC), M429 (HC), M4 (LC)	Large structural change in the region of heavy chain residues 247–253; Smaller changes in CH2 and CH3 domains	n.d.	54, 55
IgG1 (human monoclonal)	TBHP	M252 (HC), M428 (HC)	Changes in secondary and tertiary structure; Formation of (mainly submicron) aggregates	Decreased binding affinity for protein A, protein G and FcRn	48, 38

Type	Condition	Residues	Effect on structure	Effect	Ref
IgG1 (HER2)	TBHP, H_2O_2 and storage	M83 (HC), M253 (HC), W32 (LC)	Aggregation and fragmentation	n.i.	59
IgG1 (humanized MEDI-493)	ultraviolet (UV) light irradiation	W105 (HC)	No significant changes in secondary structure	Loss of target binding and biological activity	60
IgG1 (fully human)	production in CHO cell culture, H_2O_2	M, C, H	Hinge region fragmentation	n.i.	26
IgG1	Cu^{2+}/ascorbate	n.i.	Changes in secondary and tertiary structure Aggregation	Immunogenic in transgenic immune tolerant mice	56
IgG2	Cu^{2+}/ascorbate	M34 (HC) M82 (HC) M246 (HC) M352 (HC) M391 (HC) M422 (HC) H304 (HC) H427 (HC) W156 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
	H_2O_2	M34 (HC), M246 (HC), M352 (HC), M391 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58

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	Condition	Residues	Effects		References
	Thermal stress	M34 (HC) M246 (HC) M422 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
	Mechanical stress	M34 (HC) M82 (HC) M246 (HC) M352 (HC) M391 (HC) M422 (HC) W36 (LC) W47 (LC) W98 (LC) W156 (HC) W271 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
sCT	H_2O_2	n.i.	Formation of fibrillar aggregates	n.i.	66
	Storage	C	Dimerization involving Cys residues and trisulfide variants	n.d.	67
	Fe^{2+}/ascorbate/light	n.i.	Changes in secondary structure Formation of fibrillar aggregates	n.i.	61
G-CSF	H_2O_2	M1, M122, M127, M138	Conformational changes	Altered biological activity and serum half life	68, 69

	Oxidant	Residues	Structural effect	Functional effect	Ref
	TBHP	M1, M122, M127, M138	Conformational changes	Altered biological activity and serum half life	69
GH	H$_2$O$_2$	M14, M125	Subtle conformational changes	Minor effect on receptor binding and *in vitro* biological potency	73
	H$_2$O$_2$	M14, M125	Subtle conformational changes	Decreased thermal stability	74
	Cu^{2+}/ascorbate	H18, H21	Subtle conformational changes; Formation of dimers	n.i.	80
	AAPH	M14, M125, M170, L101, Y103	Aggregation	n.i.	78
Insulin	Storage	C	Aggregation	n.i.	83
	Cu^{2+}/ascorbate	H (B5, B10), F (B1, B24, B25), Y (A14, A19, B16, B26)	Changes in secondary and tertiary structure; Aggregation	Immunogenic in transgenic immune tolerant mice	9, 10

	Cu^{2+}/H_2O_2 And in vitro incubation with plasma from diabetic patients	F and Y oxidation products	n.i.	Decreased hypoglycemic activity	96
	Cu^{2+}/H_2O_2	F and Y oxidation products	Aggregation	Decrease in the insulin-dependent glucose utilization by human adipose tissue	97
IFN alpha-2a	H_2O_2	n.i.	n.i.	Reduced biological activity	99
	Storage	n.i.	n.i.	Enhanced immunogenicity	100, 101
	$Cu^{2+}/$ascorbate	n.i.	Changes in secondary structure Aggregation	Immunogenic in transgenic immune tolerant mice	(e)
IFN alpha-2b	$Cu^{2+}/$ascorbate	M16 M21 M148	Changes in secondary structure Aggregation	Immunogenic in transgenic immune tolerant mice	105
	Storage	M111	Changes in secondary structure	n.d.	104

IFN beta-1a	Storage	C	Disulfide linked aggregates	n.i.	107
	Cu^{2+}/ascorbate	n.i.	Structural changes Aggregation	n.i.	109
Oxytocin	Storage at different pH and temperature	C Y	Tri- and tetra sulfide derivatives Dityrosine linked dimers	n.i.	112
PTH	Storage	n.i.	Changes in secondary structure Aggregation	n.i.	116
	H_2O_2	M8 M18	Changes in secondary structure	n.i.	118
	H_2O_2	M8 M18	n.i.	Reduced biological activity	119
bPTH	H_2O_2	M8	n.i.	Reduced biological activity	121
pPTH	H_2O_2	M8	n.i.	Reduced biological activity	122

a: sCT: salmon calcitonin; G-CSF: granulocyte colony-stimulating factor; GH: growth hormone; PTH: parathyroid hormone.

b: TBPH: tert-butylhydroperoxide; AAPH: 2,2′- azobis(2-methylpropionamidine).

c: Amino acid residues in one letter code: C: cysteine; F: phenylalanine; H: histidine; M: methionine; W: tryptophan; Y: tyrosine.

d: Abbreviations: A and B: insulin chain A and chain B, respectively; HC and LC: IgG heavy chain and light chain, respectively; n.d.: not detected; n.i.: not investigated.

e: unpublished data by Sauerborn et al.

As oxidative modifications at the amino acid level have been extensively reviewed elsewhere [12-13, 16], we focus the discussion below on the higher-order structural consequences and the biological consequences observed for several representative protein and peptide drugs.

Consequences of protein and peptide oxidation

Monoclonal antibodies

All human IgGs feature a characteristic "Y" shape [35]: the lower part contains a single crystallizable region (Fc) critical for effector functions and half-life [36]. The upper part consists of two identical regions (Fab) that contain the complementarity determining regions (CDRs) responsible for antigen binding [37]. Most mAbs belong to the IgG1 and IgG2 subclasses, which share 97% of sequence homology [38]. The Fc region can contain up to four Met residues: Met residues at positions 252 and 428 (based on the Eu numbering system [39]) are conserved in all IgGs [40], the presence of Met 358 in IgG1 is dependent on the allele of the gene [41], while Met 397 is only present in IgG2 and IgG3 [42]. Modification of any of these Met residues may adversely affect the Fc-dependent effector function of mAbs [42].

Several authors investigated the susceptibility to oxidation of Met residues under different stress or storage conditions [43-47]. However, few studies reported the consequences of such modifications on protein structure and pharmacokinetics.

Oxidation of Met 252 and Met 428 reduced the binding with Protein A [48] (a protein often used in affinity chromatography) and the neonatal Fc receptor (FcRn) [38, 49]. This can reduce the biological half-life of the antibody, as shown by Wang et al. [50], who demonstrated that a mAb containing 80% of oxidized Met 252 features more than 4-fold reduction in the half-life in transgenic mice with human FcRn. When the percentage of Met oxidation was lower, i.e. 40%, the measured half-life was comparable to that of the native mAb.

Liu et al. [51] noticed that hydrogen peroxide-induced oxidation of Met residues in E. coli-expressed Fc, resulted in alteration of secondary and tertiary structure, evaluated by circular dichroism spectroscopy, and in a reduced melting temperature of the CH2 domain (note that Liu et al. referred to Met 33 and Met 209, which correspond to Met 252 and Met 428 on the

intact heavy chain sequence). It must be mentioned that the Fc used was produced in E. coli and thus lacks glycosylation, which is important for protein stability: Met oxidation in a glycosylated IgG1 led to similar changes in the thermal stability but conformational changes of the antibody with oligosaccharides where minor, indicating a partial protective effect of the sugar moiety [52-53]. Interestingly, also the deamidation rate of Asn 67 and Asn 96 increased, likely as a result of Met oxidation-mediated conformational changes [51]. Destabilization of the α-helix of the residues 247-253 of the Fc region of IgG1 was also observed upon hydrogen peroxide treatment [54-55]. These findings suggest that oxidation of Met residues can result in conformational changes of mAbs.

Metal catalyzed oxidation (MCO) of a monoclonal human IgG, induced by Cu^{2+}/ascorbate, generated mainly micron-sized aggregates with secondary and tertiary structure alterations that were immunogenic in a transgenic, immune-tolerant mouse model [56]. A monoclonal IgG2 was evaluated under similar stress conditions and the authors observed severe changes in secondary and tertiary structure, associated with the site specific oxidation of His 304 and His 427, besides oxidation of several Met residues and of Trp 156 [57-58]. Also particles in the size range between 0.2-10 μm were detected.

Similarly, oxidation of IgG2 with hydrogen peroxide modified the higher order structural properties of the protein and induced the formation of polydisperse aggregates. In addition to Met oxidation, Trp oxidation was observed but not the oxidation of His [57-58].

Hensel et al. suggested that the oxidation of Trp 32 (in the CDR region of the light chain) was mainly responsible for the progressive loss of target binding and biological activity [59]. Similarly, the oxidation of Trp 105, a residue in the CDR3 of the heavy chain of a humanized mAb against respiratory syncytial virus, was considered responsible for the activity loss [60]. Altogether, these results demonstrate that Met is only one of the potential targets of oxidation and oxidation frequently compromises the conformation and biological functions of monoclonal IgGs.

Calcitonin

CT is a polypeptide hormone of 32 amino acids which, in aqueous solution, assumes an unstructured conformation [61]. Mainly human and salmon

calcitonin (hCT and sCT, respectively) are used for therapeutic purposes. The two polypeptides share only 50% sequence homology, nonetheless higher order structural features are similar between the two hormones [61]. In aqueous solution, hCT tends to aggregate faster than sCT, causing the formation of fibrillar precipitates [61].

Aggregated and oxidized forms of hCT were observed *in vivo* in plasma under non-pathological conditions [62], justifying studies on the consequences of CT oxidation. Although CT contains Met, His, Phe and Tyr residues, all of which are potential oxidation targets, oxidation (during storage or forced oxidative stress) of this polypeptide hormone appears to affect mainly Met 8, the only Met residue available. Reduction of bioactivity was observed upon oxidation of Met 8 [63-64]; however, more recently it was found that the aggregation rate of Met oxidized hCT decreased [65], illustrating that oxidation not necessarily accelerates aggregation.

Aggregation of sCT accompanied by alteration of secondary structure was observed upon hydrogen peroxide treatment [66]: this suggests that mild oxidative conditions are capable of inducing structural changes in sCT.

Dimers involving Cys residues as well as a trisulfide derivative were measured in a different study investigating the stability of the hormone in aqueous solutions [67]. These findings suggest that aggregation involving disulfide scrambling of the thiol groups can be involved in sCT aggregation.

When testing the effect of hydroxyl radicals generated via a modified Fenton reaction (60-W tungsten lamp in combination with ferrous sulfate and ascorbic acid), sCT amyloid aggregates were detected. Interestingly, they were structurally similar to what was observed *in vivo* for hCT, in carcinoma medullary plaques [61].

In conclusion, *in vitro* oxidation of CT might produce fibrillar aggregates similar in structure as those observed *in vivo*. Met and Cys residues seem to be responsible for the observed structural changes. However, it is still poorly investigated if oxidation of His and Tyr, both present in hCT as well as sCT, can occur and contributes to aggregation or structural changes of this polypeptide.

Granulocyte colony-stimulating factor

Recombinant human G-CSF contains 175 amino acid residues, several of which are susceptible to oxidation [68].

Simultaneous oxidation of all four Met residues (in position 1, 122, 127 and 138) resulted in a dramatic decrease of the biological activity to 3% [69]. The biological activity of the HPLC fraction containing G-CSF with only Met 1 oxidized, was largely retained (i.e. 80% relative to G-CSF prior to oxidation), indicating that this residue is less important for the activity. In addition, engineered variants of G-CSF, where either Met 127 or Met 138 was replaced by leucine (Leu), were still sensitive to oxidation-induced inactivation. However, the variant with Leu replacement at both sites was more stable and retained *in vitro* biological activity following oxidative stress. All these experiments suggest that oxidation of Met 127 and Met 138 accounted for most of the activity loss [69].

Besides oxidation of Met residues in G-CSF, Cys oxidation is a point of concern. Under physiological conditions (37 °C, pH 7.0), G-CSF showed a significant propensity to aggregate. Several studies demonstrated that the free Cys in position 17, upon oxidation, forms a new disulfide bridge that is responsible for G-CSF aggregation [68, 70-71].

Growth hormone

Oxidative modifications of recombinant human growth hormone (hGH) have been widely described (Table I), mainly with respect to Met oxidation.

Relatively mild oxidative conditions, attained during exposure to hydrogen peroxide, have been reported to lead to selective generation of Met sulfoxides from the two most accessible Met residues in hGH (Met 14 and Met 125). Although this does not seem to induce gross conformational changes [72-73], the thermal stability of the protein dropped [74]. This may be due to the generation of Met 14 and Met 125 sulfoxides, which increases the polarity and the size of these amino acids; furthermore the new hydrogen bond networks that the protein can establish, may contribute to the observed decrease in thermal stability [74].

Cunningham et al. showed that Met 14 contributes only slightly to the binding of the hormone to its receptor [75]. In agreement with this study, the oxidation of Met 14 and Met 125 was reported to have little effect on hGH's receptor affinity and potency [73].

In contrast, Met 170 is located within the core of the native protein [76]. Nevertheless, the mass spectrometric analysis of a marketed hGH product

(Genotropin®, expressed in *E. coli K12*) revealed that 2% of the expressed protein contains several chemical modifications, including Met 170 sulfoxide [77]. This residue is located on the alpha helix IV of hGH, which is involved in one of the two receptor binding sites [76].

Steinmann et al. detected the oxidation of Met 170 (together with that of Met 14 and Met 125) during the exposure of hGH to peroxyl radicals generated from 2,2′-azobis(2-methylpropionamidine) (AAPH) [78]. In addition, the authors detected di-tyrosine, Leu 101 hydroperoxide and several oxidation products of Tyr 103. These oxidation conditions led to the formation of dimers (21%) and trimers (13%).

Light exposure resulted in the selective oxidation of His 21 [79]. Furthermore, MCO, induced by exposure to Cu^{2+}/ascorbate, specifically modified His 18 and His 21, which are both located on helix I and are critical for the integrity of the metal binding site of this hormone [80-81].

Insulin

One of the first reported experiments involving insulin oxidation dates back to 1948 when Frederick Sanger employed a mixture of hydrogen peroxide and formic acid, which generates performic acid, to fractionate insulin's A and B chains. Previously reduced Cys residues were oxidized to cysteic acid and also Tyr oxidation products were observed [82]. Since then, insulin oxidation has been extensively investigated.

Covalent aggregation of lyophilized insulin was observed upon storage at different temperatures and moisture contents [83]. Reduction of the native disulfide bridge followed by re-oxidation was responsible for new intermolecular disulfide bridges that mediate aggregate formation. Furthermore, aggregation involving Cys residues does not necessarily require the presence of this amino acid in its reduced form (i.e. free thiol groups) [83].

Therapeutic formulations of insulin, in solution or in suspension, analyzed after long term stability studies contained dimers and oligomers resulting from reduction-oxidation of Cys residues [83]. Formation of insulin aggregates with altered 3D structure was observed upon MCO using Cu^{2+}/ascorbate. In particular, the Tyr oxidation products 3,4-dihydroxyphenylalanine (DOPA) and 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH) were observed [9]. The latter, being an electrophile, was shown to be involved

in covalent cross-links with several amino groups of the insulin molecule, which led to new intra- and intermolecular cross-links. This oxidized and aggregated insulin induced anti-insulin antibodies when injected in transgenic mice immune tolerant for human insulin (unpublished data). MCO also led to the oxidation of His B5 and B10, which are important binding sites for zinc ions that play a central role in the formation of insulin's quaternary structure [84-85].

In the presence of zinc ions, insulin exists as hexamers [86], which are the main components in several long-acting therapeutic insulin formulations [87]. Oxidative stress that targets insulin's His residues involved in zinc ion binding can therefore result in unexpected pharmacokinetics.

Several studies have investigated the oxidative modifications that insulin can undergo in diabetic patients. Subjects affected by diabetes have generally high glucose plasma concentrations (hyperglycemia) and display oxidative stress associated with a decrease in the concentration of biological antioxidants such as reduced glutathione (GSH) [88]. The former event is responsible for glycation of insulin, i.e. the formaton of a covalent adduct between insulin and glucose, where insulin is oxidized (loss of hydrogen atoms from amino groups).

Glycated insulin has been measured *in vivo* and its biological activity was decreased [89]. Another important consequence of hyperglycemia is the generation of α-oxoaldehydes like glyoxal, methylglyoxal and 3-deoxyglucosone [90], which can react with insulin generating aggregates of oxidized insulin [91].

Furthermore oxidative stress is responsible for lipid peroxidation of n-3 and n-6 polyunsaturated fatty acids, which eventually generates reactive aldehydes such as 4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal [92]. The reaction between insulin and these reactive aldehydes (α, β unsaturated carbonyl compounds) occurs through Michael addition and introduces new carbonyl groups in the insulin molecule [92]. Glucose uptake as well as the hypoglycemic effect in mice was significantly reduced after treatment with insulin oxidized with reactive aldehydes, compared to treatment with native insulin [92].

More recently, based on the hypothesis that the plasma copper ion concentration is higher in diabetic patients than in normal subjects, Cheng et

al. studied the copper induced catalyzed oxidation of glycated insulin, which yielded aggregates, fragments and oxidation products [93].

Similarly, Guedes et al. investigated MCO of glycated insulin using the Fenton reaction, which induced aggregation and fragmentation of oxidized glycated insulin [94]. It is noteworthy that besides oxidation, insulin was found to be glycated on several sites including the N-terminal Gly A1, which is important for the biological activity [94-95].

Montes-Cortes et al. [96] discovered that the incubation of insulin with plasma from diabetic patients resulted in Tyr oxidation products, increased carbonyl content and decreased biological activity, similar to what observed upon Fenton oxidation of insulin [96-97]. These results suggest that a correlation between *in vivo* and *in vitro* oxidation may exist and that oxidative modifications on the insulin molecule can decrease the biological activity of this polypeptide hormone.

Interferon alpha

During storage, particularly at neutral and acidic conditions, IFNα-2a is known to undergo oxidation of Met residues [98].

Hydrogen peroxide-induced oxidation generated an IFNα-2a variant that featured reduced specific biological activity [99], but the sites of oxidation were not determined.

Immunogenicity of oxidized and aggregated IFNα-2a, formulated as lyophilized powder and stored at ambient temperature, was evaluated in patients: the oxidized form was more immunogenic than several other formulations of non-oxidized rhIFNα-2a [100-101]. Recently IFNα-2a oxidized by Cu^{2+}/ascorbate was found to undergo structural modifications and aggregation; this product was immunogenic in a transgenic mouse model immune-tolerant for human IFNα-2a (unpublished results).

In IFNα-2b, all of the 5 Met residues are sensitive to oxidation in solution under different tested storage conditions [102]. Here, Met 111 oxidizes very easily and IFNα-2b containing oxidized Met 111 has been detected in a cream for topical use [103]. The alpha-helical content of the protein containing oxidized Met 111 was slightly decreased parallel to an increase in the beta-sheet contribution; however, the biological activity was not affected [104].

MCO of IFNα-2b, where Met 16, Met 21 and Met 148 were converted into the sulfoxide derivatives, generated aggregates that were immunogenic in the transgenic immune tolerant mouse model mentioned above [105].

Interferon beta

Under mild oxidative conditions, achieved with hydrogen peroxide, Orru et al. [106] observed the oxidation of the surface exposed Met 117 in IFNβ-1a, which was the most reactive Met residue, followed by the oxidation of Met 36 and Met 1. Cys residues and the carbohydrate moiety were not modified and the biological activity of the protein was fully retained, pointing to minor consequences of Met oxidation for the activity of this cytokine [106].

Free Cys 17 in IFNβ-1a can be involved in redox chemistry as demonstrated by the detection of 2% of disulfide linked aggregates after prolonged storage [107].

Furthermore, deglycosylated IFNβ-1a was more sensitive to formation of insoluble, disulfide-linked aggregates with diminished biological activity, indicating a protective role of the carbohydrate moiety [108].

The oxidation of IFNβ-1a with Cu^{2+}/ascorbate generated covalent aggregates that contained native-like epitopes, had an average diameter of 1.6 μm and were immunogenic in transgenic mice immune tolerant for human IFNβ [109]. These aggregates were shown to be cross-linked through 1,4 and 1,6-type addition at Tyr oxidation products [110]. Oxidation mediated by hydrogen peroxide of IFNβ-1a also yielded immunogenic IFNβ-1a aggregates, but the percentage of monomeric IFNβ-1a was higher compared to the MCO protein [109].

Oxytocin

Oxytocin is a small peptide which contains a six-amino acid ring (Cys1, Tyr2, Ile3, Gln4, Asn5, Cys6) and a tail of three amino acids (Pro7, Leu8, Gly9-NH2) [111]. As for CT, oxytocin formed tri- and tetrasulfide derivatives (introduction of one and two sulfur atoms, respectively, into its chemical structure) under accelerated degradation conditions at different pH and temperature [112].

Besides degradation involving sulfur atoms, heat stressed oxytocin formulations at pH 4.5, 7.0 and 9.0, generated also di-tyrosine-linked dimers, albeit at low percentages [112].

Rosei et al. showed that Tyr 2 in the oxytocin molecule, even though it is located internally in the primary sequence, functions better as hydrogen donor than free Tyr [113]. Hence, Tyr radicals, which are precursors in the generation of di-tyrosines [114], can be easily formed in the oxytocin molecule.

Parathyroid hormone

Synthetic parathyroid hormone contains two Met, three His, one Trp and one Phe, residues which are particularly oxidation-sensitive under several applied experimental conditions [34].

Oxidation of this hormone has been detected in blood from patients with renal disease [115]. During long term storage of hPTH (1-34) up to 24 weeks at room temperature, oxidation-induced aggregation and loss of secondary structure were observed. Although the oxidation sites were not determined, it was found that sucrose substantially reduced hPTH (1-34) oxidation. This protective effect was due to a more compact conformation that the hormone assumed in presence of the sugar, where amino acid residues sensitive to oxidation are more buried [116].

hPTH (1-34) features minimal tertiary structure [117], but the secondary structure is well defined and consists of approximately 33% alpha-helical content and 32% ß-sheet [118]. Circular dichroism spectroscopic studies indicated that most of the secondary structure resides in the N-terminal region of this hormone, in agreement with the findings that oxidation of Met 8, close to the N-terminal region, produces substantial changes, while oxidation of Met 18 has a small impact on the secondary structure [118]. This finding correlated well with the observed alteration in biological activity: oxidation of Met 8, caused a remarkably larger suppression of the activity when compared to that of Met 18 [119].

More recently it was discovered that oxidation of Met 8 (into a sulfide radical cation during Fenton oxidation) results in the specific hydrolysis of the peptide bond between Met 8 and His 9, suggesting that also fragmentation of PTH (1-34) can occur during oxidation catalyzed by iron (II) [120].

A similar decreased activity by oxidation was observed for bovine [121] and porcine PTH, which both share Met 8 (but not Met 18) with the human counterpart [122]. Thus, the region around Met 8 is important for the activity. Nonetheless Met 18 in human PTH is another receptor recognition site [123], which would explain why the activity further decreased when both Met 8 and Met 18 are oxidized [119]. Based on these results the native secondary structure seems to be essential for receptor binding, as it is strongly perturbed upon oxidation of Met residues [118]. Additional studies indicated that Met 18 oxidizes more easily than Met 8, probably because PTH assumes a secondary structure that protects Met 8 against oxidation. Indeed, unfolding of the protein with 3 M guanidinium hydrochloride eliminated this difference, as it generates similarly surface-exposed Met residues [124].

Conclusion

The structural and biological consequences of several therapeutic proteins and peptides were reviewed. Redox chemistry of Cys residues is widely involved in the generation of new intra- and intermolecular covalent bonds, as observed for IFNβ-1a, insulin, calcitonin and oxytocin.

Met oxidation usually involves solvent-exposed residues and often results in altered protein conformation and biological activity, even when the aggregation state of the protein is not affected.

His oxidation is generally catalyzed by trace metals that induce site-specific oxidation and can have drastic consequences on the pharmacokinetics or the activity of the protein, as observed for insulin and GH.

Phe and Tyr oxidation can yield Tyr oxidation products, which are electrophiles prone to 1,4- and 1,6-type addition: such modifications mediated aggregates formation in insulin and IFNβ-1a. Oxidation-induced aggregation of a monoclonal IgG, IFNα-2a, IFNα-2b and IFNβ-1a probably occurred via the same mechanism. All these oxidized and aggregated products were found to be more immunogenic than their native counterparts in mouse models, and for IFNα-2a also in human patients.

Trp oxidation, although occurring in the minority of the studied proteins, can also be responsible for bio-activity loss, as observed in some mAbs.

In conclusion, oxidation of peptides and proteins is an important degradation pathway. From the case studies discussed in this review, it is

clear that oxidation not only leads to changes in the primary structure, but also can perturb higher-order structures and induce aggregation, which in turn can have important biological consequences, such as altered pharmacokinetics, loss of function and enhanced immunogenicity. Therefore, it cannot be emphasized enough that control of oxidation during production, purification, formulation, transportation, storage and use of therapeutic proteins and peptides is of utmost importance for their quality, safety and efficacy.

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Chapter 3

Plain and mono-PEGylated recombinant human insulin exhibit similar stress-induced aggregation profiles

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Abstract

PEGylation has been suggested to improve the stability of insulin, but evidence for that is scarce. Here we compared the forced aggregation behavior of insulin and mono-PEGylated insulin (PEG-insulin). Therefore, recombinant human insulin was conjugated on Lys B29 with 5 kDa PEG. PEG-insulin was purified by size exclusion chromatography (SEC) and characterized by mass spectrometry. Next, insulin and PEG-insulin were subjected to heating at 75 °C, metal-catalyzed oxidation and glutaraldehyde cross-linking. The products were characterized physicochemically by complementary analytical methods. Mono-PEGylation of insulin was confirmed by SEC and mass spectrometry. Under each of the applied stress conditions, insulin and PEG-insulin showed comparable degradation profiles. All the stressed samples showed submicron aggregates in the size range between 50-500 nm. Covalent aggregates and conformational changes were found for both oxidized products. Insulin and its PEGylated counterpart also exhibited similar characteristics when exposed to heat stress, i.e. slightly changed secondary and tertiary structures, covalent aggregates with partially intact epitopes and separation of chain A from chain B. Glutaraldehyde-treated insulin and PEG-insulin both contained covalent and noncovalent aggregates with intact epitopes, showed partially perturbed secondary structure and substantial loss of tertiary structure. From these results we conclude that PEGylation does not protect insulin against forced aggregation.

Introduction

Throughout almost one century, different production platforms, purification protocols and formulations have been investigated in order to produce highly purified insulin with high physico-chemical stability [1]. Still, none of the current insulin formulations is capable of fully protecting this small polypeptide hormone against degradation. In particular, insulin aggregation is hard to avoid and the presence of covalent aggregates in insulin products has been associated with the induction of anti-insulin antibodies from which systemic hypersensitivity and insulin resistance may arise [2-4].

With the progress made in organic and amino acid chemistry, the chemical modification of proteins has become one of the methods to improve the half-life of a therapeutic protein. In particular, PEGylation has been widely used to achieve this aim and PEGylation has been proposed also as a way to increase the stability of biopharmaceutical products [5-6]. A great number of procedures and chemicals have been developed to achieve site-specific modifications [7].

The influence of PEG conjugation to insulin on its stability with respect to the formation of fibrils has been investigated by several authors [8-10]. Briefly, it was found that PEGylation on lysine (Lys) B29 or phenylalanine (Phe) B1 improved insulin's long-term physical stability, as evaluated by a shaking testing done at 100 strokes/min and 37 °C, parallel to an enhancement of its plasma half-life. The higher the molecular weight of PEG (ranging between 750 Da and 2 kDa), the more stable the conjugated protein was found to be. However, when conjugating 5 kDa PEG on Phe B1, a slight decrease in the biological activity has been found [6]. A similar decrease in the activity of the hormone was observed when two 600 Da or 2 kDa PEG chains were covalently bound to the protein [6]. However, no literature is available concerning the behavior of PEGylated-insulin when exposed to other stress factors.

The aim of this work was to investigate the effect of three different stress methods on the stability of PEGylated-insulin in comparison to unmodified insulin, with a special emphasis on the formation of amorphous, non-fibrillar aggregates. To this end, mono-PEGylated insulin (PEG-insulin) was prepared by site-specific linkage of 5 kDa PEG onto Lys B29 of recombinant human insulin. Both proteins, insulin and PEG-insulin, were exposed to three stress conditions, i.e. thermal stress, oxidation by Cu^{2+}/ascorbate, and cross-linking with glutaraldehyde. By using complementary physicochemical analytical

methods, we show that the sensitivity of PEG-insulin to these stress factors is comparable to that of its unmodified counterpart.

Materials and Methods

Materials

Recombinant human insulin containing 0.4% (w/w) zinc ions was provided by Schering Plough, Oss, The Netherlands. Glutaraldehyde, sodium borohydride, L-ascorbic acid, copper (II) chloride, arginine, disodium hydrogen phosphate, boric acid and ammonium bicarbonate were purchased from Sigma–Aldrich, Germany. Alpha-metoxy omega-carboxylic acid succinimidyl ester poly(ethylene glycol) (MeO-PEG-NHS, 5 kDa) was purchased from Iris Biotech GmbH, Germany. Glacial acetic acid and acetonitrile were purchased from Boom, The Netherlands. 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid (SA) peptide/protein calibrations standards for MALDI-TOF MS were purchased from Bruker Daltonik GmbH, Bremen, Germany. Deionized water for MS measurements was obtained from a Milli-Q water purification system (Millipore, USA). All chemicals were of analytical grade and used without further purification.

Synthesis and purification of PEG-insulin

Ten mg of insulin were dissolved in 0.1 M hydrochloric acid and brought to a final volume of 1 mL with 60% acetonitrile/40% H_3BO_3 0.1 M, pH 10. Under these alkaline conditions the nucleophilicity of the ε-amino group of the Lys B29 is higher than that of the α-amino groups of Gly A1 and Phe B1, so the conjugation should preferentially occur in position B29 [9].

A 3-fold molar excess of MEO-PEG-NHS, 5 kDa, was added in three times, 1 equivalent every 15 minutes, to the insulin solution. After 45 minutes the reaction mixture was purified by SEC, using an Insulin HMWP Column, 7.8 x 300 mm (Waters) and an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a UV detector set at 276 nm. The mobile phase was composed of a mixture of 1 g/L L-arginine aqueous solution:acetonitrile: glacial acetic acid 65:20:15 (v/v/v) as reported in the United States and European pharmacopeias [11-12]. The elution buffer was freshly prepared, filtered and degassed prior to use.

After purification, PEG-insulin was concentrated to about 0.5 mg/mL using centrifugal filter units (Ultra 15 mL, 10 kDa, Amicon, Millipore, USA) and dialyzed against 10 mM sodium phosphate, pH 7.4 (PB), using Slide-A-Lyzer dialysis cassettes with 10 kDa cut-off (Biorad, The Netherlands).

Reduction and alkylation of PEG-insulin

To confirm that PEG was conjugated to chain B, first sodium phosphate buffer was exchanged with 0.1 M ammonium bicarbonate (ABI), pH 8, using centrifugal filter units. Next PEG-insulin was reduced by a 50-fold excess of DTT and alkylated by a 100-fold excess of iodoacetamide with respect to the moles of disulfides contained in the insulin molecule, as reported by Tuesca et al [13].

Digestion of PEG-insulin and insulin

To confirm the site of conjugation, 0.5 mg/mL of PEG-insulin and insulin in 0.1 M ABI, pH 8, were treated with a 0.25 mL of gel immobilized trypsin (Pierce Chemical Company, Rockville, IL). The mixture was incubated overnight at 37 °C. The sample was shaken for ten seconds at 300 rpm every five minute using a Thermomixer (Eppendorf, USA). The immobilized trypsin was then removed from the solution by centrifugation and the supernatant was recovered, concentrated with centrifugal filter units (Amicon, Ultra 15 mL, 3 kDa) and kept at 4 °C until analysis. MS results were eventually compared.

Forced degradation of insulin and PEG-insulin

All the formulations and the dilutions were made in PB. PEG-insulin was obtained as described above. Insulin (not PEGylated) was first dissolved in 0.1 M hydrochloric acid and diluted in PB. The pH was adjusted to 7.4 using 0.1 M sodium hydroxide and the insulin concentration was determined by UV spectroscopy, using a molecular weight of 5.8 kDa and an extinction coefficient of 6200 $M^{-1}\,cm^{-1}$ at 276 nm [14]. Further dilution in PB was done to obtain a final insulin concentration of 0.5 mg/mL.

Heat treatment was done by heating solutions of 0.5 mg/mL insulin and PEG-insulin, 1 mL in 1.5-mL safe-lock eppendorf tubes, at 75 °C for 1 hour using a water bath [15].

Metal-catalyzed oxidation was performed by adding to solutions of 0.5 mg/mL insulin and PEG-insulin, 2.5 mL in 5-mL glass light-resistant vials, 0.4 mM $CuCl_2$ in PB, to a final concentration of 40 µM. The reaction was allowed to proceed for 10 minutes and subsequently 40 mM L-ascorbic acid in PB was added to a final concentration of 4 mM. After three hours of incubation at room temperature the reaction was quenched by adding 100 mM EDTA in PB, to a final concentration of 1 mM [16]. The oxidized samples were dialyzed at 4 °C against PB for 24 hours.

Cross-linking with glutaraldehyde was achieved by incubating for 2 minutes 2.5 mL (in 5-mL glass light-resistant vials) of 0.5 mg/mL insulin and PEG-insulin solutions, with glutaraldehyde (final concentration of 4.23 mM). After 2 minutes, 11 µL of 12% (w/w) $NaBH_4$ in 14 M NaOH, diluted 30 times in water, was added to a final concentration of 646 µM and the mixtures were left in the dark at room temperature for 20 hours [17]. Next, the solutions were dialyzed at 4 °C against PB for 24 hours.

UV spectroscopy

UV/VIS measurements were performed with an Agilent 8453 UV/VIS spectrophotometer (Waldbronn, Germany), which included a Peltier element for temperature control. Quartz cells with a path length of 1 cm were used for all measurements. Scans were taken from 200-900 nm with 1 nm intervals at 25 °C.

Near-UV and far-UV circular dichroism (CD) spectroscopy

Near-UV CD and far-UV CD spectra were recorded from 250 to 320 nm and 190 to 250 nm, respectively, using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-cm (near-UV CD) and a 1-mm (far-UV CD) path length quartz cuvette at 20 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 6 repeated scans and background

corrected with the corresponding buffer spectrum. The CD signals were converted to molar ellipticity per amino acid residue.

Nanoparticle tracking analysis (NTA)

NTA measurements were performed at room temperature with a NanoSight LM20 (NanoSight, Amesbury, United Kingdom), equipped with a sample chamber with a 640-nm laser and a Viton fluoroelastomer O-ring, as described previously by Filipe et al [18]. Briefly, the samples were injected in the sample chamber with sterile syringes (BD Discardit II, New Jersey, USA) until the liquid reached the tip of the nozzle. The software used for capturing and analyzing the data was the NTA 2.0 Build 127. The samples were measured for 60 s with manual shutter and gain adjustments. The "single shutter and gain mode" was used to capture the protein aggregates. The mean size was calculated by using the NTA software.

Light obscuration analysis

Light obscuration analysis was performed using a PAMAS SVSS-C (PAMAS GmbH, Bad Salzuflen, Germany). The pre-run volume was 0.3 mL and for each sample, 3 measurements were done, using a volume of 0.2 mL. Between each measurement, the instrument was washed with Milli-Q water, followed by 30% (v) ethanol in water if necessary. After cleaning, water was measured to confirm that the instrument was cleaned (max. 50 particles/mL, 1μm). For the measurement the samples were diluted 100-fold in PB and the blank was subtracted.

Intrinsic and extrinsic steady state fluorescence spectroscopy

Intrinsic fluorescence was measured in 96-well plates using the plate reader unit of the FS920 fluorescence spectrometer (Edinburgh Instruments). For the measurement, all the formulations were diluted to a concentration of 0.1 mg/mL to avoid inner filter effects. Tyrosine was selectively excited at 275 nm. The emission spectra were recorded from 290 to 400 nm using a step size of 2 nm, gain of 145, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz. The extrinsic fluorescent dyes 4,4'-dianilino-1,1'-

binaphtyl-5,5'-disulfonic acid (Bis-ANS) and thioflavin T (ThT) were employed to characterize the aggregates [19].

For measurements of Bis-ANS fluorescence, 10 µL of an aqueous solution of 42 µM Bis-ANS were added to 200 µL of 0.1 mg/mL (PEGylated) insulin sample. The concentration of the stock solution was determined by using an extinction coefficient of 16760 M^{-1} cm^{-1} in water at 385 nm. Each sample was excited at 385 nm and the emission spectra were recorded between 400 and 550 nm, using a step size of 2 nm, gain of 150, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz.

For measurements of ThT fluorescence, 10 µL of a 210 µM aqueous solution of ThT were added to 200 µL of 0.1 mg/mL (PEGylated) insulin sample. The determination of the concentration of the stock solution was done using an extinction coefficient of 36000 M^{-1} cm^{-1} in water at 412 nm. Each sample was excited at 450 nm and the emission spectra were recorded between 465 and 600 nm, using a step size of 2 nm, gain of 130, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gradient gels (10-20% tris-tricine) were purchased from Bio-Rad and run under reducing (sample buffer containing 5% (v/v) ß-mercaptoethanol) and nonreducing (sample buffer without ß-mercaptoethanol) conditions at 100 V at room temperature. The cathode electrophoresis buffer was 0.1 M tris(hydroxymethyl) aminomethane, 0.1 M tricine, and 3 mM SDS, pH 8.3. The anode electrophoresis buffer was 0.1 M Tris pH 8.9. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples were boiled at 95 °C for 2 minutes and then centrifuged for 1 minute at 13000 rpm, before application to the gel. A polypeptide marker solution (Biorad, The Netherlands) was included on the gel for apparent molecular weight determination.

Western blotting

Proteins run on SDS-PAGE gels were blotted onto a polyvinylidene difluoride (PVDF) immuno blotting membrane, overnight at 4 °C using a mini trans-blot

system with a voltage applied of 30 V (Biorad, The Netherlands). Blots were blocked for 2 hours at room temperature with 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) with constant orbital shaking. After washing with 0.1% (v/v) Tween 20 in PBS and with water, the blots were incubated with polyclonal guinea pig anti-human insulin antibody (Abcam) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled goat polyclonal anti-guinea pig IgG (Abcam) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water and incubated in a solution of 4-chloro-1-naphtol (Sigma-Aldrich) in methanol (20% (v/v)), water, and H_2O_2 (0.015% (v/v)). After color development the blots were stored in water overnight in the dark to increase the intensity of the bands.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on BIFLEX MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Specifically, 1 µL of sample solution and 1 µL of matrix solution were mixed in an Eppendorf tube and applied onto the MALDI sample plate and let to dry at room temperature. Saturated DHB was prepared in a mixture of 50% acetonitrile, 50% deionized water, and 0.1% trifluoroacetic acid as matrix solutions. Analysis was performed in the linear positive mode with delayed extraction of 200 ns. The samples were irradiated by a 337 nm pulsed nitrogen laser. The laser intensity was optimized to give the best signal-to-noise ratio for each sample. The acceleration voltage was 19 kV. Each mass spectrum was generated by averaging ~300 laser shots. MALDI-TOF MS evaluation was performed with FlexAnalysis Software (Bruker Daltonics) by a centroid peak detection algorithm with a signal-to-noise threshold of 3, a peak width of 1 m/z, a height of 80%. External calibration was performed by using a solution of peptide and protein calibration standards from Bruker Daltonics.

Results and Discussion

For the preparation of PEG-insulin we used PEG with a chain length of 5 kDa, because shorter PEGs are likely less stabilizing [10] and PEG with a longer chain length would compromise insulin's specific activity [8]. In this section are reported the results related with the synthesis, purification and characterization of PEG-insulin, followed by the characterization of the aggregates. SEC, NTA, light obscuration and SDS-PAGE were used for investigating the size and (non)covalent nature of the aggregates; UV, CD, and fluorescence spectroscopy for obtaining information about protein conformation and Western blotting for studying the integrity of epitopes.

Synthesis and purification of mono-PEGylated insulin

As can be seen from Figure 1, the yield of PEG-insulin, as determined by SEC and based on integrated peak area, progressively increased with the addition of MeO-PEG-NHS, while the percentage of unmodified insulin decreased. Beyond the addition of 3 equivalents of PEG, only a substantial increase of bi-PEGylated insulin, but not PEG-insulin, was achieved (results not shown).

The crude reaction mixture was purified by collecting the SEC fraction eluting between 15 and 17 minutes. The same SEC procedure was used for preparative and quantitative purposes. By re-analyzing the purified fraction on SEC, the final percentage of PEG-insulin was found to be 99.0 ± 0.4% (Figure 1, Table I).
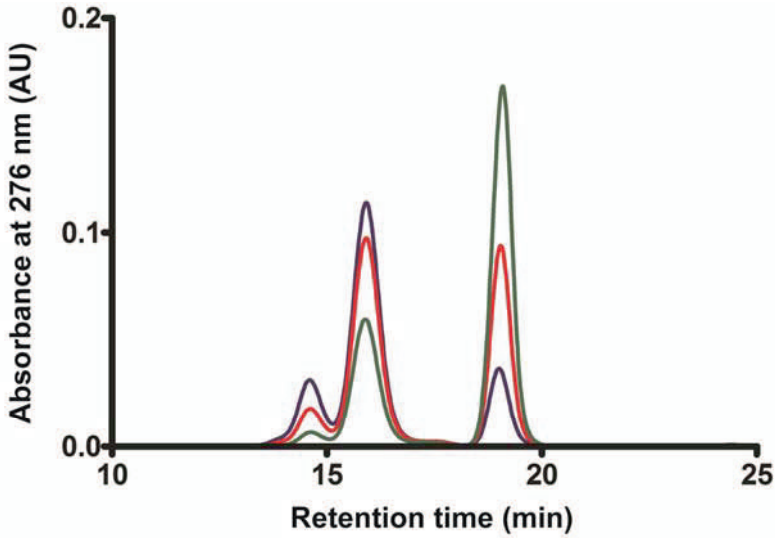
Figure 1. Synthesis of PEG-insulin as monitored by SEC: chromatograms of the reaction mixture after adding 1 eq. (green), 2 eq. (red), 3 eq. (blue) of PEG 5 kDa. After adding each equivalent, the reaction mixture was allowed to equilibrate for 15 minutes.

Table I. Summary of SEC analysis of unstressed insulin and PEG-insulin* (in %).

Sample	Monomer	Dimer	Trimer	Larger aggregates	Fragments	Insoluble
Insulin	99.3 ± 0.1	0.7 ± 0.1	nd	nd	nd	nd
PEG-insulin	99.0 ± 0.4	0.8 ± 0.3	nd	nd	0.2 ± 0.0	nd
HI	89.5 ± 0.3	5.1 ± 0.2	nd	nd	2.6 ± 0.1	2.9 ± 0.3
HPI	85.4 ± 2.5	2.6 ± 0.4	nd	nd	5.3 ± 0.8	6.7 ± 2.1
MI	73.6 ± 0.2	20.3 ± 0.2	6.1 ± 0.3	nd	nd	nd
MPI	64.2 ± 10.3	13.3 ± 1.5	nd	nd	22.5 ± 11.7	nd
CRI**	70.7 ± 0.3	28.2 ± 0.5	29.1 ± 0.1	51.4 ± 1.8	2.5 ± 0.0	nd
CRPI**	133.3 ± 4.8	16.3 ± 1.1	nd	nd	1.7 ± 1.0	nd

* Percentages are based on the AUC of the peaks. Errors are the deviation from the average between two batches. nd = not detectable.
** For glutaraldehyde-treated samples, the total AUC was higher when compared with the native protein, probably due to changes in the molar extinction coefficient or extensive light scattering.

Mass spectrometric analysis of mono-PEGylated insulin

MALDI-TOF MS analysis revealed the presence of mono-PEGylated insulin, showing a peak centered at *m/z* ~10600 (Figure 2, red spectrum). The typical distribution of peaks 44 Da apart from each other is related to the polydispersity of the PEG, i.e. the varying number of ethylene oxide monomer units in the polymer chain.

Confirmation of the site of conjugation was achieved in two steps. First, PEG-insulin was reduced, alkylated, purified by SEC and analyzed with MALDI-TOF MS to prove that PEG is indeed bound to chain B but not chain A. The charge distribution centered at *m/z* ~8400 matches well the calculated mass centered around 8359 Da for a fully alkylated insulin B chain conjugated with PEG 5 kDa (Figure 2, black spectrum).
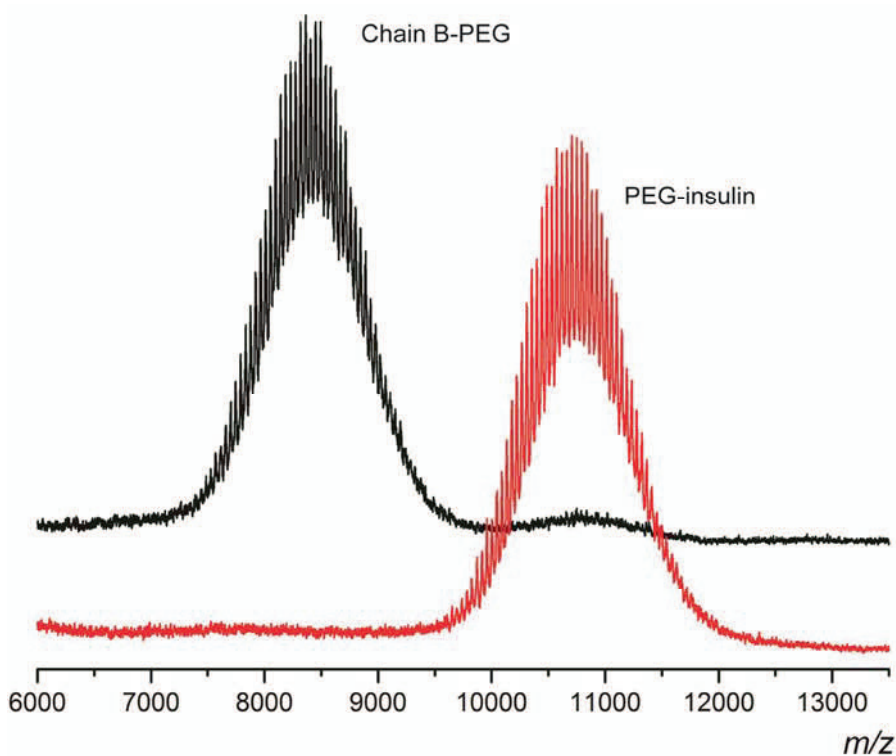
Figure 2. Matrix-assisted laser desorption/ionization time of flight mass spectrometry spectra of PEG–insulin (red) and chain B of reduced/alkylated PEG–insulin purified by size-exclusion chromatography (black).

There was no evidence of any PEG conjugated to chain A (results not shown).

Second, plain insulin as well as the conjugate was subjected to trypsin digestion to confirm that PEGylation had occurred at the C-terminal end of the B-chain, more precisely on Lys B-29. Trypsin is a common enzyme used for the selective cleavage of peptides/proteins at the carboxyl side of the amino acid residues Lys (K) and arginine (R). As can be seen in Figure 3, only chain B of insulin has two sites that can readily be cleaved by trypsin.
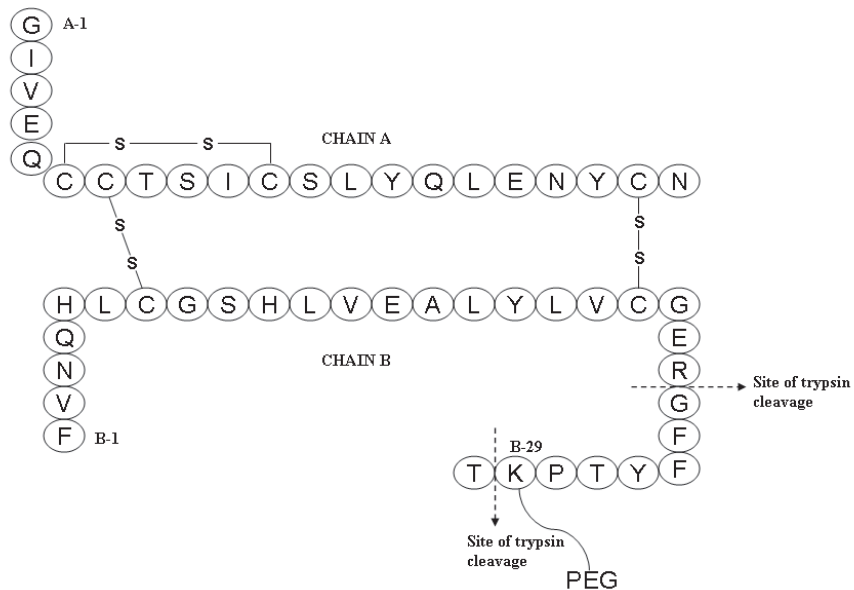
Figure 3. Primary structure of human insulin, including the theoretical cleavage sites of trypsin and the site of PEG conjugation.

The site of the conjugation could be confirmed indirectly by detecting the peptidic fragment $(A_1-A_{21})-(B_1-B_{22})$ for both the unmodified insulin (Figure 4, panel A) and PEG-insulin (Figure 4, panel B).

For unmodified insulin, the peak at 4869.4 Da depicted in Figure 4, panel A, corresponds well to the calculated molecular weight of 4866.2 Da for chain A-(B_1-B_{22}) fragment. The peaks at 5710.5 Da and 5812.1 Da correspond to chain A-(B_1-B_{29}) and undigested insulin, respectively. In the spectrum of plain insulin also masses of 959.4 Da and 858.4 Da corresponding to $B_{23}-B_{30}$ and $B_{23}-B_{29}$, respectively, could be discerned (data not shown). The spectrum of

digested PEG-insulin (without reduction/alkylation) showed a main peak at 4869.7 Da (Figure 4, panel B) belonging to chain A-(B_1-B_{22}), indicating that the PEG conjugation was definitely not on chain A. Moreover, beneath the main peak a low-signal peak distribution with the PEG profile (44-Da spacing) can be seen, most likely corresponding to the peptide fragments (B_{23}-B_{30})-PEG and (B_{23}-B_{29})-PEG. For comparison, the reduced and alkylated PEG-insulin was also digested by trypsin under the same conditions.
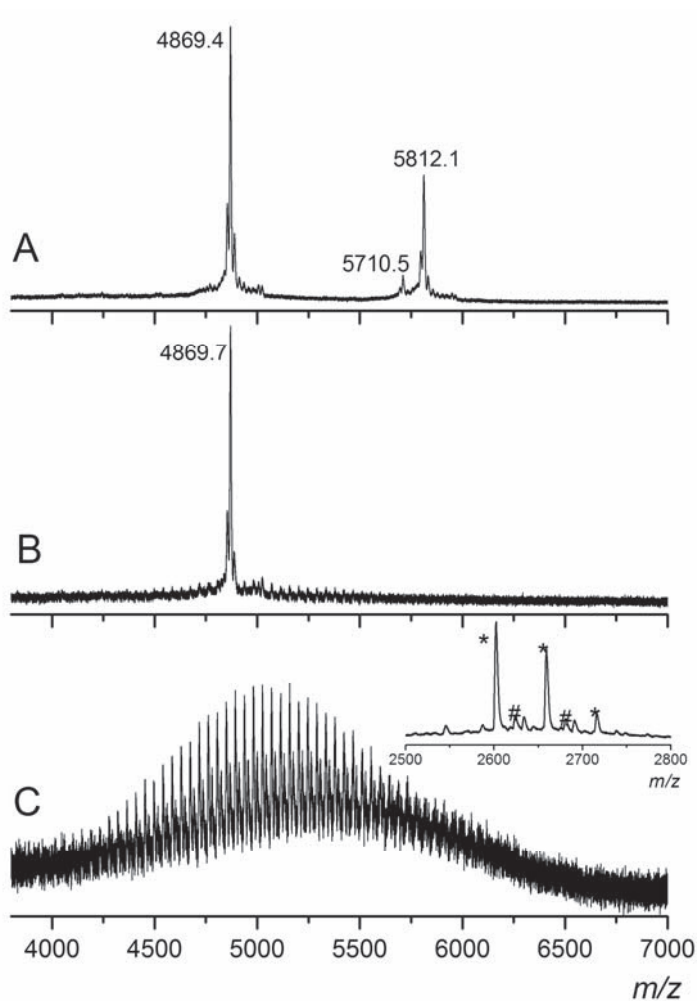
Figure 4. Comparison of MALDI-TOF MS spectra of A) digested insulin, B) digested PEG-insulin and C) digested PEG-insulin after reduction/alkylation. The inset in panel C shows the fully and over-alkylated chain B_1-B_{22} (*) and chain A (#) peaks.

As can be seen in Figure 4, panel C, the intense 4869 Da peak belonging to chain A-(B_1-B_{22}) had disappeared. Instead, a series of overlaying Gaussian peak distributions were detected. In Figure 4, panel C, the overlapping charge distributions can be attributed to the (B_{23}-B_{30})-PEG and (B_{23}-B_{29})-PEG fragments and free PEG, with theoretical masses centered around 5777 Da, 5676 and 4933 Da, respectively. The inset in panel C of Figure 4, indicates that there is no PEG attached to chain (B_1-B_{22}) (*) and chain A (#) fragments.

Altogether, these results show that we successfully synthesized insulin that is mono-PEGylated at Lys (B_{29}).

Characterization of insulin and PEG-insulin

SEC of native insulin and PEG-insulin showed a main peak of monomeric protein which in both cases represented more than 99% of the protein (Table I and Figure 5, panel A).

SDS-PAGE under nonreducing conditions showed a monomer band with an apparent molecular weight of approximately 6 kDa and 12.5 kDa for plain and PEGylated insulin, respectively (Figure 5, panel B, left). The broad band for PEG-insulin can be ascribed to the heterogeneous molecular weight of the PEG (cf. the MS results, Figure 2). The higher apparent molecular weight for the PEGylated insulin in SDS-PAGE as compared to the MS data is due to the fact that PEG hardly interacts with SDS, resulting in a lower electrophoretic mobility of PEG-insulin as compared to a non-PEGylated protein with the same molecular weight. Western blotting showed that both plain and PEG-insulin reacted with polyclonal anti-human insulin antibody (Figure 5, panel B, right).

NTA is an emerging technique which is particularly suitable to determine the particle size in polydisperse samples [18]. Since insulin monomers and small oligomers do not scatter enough light to be detected by this method, only larger nano-sized aggregates are selectively detected. For the native proteins, relatively low counts between 0.5-$1.0*10^8$ particles per mL, with a size distribution centered at ~100 nm, were detected (Figure 5, panel C, Table II).
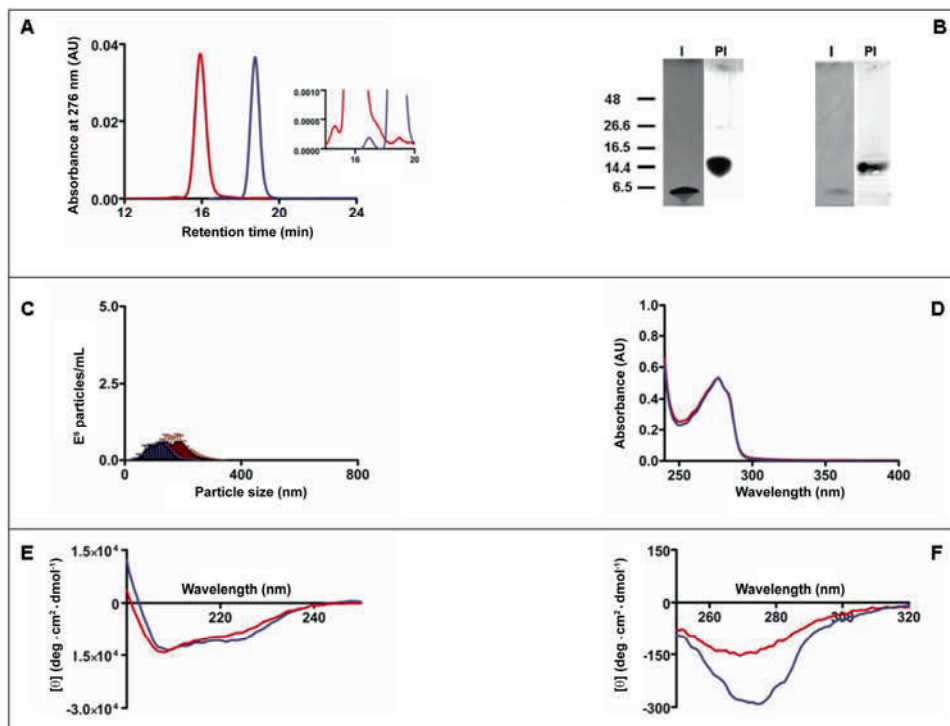
Figure 5. Main characteristics of insulin (I) (blue color) and PEG-insulin (PI) (red color) samples: (A) SEC (the inset shows the zoom in of insulin and PEG-insulin for a better correlation with the % reported in table I), (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

Subvisible particles in the range between 1 and 200 μm were analyzed by light obscuration. For both insulin and PEG-insulin, approximately 100-200*10^3 particles per mL were found (Table II), mainly in the size range 1-3 μm.

The UV spectra of proteins can give important information regarding the presence of aggregates in the solution. The decrease in A280/A260 ratio in particular indicates the presence of aggregates [20]. The ratio found for insulin and PEG-insulin obtained from the UV spectra (Figure 5, panel D) was similar (Table III).

Far-UV CD was employed to investigate the secondary structure. PEG-insulin showed a slightly higher $\theta_{208nm}/\theta_{223nm}$ ratio (Figure 5, panel E and Table III) as compared to plain insulin. This change has been observed by other authors

and has been assigned to a slight increase in antiparallel *ß*-structure content [9, 21].

Table II. Concentration and mean size of the particles detected with NTA and light obscuration*.

Sample	Nanoparticle Tracking Analysis		Light Obscuration (10^3/mL)		
	10^8/mL	Mean size (nm)	1-10 µm	10-25 µm	> 25 µm
Insulin	0.52 ± 0.04	116.5 ± 5.5	155 ± 38	3.35 ± 1.66	0.83 ± 0.83
PEG-insulin	0.78 ± 0.26	168.0 ± 3.0	286 ± 35	-	0.41 ± 0.41
HI	1.14 ± 0.12	197.5 ± 22.5	172 ± 37	-	-
HPI	1.96 ± 0.5	242.5 ± 48.5	215 ± 19	0.41 ± 0.41	-
MI	1.05 ± 0.1	193.0 ± 1.0	138 ± 3	2.50 ± 0.83	2.91 ± 1.25
MPI	4.65 ± 1.39	160.0 ± 20.0	381 ± 68	0.42 ± 0.41	-
CRI	7.00 ± 1.92	248.0 ± 10.0	415 ± 95	4.58 ± 0.41	3.74 ± 0.41
CRPI	5.50 ± 0.50	185.5 ± 18.5	403 ± 3	2.92 ± 2.08	0.41 ± 0.41

* Concentration obtained by NTA is cumulative and is reported as 10^8 (E^8 instead of E^6 used in Figures 5-8, panel C). Errors are the deviation from the average between two batches. - = no particles detected.

Additional information concerning insulin's structure was obtained by analyzing its near-UV CD spectrum in the region 260–320 nm.

The signal in this region is due to the absorbance of insulin's tyrosine, phenylalanine and disulfides, and is substantially affected by its self association state [22-23]. In particular, reduction of the 273 nm CD-band is observed when insulin is dissociated [24-25]. Plain insulin presented a higher negative molar ellipticity band when compared with PEG-insulin, which can be explained by the association state of plain insulin differing from that of PEG-insulin, since in the free protein hexamer prevails [26] (Figure 5, panel F and Table III), whereas the conjugated protein appeared to be in the dimeric state, as reported by Hinds et al [9].

Intrinsic tyrosine fluorescence spectroscopy was used to further analyze the tertiary structure of the proteins. Upon excitation at 275 nm, native insulin showed an emission maximum at 299 ± 1 nm. PEG-insulin showed a similar spectrum concerning the position of the emission maximum and the intensity (Table III).

These results indicate that both plain insulin and PEG-insulin have structural properties that are consistent with earlier reports, PEG-insulin exhibiting a lower association state than plain insulin.

Table III. Summary of spectroscopic measurements of unstressed and stressed insulin and PEG-insulin*.

Sample	UV	Circular Dichroism			Intrinsic Fluorescence	Bis-ANS Fluorescence
	A 280/260	[θ]208/223	% alpha helix	[θ]273	Intensity (a.u.)	Intensity (a.u.)
Insulin	1.56 ± 0.00	1.23 ± 0.00	39.2 ± 3.7	-314.7 ± 29.4	12.7 ± 0.3	2.25 ± 0.05
PEG-insulin	1.52 ± 0.06	1.57 ± 0.05	33.5 ± 1.5	-148.3 ± 2.9	12.9 ± 0.5	3.97 ± 0.92
HI	1.42 ± 0.01	1.31 ± 0.02	33.7 ± 0.3	-250.6 ± 0.4	10.9 ± 0.0	15.76 ± 1.48
HPI	1.45 ± 0.02	1.63 ± 0.02	30.9 ± 1.9	-122.4 ± 8.4	12.7 ± 1.9	6.85 ± 1.49
MI	1.43 ± 0.01	1.37 ± 0.04	26.8 ± 3.2	-113.4 ± 10.4	7.4 ± 1.0	5.91 ± 0.04
MPI	1.26 ± 0.00	1.87 ± 0.10	10.2 ± 0.0	-55.5 ± 19.8	6.3 ± 1.9	5.88 ± 0.06
CRI	1.03 ± 0.00	1.54 ± 0.01	31.6 ± 0.9	15.5 ± 7.4	7.3 ± 0.4	5.76 ± 0.31
CRPI	1.08 ± 0.00	1.58 ± 0.07	30.7 ± 0.5	50.2 ± 11.3	12.2 ± 0.0	5.58 ± 0.62

* Molar ellipticity in (deg* cm²* dmol⁻¹), % of alpha helix calculated as reported by Pocker et al [21]. Errors are the deviation from the average between two batches.

Characterization of heat-stressed insulin and PEG-insulin

Analysis by SEC showed that heat-stressed insulin (HI) and PEG-insulin (HPI) samples contained small amounts of dimers (Figure 6, panel A, Table I). Whereas the dimer content was slightly higher for heated insulin, heated PEG-insulin showed a larger fraction of unrecovered material (Table I), likely insoluble aggregates. Moreover, in both samples a fragment eluting at ~21 min was observed (Figure 6, panel A), which was identified as chain A by MALDI-TOF MS (not shown).
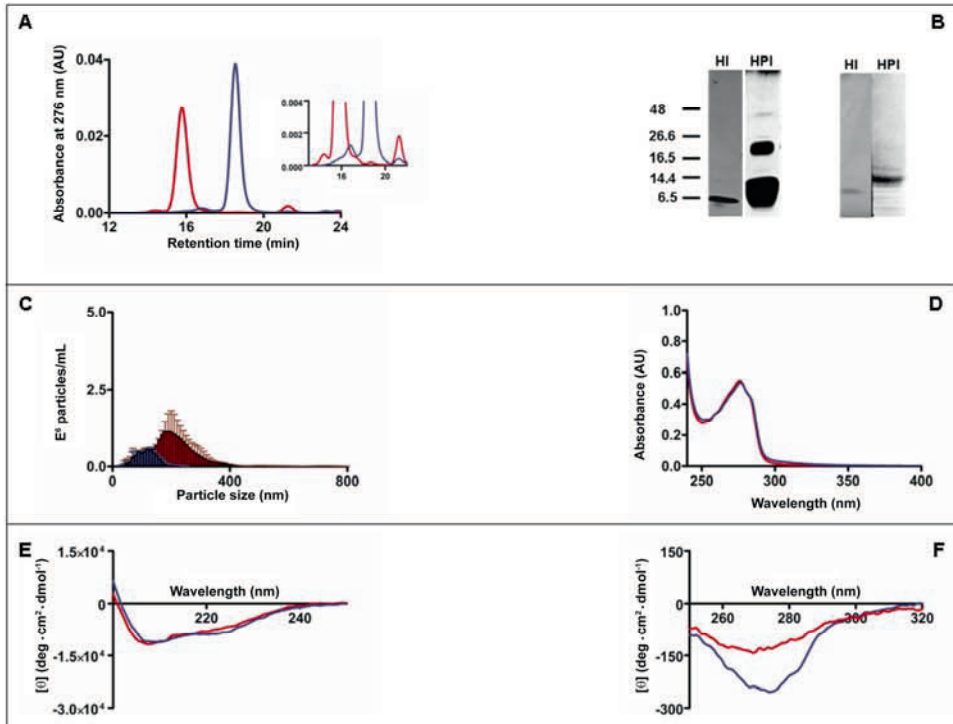
Figure 6. Main characteristics of heated insulin (HI) (blue color) and heated PEG-insulin (HPI) (red color) samples: (A) SEC (the inset shows the zoom in of insulin and PEG-insulin for a better correlation with the % reported in table I), (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

SDS-PAGE of HI showed a clear monomer band under nonreducing conditions, indicating the absence of covalent aggregates. For HPI, besides the monomer, a band with an apparent molecular weight of approximately 25 kDa and less intense bands with higher molecular weight were detected (Figure 6, panel B, left), indicating the presence of covalent aggregates. In Western blotting analysis only the monomer was detected, both for HI and HPI (Figure 6, panel B, right).

In NTA both HI and HPI showed an increase in particle concentration compared to the proteins before heat treatment (Table II). The size distribution of HPI was centered at about 200 nm, slightly larger than for HI (Figure 6, panel C). Light obscuration did not present major differences in subvisible particle counts with respect to the native proteins (Table II).

The UV spectra practically overlapped with those of the native proteins, although a small decrease in A280/A260 ratio was observed (Figure 6, panel D and Table III), consistent with the presence of small amount of aggregates in both HI and HPI.

Far-UV CD spectra of HI and HPI (Figure 6, panel E) were characterized by a slight increase in the 208/223 nm ratio, a slightly reduced intensity and consequently a decrease in the calculated alpha helix (Table III). The CD signals in the near-UV region (Figure 6, panel F) also were slightly reduced (Table III), indicating that HI and HPI also underwent small changes in the tertiary and quaternary structures.

The fluorescence emission spectrum was not shifted, but the intensity of HI (but not HPI) had slightly decreased (Table III).

Bis-ANS fluorescence was measured as a sensitive probe for the formation of new hydrophobic pockets in heat-stressed proteins [19, 27]. For HI and HPI Bis-ANS fluorescence was increased by approximately 5 and 2 fold, respectively (Table III), indicating a moderate increase in hydrophobicity after heating.

Thioflavin T fluorescence of the stressed samples was as low as that of native insulin and PEG-insulin (data not shown), indicating that the samples were free from fibrils.

In conclusion, the heated samples, insulin and PEG-insulin, showed similar degradation profiles.

Characterization of oxidized insulin and PEG-insulin

Metal catalyzed oxidized insulin (MI) and PEG-insulin (MPI) showed similar loss of monomer, as analyzed by SEC (Figure 7, panel A, Table I). Both MI and MPI contained substantial amounts of oligomers, mainly dimers. Besides, MPI but not MI, showed considerable fragmentation, which is likely related with the ability of radical oxidants to cleave the C-C bond of PEG [24]. Insoluble aggregates, based on the AUC of the peaks, were not found.

Nonreducing SDS-PAGE showed that both MI and MPI contained various covalent aggregates (Figure 7, panel B, left). Whereas the multimeric species present in MI reacted with the antiserum in Western blotting, the blot of MPI did not show any stained bands. This can be due to an extensive loss of the native epitopes and/or less efficient transfer to the PVDF membrane of the oligomeric PEGylated species (Figure 7, panel B, right).

Both stressed samples showed elevated levels of submicron aggregates when analyzed with NTA, mainly in the range size between 150-250 nm (Figure 7, panel C and Table II). This effect was most pronounced for MPI. Subvisible particle counts by light obscuration were similar to those for the unstressed proteins (Table II).
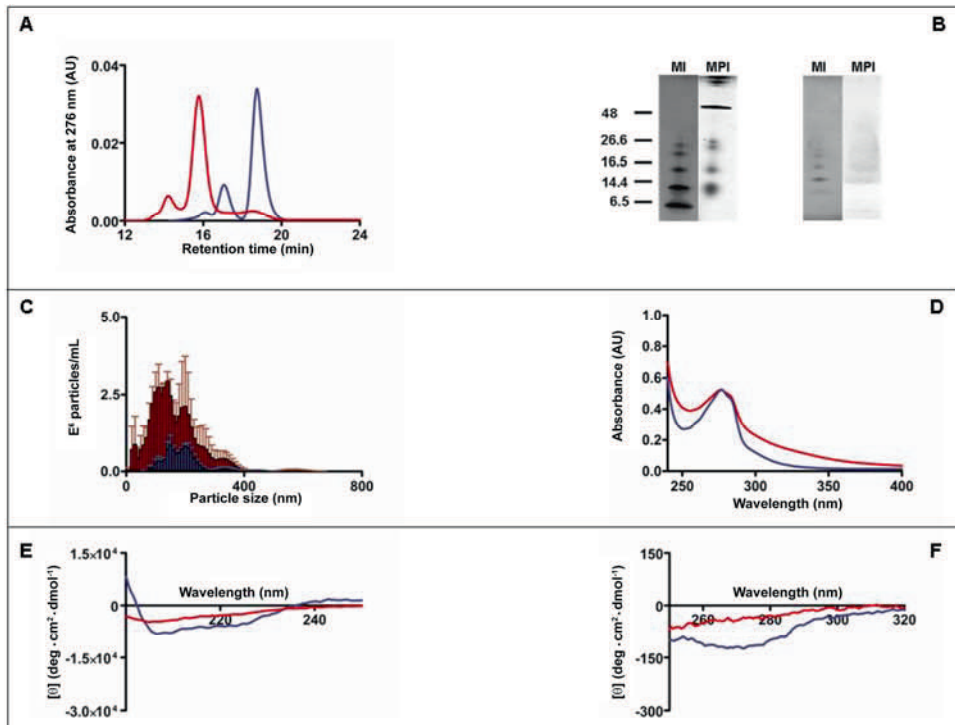

Figure 7. Main characteristics of oxidized insulin (MI) (blue color) and oxidized PEG-insulin (MPI) (red color) samples: (A) SEC, (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

The UV spectra of MI and MPI in Figure 7, panel D showed an increase in light scattering, indicating the presence of particles. This is clearly reflected in the reduced A280/A260 ratio compared to the unstressed compounds (Table III).

The far-UV CD spectrum of MI showed a reduction of the intensity of the entire spectrum with an increase in the 208/223 nm ratio (Figure 7, E), indicating a substantial decrease in alpha-helical content (Table III). MPI

showed an even more extensive reduction in the alpha helix content. Also the near-UV CD signals of MI and MPI were much smaller as compared to the unstressed proteins, providing evidence that both proteins had undergone considerable loss of tertiary and quaternary structure.

Bis-ANS fluorescence (Table III) was approximately 2 fold higher than for the native proteins, indicating a moderate increase in hydrophobicity. Thioflavin T fluorescence of the stressed samples was as low as that of native insulin and PEG-insulin (data not shown), indicating that the samples were free from fibrils.

Concluding, when oxidation mediated by radicals occurs, PEG might promotes rather than prevents aggregation and loss of insulin's structural features.

Characterization of glutaraldehyde-treated insulin and PEG-insulin

According to SEC, glutaraldehyde-treated insulin (CRI) showed an increase in dimer, trimer and some bigger oligomers, whereas glutaraldehyde-treated PEG-insulin (CRPI) showed a small decrease in the monomer content, parallel to the formation of dimer, (Figure 8, panel A, Table I). PEG only partially prevented the formation of bigger oligomers. Insoluble aggregates, based on the AUC of the peaks, were not found.

Nonreducing SDS-PAGE confirmed the formation of covalent oligomeric species in both CRI and CRPI (Figure 8, panel B, left). Reducing SDS-PAGE confirmed the presence of nonreducible species, in contrast with the non-stressed, heat-stressed and oxidized products (results not shown).

Western blotting revealed that most of the multimeric species in CRPI and CRI contained native epitopes (Figure 8, panel B, right), whereas the monomers in both cases react only weakly with the antiserum.

NTA showed that both CRI and CRPI contained elevated levels of submicron particles, with sizes up to ~700 nm (Figure 8, panel C, Table II). Only a minimal increase in the concentration of subvisible particle content, but not size, was observed by light obscuration analysis (Table II).

The UV spectra (Figure 8, panel D) showed a substantial reduction of the A280/A260 ratio for both CRI and CRPI (Table III), which is consistent with extensive aggregation observed with other methods.
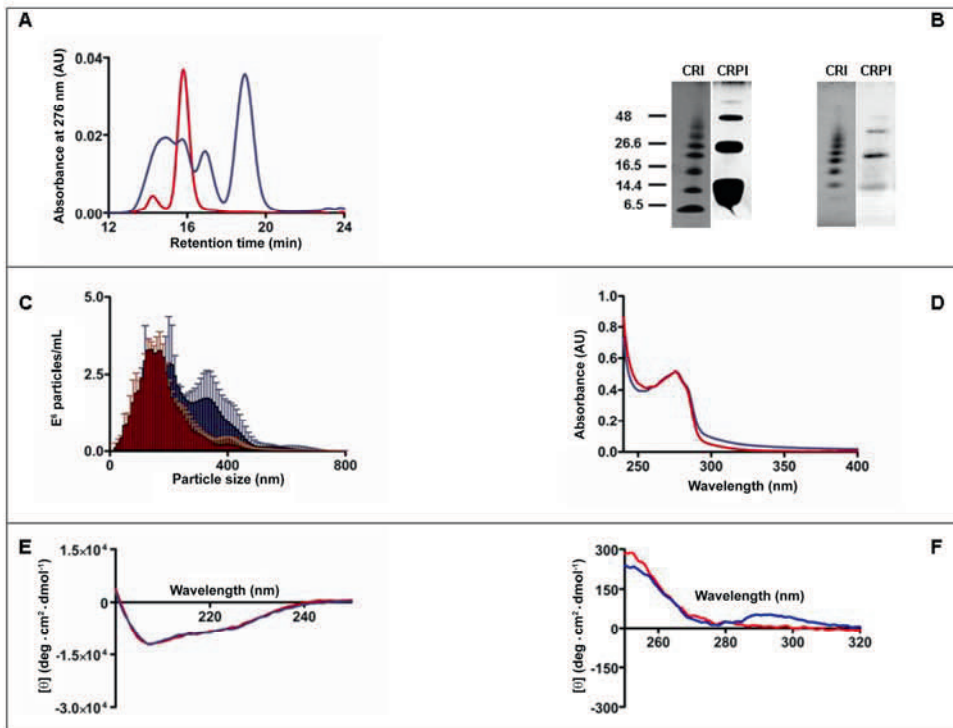
Figure 8. Main characteristics of cross-linked insulin (CRI) (blue color) and cross-linked PEG-insulin (CRPI) (red color) samples: (A) SEC, (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

Far-UV CD (Figure 8, panel E, Table III) indicated a loss of alpha helix content for both CRI and CRPI. The near-UV region of the spectrum showed major changes (Figure 8, panel F), indicating major perturbations of the tertiary/quaternary structures.

The extrinsic fluorescence measured with Bis-ANS (Table III) indicates that new hydrophobic surfaces were created in the cross-linked samples.

Thioflavin T fluorescence was not enhanced (not shown), indicating the absence of fibrillar species.

In conclusion, PEG is not capable to preserve insulin's structural features when the protein is treated with cross-linking agent as glutaraldehyde.

Conclusion

With this study, we demonstrated that exposure of insulin and PEG-insulin to three different stress factors, i.e. short heat exposure, metal catalyzed oxidation and cross-linking with glutaraldehyde, results in afibrillar aggregates with characteristics that strongly depend on the stress factor. In contrast, the quality of the aggregates, as shown by SEC, SDS-PAGE, Western blotting and NTA, and the altered structural properties of the proteins, as observed by CD and extrinsic fluorescence, were remarkably similar for insulin and PEGylated insulin, indicating that PEGylation neither positively nor negatively affects the sensitivity of insulin to the stress factors applied.

Acknowledgements

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Chapter 4

Chemical modifications in aggregates of recombinant human insulin induced by metal-catalyzed oxidation: covalent cross-linking via Michael addition to tyrosine oxidation products

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Abstract

Purpose. To elucidate the chemical modifications in covalent aggregates of recombinant human insulin induced by metal catalyzed oxidation (MCO).

Methods. Insulin was exposed for 3 hours at room temperature to the oxidative system copper(II)/ascorbate. Chemical derivatization with 4-(aminomethyl) benzenesulfonic acid (ABS) was performed to detect 3,4-dihydroxyphenylalanine (DOPA) formation. Electrospray ionization-mass spectrometry (ESI-MS) was employed to localize the amino acids targeted by oxidation and the cross-links involved in insulin aggregation. Oxidation at different pH and temperature was monitored with size exclusion chromatography (SEC) and ESI-MS analysis to further investigate the chemical mechanism(s), to estimate the aggregates content and to quantify DOPA in aggregated insulin.

Results. The results implicate the formation of DOPA and 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH), followed by Michael addition, as responsible for new cross-links resulting in covalent aggregation of insulin during MCO. Michael addition products were detected between DOCH at positions B16, B26, A14, and A19, and free amino groups of the N-terminal amino acids Phe B1 and Gly A1, and side chains of Lys B29, His B5 and His B10. Fragments originating from peptide bond hydrolysis were also detected.

Conclusion. MCO of insulin leads to covalent aggregation through cross-linking via Michael addition to tyrosine oxidation products.

Introduction

During pharmaceutical production and storage, therapeutic proteins can be exposed to components that are able to induce metal-catalyzed oxidation (MCO) [1], i.e. redox active transition metals, peroxides and reductants [2-3]. MCO of therapeutic proteins [4] such as recombinant human interferon alfa and recombinant human interferon beta has been reported to form highly immunogenic aggregates [5-7], providing evidence that protein oxidation can potentially lead to severe side reactions and loss of therapeutic effect.

Furthermore, it has been shown that in diabetic complications [8], copper ion concentrations are higher than in normal subjects [9]. Therefore, MCO is a potential cause of insulin degradation *in vivo* as well, when the production of reactive oxygen species (ROS) exceeds the endogenous antioxidant defense.

Montes-Cortes et al. [10] showed that the carbonyl content of human insulin and the hydroxylation of phenylalanine, based on the formazan assay [11], is increased after exposure of insulin to the plasma of diabetic patients, which can contain high concentrations of oxidants [12].

Moreover, in a recent paper we showed that MCO induces significant covalent aggregation of insulin *in vitro* [13]. However, the underlying mechanisms of insulin aggregation through MCO are still unknown. Previous reports on the MCO of human insulin and glycated insulin indicate that both histidine residues are easily oxidized to 2-oxo histidine [2, 9].

Here we provide a more detailed analysis of the chemical modifications that occur during the MCO of recombinant human insulin, induced by Cu^{2+} and L-ascorbic acid as a representative electron donor [14-16], focusing on the amino acids that are modified and on the chemical mechanisms that lead to cross-linked insulin, as a consequence of the modifications in the primary structure.

Since previous studies have demonstrated the presence of DOPA in proteins like cataractous lens proteins [17] and in atherosclerotic lesions [18], emphasis was placed on the detection of oxidation products arising from the four Tyr and the three Phe contained in insulin and, especially, on the formation of 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH), which constitutes a Michael acceptor for nucleophilic addition. Figure 1, panel A, displays the primary structure of insulin, the Glu-C digestion sites, the amino acids (in blue) with nucleophilic function, and the amino acids (in red) that can be oxidized to 3,4-dihydroxyphenylalanine (DOPA) and DOCH.
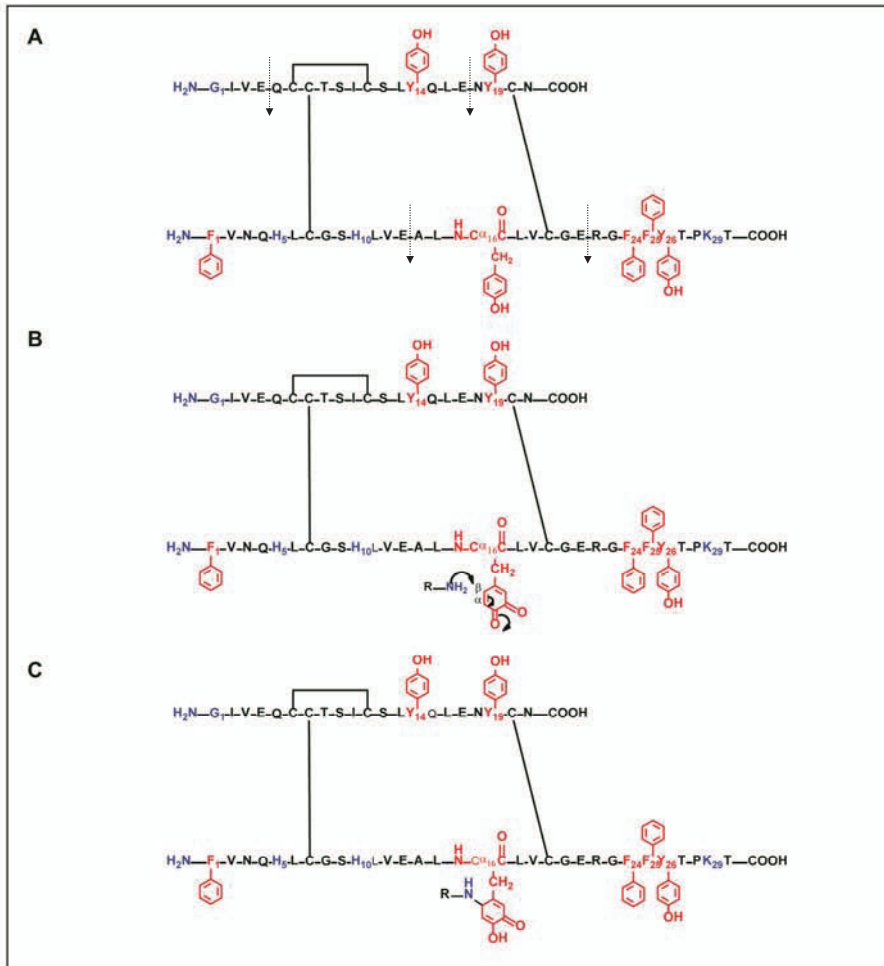
Figure 1. (A) primary structure of human insulin with the Glu-C endoproteinase digestion sites (dashed arrows). In blue are shown the nucleophilic amino acids which can be involved in Michael addition. In red the amino acids that can be oxidized to DOPA and DOCH. (B) Michael addition on oxidized human insulin. The amino group that initiates a nucleophilic reaction with the α,β unsaturated carbonyl compound (DOCH), represents one of the nucleophilic amino acids of human insulin (in blue). (C) Product of Michael addition on oxidized insulin (note that the hydrogen on the alpha carbon at position 16 was omitted for graphic requirements).

In the same Figure, panel B representatively illustrates the Michael addition on DOCH at position B16, and panel C shows the product of Michael addition on oxidized insulin (note that the hydrogen on the alpha carbon at position 16 was omitted for graphic requirements). Complementary detection of DOPA

and DOCH was achieved using mass spectrometry and fluorogenic tagging with 4 (aminomethyl)benzene sulfonic acid (ABS) (Scheme 1), according to the method reported recently by Sharov et al [19-20].
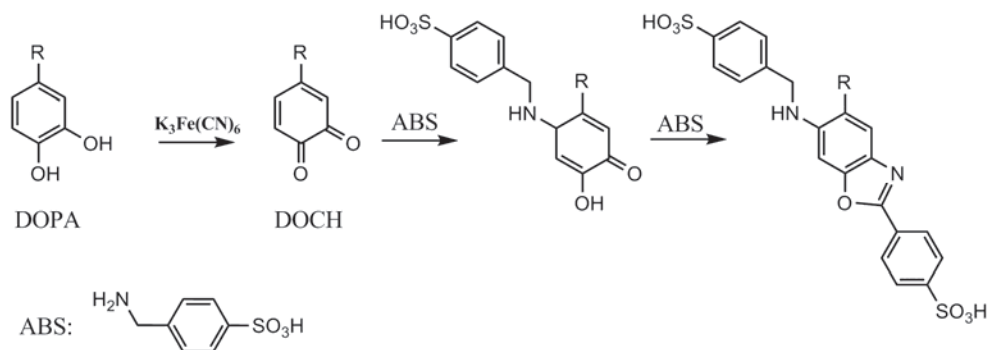
Scheme 1. Chemical derivatization of DOPA and DOCH with ABS.

Our results indicate that all aromatic amino acids of insulin, i.e. His, Tyr and Phe, are subject to MCO, predominantly yielding mono- and/or dihydroxylation products (or their respective oxidation products, i.e. DOCH from DOPA). Important for the mechanistic analysis of covalent protein aggregation, we detected cross-links between DOCH at positions B16, B26, A14, and A19 and free amino groups of the N-terminal amino acids Phe B1 and Gly A1, and the side chains of Lys B29, His B5 and His B10.

Materials and Methods

Materials

Recombinant human insulin containing 0.4% (w/w) zinc was provided by Merck (Oss, The Netherlands). L-ascorbic acid, ethylenediaminetetraacetic acid sodium salt (EDTA), copper dichloride, arginine, monobasic and dibasic sodium hydrogen phosphate, ammonium bicarbonate (ABI), sodium citrate and citric acid, dithiothreitol (DTT), iodoacetamide (IAM) and the solvents glacial acetic acid and acetonitrile were purchased from Sigma–Aldrich (St.Louis, MO, USA). Millipore Q water was used for the preparation of all the formulations and solutions. All chemicals were of analytical grade and used without further purification. ABS was synthesized according to a published

procedure [21]. Glu-C endoproteinase used in this study was purchased from Promega (Madison, WI, USA). Centrifugal filter units with a volume capacity of 4 mL and a molecular weight cut-off of 3 kDa were purchased from Millipore (Billerica, MA, USA). Cassette dialysis slides with a 2 kDa cut-off were purchased from Thermo Scientific (Asheville, NC, USA).

Metal-catalyzed oxidation of insulin

Insulin oxidation was performed at three pH values: pH 7.4 in 50 mM sodium phosphate buffer (PB), pH 3.0 in 50 mM sodium citrate buffer (CB) and at pH 8.0 in 250 mM ammonium bicarbonate (ABI). For all the oxidation reactions, insulin was first dissolved in 0.1 M hydrochloric acid and then diluted into the corresponding buffer. Depending on the desired pH, 0.1 M sodium hydroxide was used. When CB was used, the final pH of 3 was achieved without further addition of base. Insulin concentration was measured by UV spectroscopy using a molecular weight of 5.8 kDa and an extinction coefficient of 6200 M^{-1} cm^{-1} at 276 nm [22]. Further dilutions in PB, ABI or CB were performed to obtain a final insulin concentration of 1 mg/mL. Controls included 1 mg/mL of insulin in 50 mM PB, pH 7.4; in 50 mM CB, pH 3.0; and in 250 mM ABI, pH 8.0. MCO was performed by addition to 1 mL of 1 mg/mL insulin, 100 µL of 0.4 mM $CuCl_2$ in 50 mM PB, pH 7.4, or in 50 mM CB, pH 3, or in 250 mM ABI, pH 8, depending on the desired pH, to a final concentration of 40 µM $CuCl_2$. The reaction was performed in 2-mL Eppendorf tubes covered with aluminum foil to protect the reaction mixture from light. After 10 minutes of incubation of insulin with Cu^{2+}, to allow copper to bind to insulin, the oxidation reaction was started by the addition of 110 µL of 40 mM L-ascorbic acid in 50 mM PB, pH 7.4, or in 50 mM CB, pH 3, or in 250 mM ABI, pH 8, depending on the desired pH, to a final concentration of 4 mM. The reaction was quenched after 3 hours of incubation at room temperature by adding 12.1 µL of a 100 mM EDTA in 50 mM PB, pH 7.4, or in 50 mM CB, pH 3, or in 250 mM ABI, pH 8, depending on the desired pH, to a final concentration of 1 mM [15]. To monitor the presence of protein fragments, the oxidation was also performed (in PB with the same amount of copper and L-ascorbic acid) at room temperature and at 37 °C for 24 hours before quenching. The oxidized samples were extensively dialyzed at 4 °C against 50 mM ammonium bicarbonate (ABI) buffer, pH 8.0, for 24 h. In another experiment, the oxidized

sample in 50 mM sodium citrate buffer pH 3, was extensively dialyzed at 4 °C in 50 mM sodium phosphate buffer, pH 7.4, using centrifugal filter units. After that the sample was left to equilibrate for 12 hours at room temperature.

ABS-derivatization of undigested samples

Solutions of native insulin and insulin oxidized at pH 3.0 (in CB) and pH 7.4 (in PB), dialyzed into 50 mM ABI, were treated with 0.1 M sodium hydroxide to a final pH of 9.0. Subsequently, to 100 μL of these solutions, 11 μL of a solution of 100 mM ABS dissolved in water were added to a final concentration of 10 mM. Next, 1.1 μL of 50 mM $K_3Fe(CN)_6$ dissolved in water was added to a final concentration of 0.5 mM. The reaction was conducted for 1 hour at room temperature before performing SEC analysis. Controls for non-specific fluorescence included derivatization reagents (without protein) incubated under the same conditions and non-derivatized protein.

Reduction, alkylation and ABS-derivatization of insulin

Solutions of native insulin and oxidized insulin, dialyzed into 50 mM ABI, pH 8.0, were reduced using 50 mM dithiothreitol (DTT), freshly prepared in 50 mM ABI, pH 8.0, added to a final concentration of 5 mM. The samples were incubated for 45 minutes at 45 °C using a thermo heating bath (Thermo Scientific, Asheville, NC, USA). Subsequently, 200 mM iodoacetamide (IAM), freshly prepared in 50 mM ABI, pH 8.0, was added to a final concentration of 20 mM. The pH of 100 μL of dialyzed, reduced and alkylated insulin samples was adjusted to 9.0 with 0.1 M sodium hydroxide. Then, 11 μL of a solution of 100 mM ABS dissolved in water were added to a final concentration of 10 mM. Subsequently, 1.11 μL of 50 mM $K_3Fe(CN)_6$ dissolved in water was added to a final concentration of 0.5 mM. The reaction was conducted for 1 hour at room temperature before performing digestion with Glu-C.

Glu-C digestion

The proteolytic digestion was performed after reduction, alkylation and ABS-derivatization of insulin, by incubating the samples with Glu-C endoproteinase in a ratio insulin: Glu-C endoproteinase 10:1 (w/w), overnight at 37 °C.

Size-exclusion chromatography

An Insulin HMWP column (7.8 × 300 mm, Waters, Milford, MA, USA) was connected to a Shimadzu HPLC system (UFLC Shimadzu Instrument equipped with two LC-20AT pumps, Columbia, MD) coupled to a photo-diode array detector (Shimadzu, SPD-M20A) and a fluorescence detector (Shimadzu, RF-20A). The photo diode array detector allowed for the recording of the UV spectrum in the region between 200-800 nm. Undigested insulin samples, without ABS treatment, were injected to calculate the percentages of the aggregates and to measure tyrosine fluorescence. Moreover, undigested insulin samples that were treated with ABS were injected to measure the benzoxazole fluorescence, which can be formed only in presence of DOPA and/or DOCH. The percentage of aggregates was calculated based on peak areas of the UV peaks at 276 nm (chromatograms not shown), as reported by Hermeling et al [5-6]. The fluorescence detector was set at various different excitation (Ex) and emission (Em) wavelengths, depending on the type of analysis: Ex 275 nm/Em 302 nm was used for monitoring Tyr fluorescence and Ex 360/Em 490 nm was used for the detection of the benzoxazole fluorescence of the ABS-derivatized samples. The mobile phase was composed of a mixture of 1 g/L L-arginine in water/acetonitrile/glacial acetic acid 65:20:15 (v/v/v) as reported in the United States and European pharmacopeias [23-24]. The elution buffer was freshly prepared, filtered using a regenerated cellulose filter (Sartorius Stedim Biotech, Arvada, CO) and degassed prior to use.

Steady-state fluorescence spectroscopy

The benzoxazole fluorescence of the ABS-derivatized samples was measured upon excitation at 360 nm. The emission spectra were recorded from 490 nm to 600 nm with a 5-nm bandwidth on a Shimadzu RF-5000U spectrofluorometer. To monitor the presence of dityrosine, the non-derivatized, oxidized samples were analyzed after dialysis in 50 mM ABI, pH 8.0, using an excitation wavelength of 315 nm and detection at 420 nm [25]. After ABS-derivatization and dialysis, sample volumes were diluted with 50 mM ABI to 0.5 mL. Spectra were recorded in 0.5 mL 1-cm path length fluorescence cuvettes (Hellma, Plainview, NY, USA). Controls for non-specific

fluorescence included samples without a substrate or reagents incubated under the same conditions.

Mass spectrometry

Digested and non digested peptides were analyzed by means of an LTQ-FT hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo-Finnigan, Bremen, Germany) and a SYNAPT-G2 (Waters Corporation, Milford, MA), both located in the Mass Spectrometry Laboratory of the University of Kansas, under the conditions as described by Ikehata et al [26]. In short, peptides were separated on a reversed-phase LC Packings PepMap C18 column (0.300 × 150 mm) at a flow rate 10 µL/min with a linear gradient from 0 to 65% acetonitrile in 0.06% aqueous formic acid over a period of 55 min using LC Packing Ultimate Chromatograph (Dionex). LC-MS experiments were performed in a data-dependent acquisition manner using Xcalibur 2.0 software (Thermo Scientific). Five most intensive precursor ions in a survey MS1 mass spectrum acquired over a mass range of 300–2000 m/z were selected and fragmented in the linear ion trap by collision-induced dissociation. The ion selection threshold was 500 counts. The MS/MS spectra obtained were analyzed with the software MassMatrix [27-30]. MassMatrix was used to generate the theoretical fragment tables of the b and y ions of the different oxidized and cross-linked products. The theoretical fragments were compared to the experimental MS/MS spectra to validate the structures, which were taken into consideration only if the difference between the theoretical and the experimental m/z of the parent ion (and the fragment ions) was strictly below 0.1 Da. The SYNAPT-G2 instrument was operated for maximum resolution with all lenses optimized on the $[M + 2H]^{2+}$ ion from the [Glu][1]-fibrinopeptide B. The cone voltage was 30 V and Ar was admitted to the collision cell. The spectra were acquired using a mass range of 50-2000 m/z. The data were accumulated for 0.7 sec per cycle. The CID data, at the MS^2 level, acquired with the FT-ICR instrument were obtained after an attenuation of the parent ion of 35%. The mass window to collect the parent ion was fixed to 0.1 Da. Deconvolution of the electrospray ionization data of the undigested insulin was obtained using the maximum entropy distribution algorithm implemented in the Masslynx MaxEnt software (Waters Corporation, Milford, MA) using an adduct of 1 proton. Assuming a normal statistical distribution of

the noise, a uniform Gaussian with a width at half height of 0.5 Da was used. A number of fifty iterations and a range between 0-36000 Da were used to build the most probable mass spectrum of the parent ions.

Results

To guide the reader through the results, these will be presented in three sections: 1) Aggregation profile of oxidized insulin and elucidation of aggregation mechanism, 2) MS analysis of undigested insulin samples, and 3) identification of chemical modifications by MS/MS analysis of reduced, alkylated, ABS-derivatized and digested samples.

Aggregation profile of oxidized insulin and elucidation of aggregation mechanism

Investigation of the chemical mechanisms, as well as the aggregation profile, was achieved with SEC. This technique was employed for several purposes: to detect and quantify the percentage of monomer loss, and the formation of covalent dimers and high-molecular-weight oligomers (HMWO) during MCO, by using UV detection at 276 nm (note that the composition of the mobile phase does not allow us to estimate the amount of non-covalent aggregates since these will be dissociated because of the organic solvent and the low pH of the eluent [31]); to monitor Tyr fluorescence in oxidized insulin, by using an excitation wavelength of 275 nm with emission set at 302 nm, and to detect the development of DOPA and DOCH through derivatization with ABS.

Furthermore, we used SEC to monitor the presence of fragments after prolonged oxidation. The percentages found (Table I) are in agreement with our previous results [13]. MCO under our applied standard conditions leads to a loss of 25.6% of monomer and the formation of 18.8% of dimer and 6.8% of HMWO (Figure 2, panel A). The derivatization with ABS, followed by SEC analysis, allowed the detection of DOPA and DOCH, in the monomer, as well as in the aggregates (Figure 2, panel B).

The overall results point to the presence of Tyr and Phe oxidation products in all insulin species formed during MCO. A much weaker overall fluorescent signal was detected for the derivatized non-oxidized insulin (Figure 2, panel B), suggesting that a low percentage of DOPA was already present in the

starting material, which could have been oxidized during production, storage and handling.

The fluorescence spectra of the ABS derivatized samples (data not shown) were in agreement with the fluorescence detection performed with SEC: a low but measurable fluorescence emission was found for the native insulin derivatized with ABS and a 6-fold higher fluorescence signal was obtained for the oxidized insulin derivatized with ABS.

Aggregated insulin was almost completely absent when MCO was performed at pH 3.0 (panel C of Figure 2). This result suggests that the aggregation process, which appears to be mediated by nucleophilic reactions with DOCH, is inhibited at lower pH, likely due to protonation of free amino groups and His residues.

Nevertheless, DOPA and DOCH were detected after derivatization with ABS (Figure 2, panel D), indicating that the lower pH did not inhibit insulin oxidation. Hence, it may be possible that insulin oxidized at pH 3.0 would still aggregate if the pH is increased after oxidation. To test this hypothesis, the buffer of the insulin sample oxidized at pH 3.0 was subsequently exchanged with 50 mM sodium phosphate buffer, pH 7.4, using centrifugal filter units. The sample was left to equilibrate for 12 hours at room temperature before SEC analysis.

Figure 2, panel E and Table I represent the chromatograms and the percentages of aggregates for this experiment, displaying an increased amount of insulin dimer and trimer after the buffer change.

Altogether these results indicate that Michael addition, the addition of a nucleophile to an α,β-unsaturated carbonyl compound [32], may be the main mechanism of covalent insulin aggregation during MCO. Moreover, this experiment disproves a major role of S-S scrambling in insulin aggregation during MCO. However, the occurrence of non-covalent (besides covalent) aggregation cannot be fully excluded.
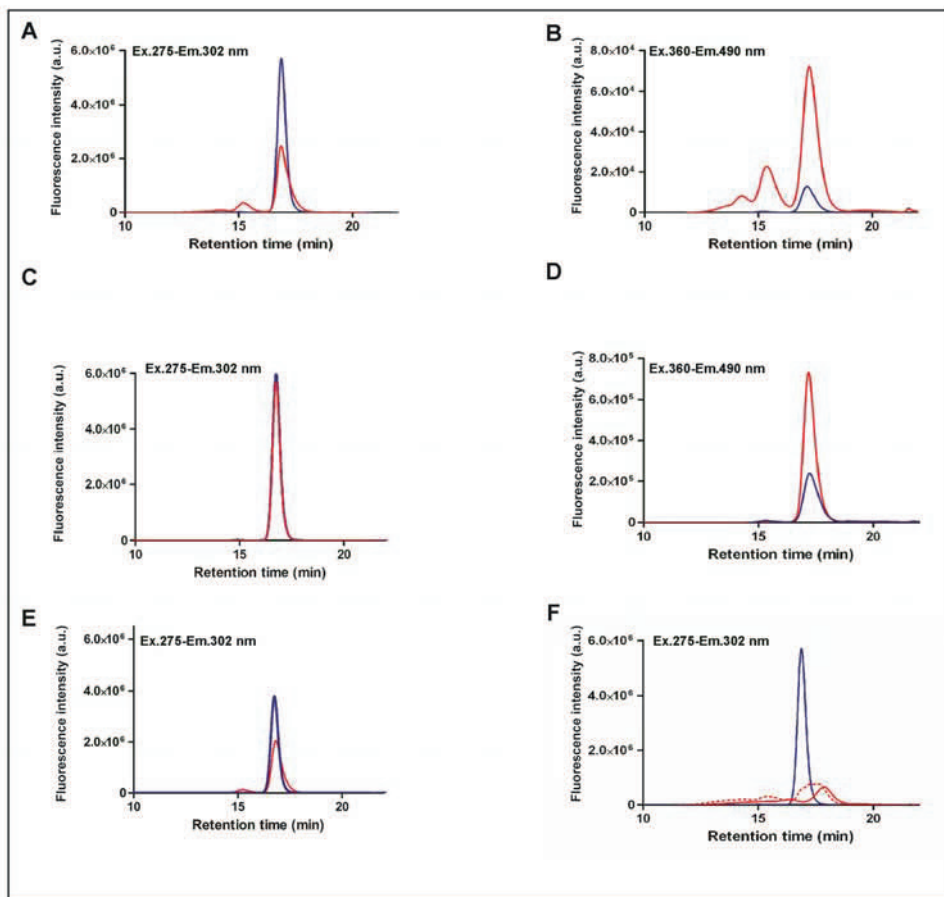
Figure 2. SEC with intrinsic tyrosine fluorescence detection (panels A, C, E, F; Ex 275 nm-Em 302 nm) and ABS fluorescence detection (panels B, D): (A) insulin oxidized in PB 50 mM, pH 7.4, for 3 hours at room temperature (in red) and its control, untreated insulin (in blue); (B) oxidized insulin (pH 7.4), derivatized with ABS (in red) and its control, untreated insulin derivatized with ABS (in blue); (C) insulin oxidized in 50 mM sodium citrate, pH 3.0, for 3 hours at room temperature (in red) and its control, untreated insulin (in blue); (D) oxidized insulin (pH 3.0), derivatized with ABS (in red) and its control, untreated insulin derivatized with ABS (in blue); (E) insulin oxidized in 50 mM sodium citrate, pH 3.0, for 3 hours at room temperature, exchanged in 50 mM PB, pH 7.4, for 12 hours at room temperature (in red) and its control, oxidized insulin (pH 3) (in blue); (F) insulin oxidized in 50 mM PB, pH 7.4, for 24 hours, at room temperature (dotted red line) and at 37 °C (solid red line), untreated insulin (blue line).

Table I. Percentages of monomer, dimer and high-molecular-weight oligomers (HMWO) for the insulin oxidized under different conditions and native insulin.§

Sample	%Monomer	%Dimer	%HMWO
Oxidized insulin in PB, pH 7.4	74.4 ± 0.9	18.8 ± 1.4	6.8 ± 1.2
Oxidized insulin in ABI, pH 8.0	96.1 ± 0.7	3.9 ± 0.7	ND#
Oxidized insulin in sodium citrate, pH 3	99.2 ± 0.3	0.8 ± 0.3	ND
Oxidized insulin in sodium citrate, pH 3.0, spiked into PB, pH 7.4	88.8 ± 1.3	9.3 ± 1.1	1.9 ± 0.1
Native insulin in PB, pH 7.4	99.4 ± 0.2	0.6 ± 0.2	ND

§ Based on SEC analysis with UV detection at 276 nm; data represent mean ± standard deviation of 3 individual batches
# ND = not detected

Additional evidence for Michael addition was provided in an experiment in which we performed the oxidation of insulin at pH 8.0, in the presence of a relatively high concentration (0.25 M) of ammonium bicarbonate. Under these conditions ammonia competes with nucleophilic functional groups of insulin (i.e. the N-terminus of Gly A1 and Phe B1, and the side chains of His B5, B10 and Lys B29) for the addition to DOCH, thereby inhibiting aggregate formation, as shown in Figure 3, panel A and indicated by the low percentages of covalent aggregates measured (Table I). This was confirmed with FT-ICR MS analysis, performed on oxidized insulin in ABI, after reduction, alkylation and Glu-C digestion. As can be seen in the chemical structure representatively depicted in panel B of Figure 3, two amino groups (highlighted in blue) are covalently bound to the first Phe residue and one amino group is covalently bound to the second Phe residue, indicating that Michael addition occurred between both oxidized Phe residues and ammonia originating from the buffer.
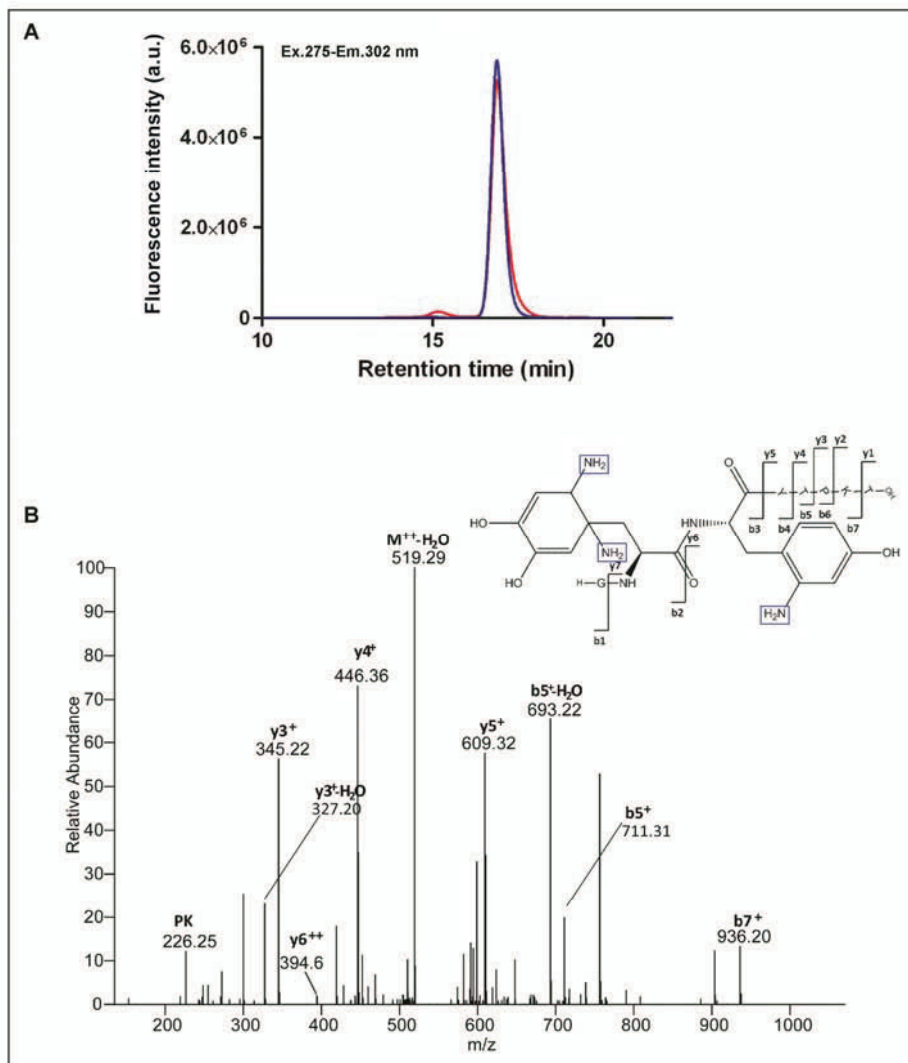
Figure 3. (A) SEC with intrinsic tyrosine fluorescence detection (Ex 275 nm-Em 302 nm) of insulin oxidized for 3 hours at room temperature in ABI 0.25 M (in red) and its control (in blue). (B) MS/MS of peptidic fragment obtained from oxidized insulin in ABI 0.25 M, reduced (DTT), alkylated (IAM), and digested with Glu-C. The amino groups added through Michael addition to the oxidized phenylalanine are highlighted in blue.

These Michael addition products are generated by addition of the nucleophiles to the two β-type carbon atoms in the oxidation product DOCH, and are consistent with the mass increases of the respective original Phe

residues; however, due to the low abundance of sample we did not confirm the regiochemistry of nucleophile addition by NMR. The most relevant ions in the MS/MS spectra, which allow to confirm the addition of ammonia to DOCH, are b5$^+$, y6^{++} and y5$^+$. The first, with m/z 711.31 of a singly charged ion, shows the sequence G$_{B23}$FFYT$_{B27}$ plus the additional molecular weight of three oxygen atoms and three ammonia molecules. These data suggest that the sequence PKT is neither oxidized, nor derivatized with amino groups. The doubly charged ion y6^{++}, with m/z 394.6, related to the sequence F$_{B25}$YTPKT$_{B30}$, shows that one oxygen and one ammonia molecule are incorporated into this sequence. Of course the Tyr residue could be the target for this incorporation; however, the ion y5$^+$ argues against this hypothesis where the singly charged ion with m/z 609.32 represents the unmodified sequence Y$_{B26}$TPKT$_{B30}$.

To evaluate the potential of MCO to induce fragmentation, already reported by other authors for the MCO of glycated insulin [9, 33], and by our group for the MCO of PEGylated insulin [13], human insulin was oxidized in 50 mM PB, pH 7.4, for 24 hours at room temperature and 37 °C. Figure 2, panel F clearly shows the elution of species after the monomer peak, which indicates the formation of lower molecular weight species.

MS analysis of undigested insulin samples

The FT-ICR MS analysis presented in this section was performed on oxidized insulin and native insulin (our control), prior to reduction, alkylation, ABS-derivatization and digestion with Glu-C. This analysis was executed to monitor the formation of oxidized monomers and to confirm the MCO-induced fragmentation, as well as to quantify these species relative to native insulin.

Oxidation

Human insulin contains three Phe and four Tyr residues. Oxidation of Phe to DOPA would require the incorporation of two oxygen atoms. Instead, oxidation of Tyr to DOPA would be represented by the incorporation of one oxygen atom. Hence, it should be possible to detect mass increases corresponding to ten atoms of oxygen per monomer, considering DOPA as oxidation product of Phe and Tyr. Based on this number, after deconvoluting each MS spectrum by using the maximum entropy distribution algorithm

(Figure 4), we added up the intensities observed for all monomers containing additional oxygen atoms (n=0-10) (i.e., for the masses 5804.63+16, 5804.63+32+...+5804.63+160 Da).
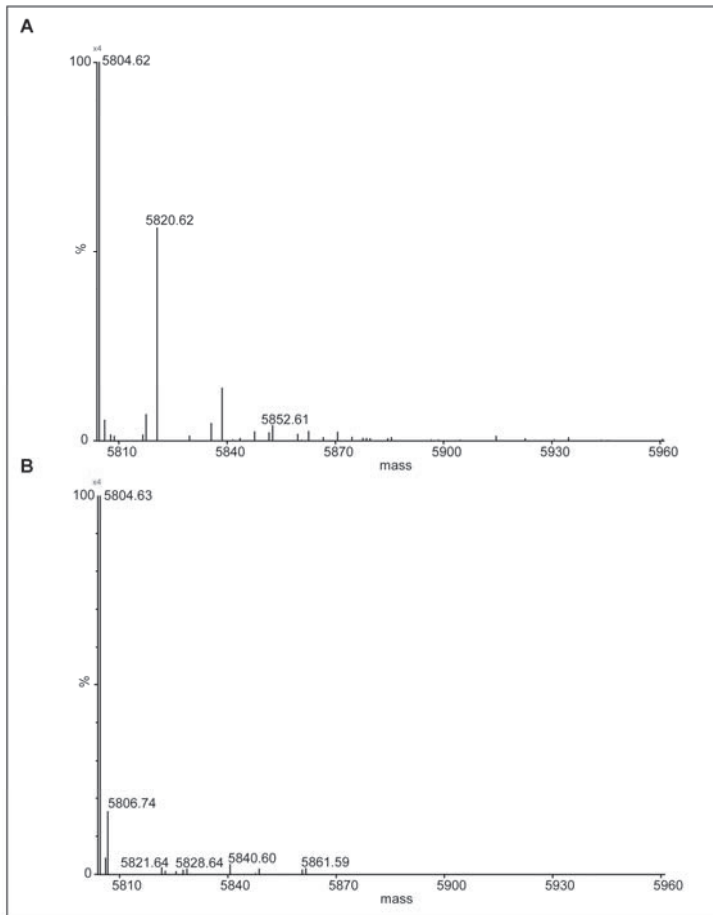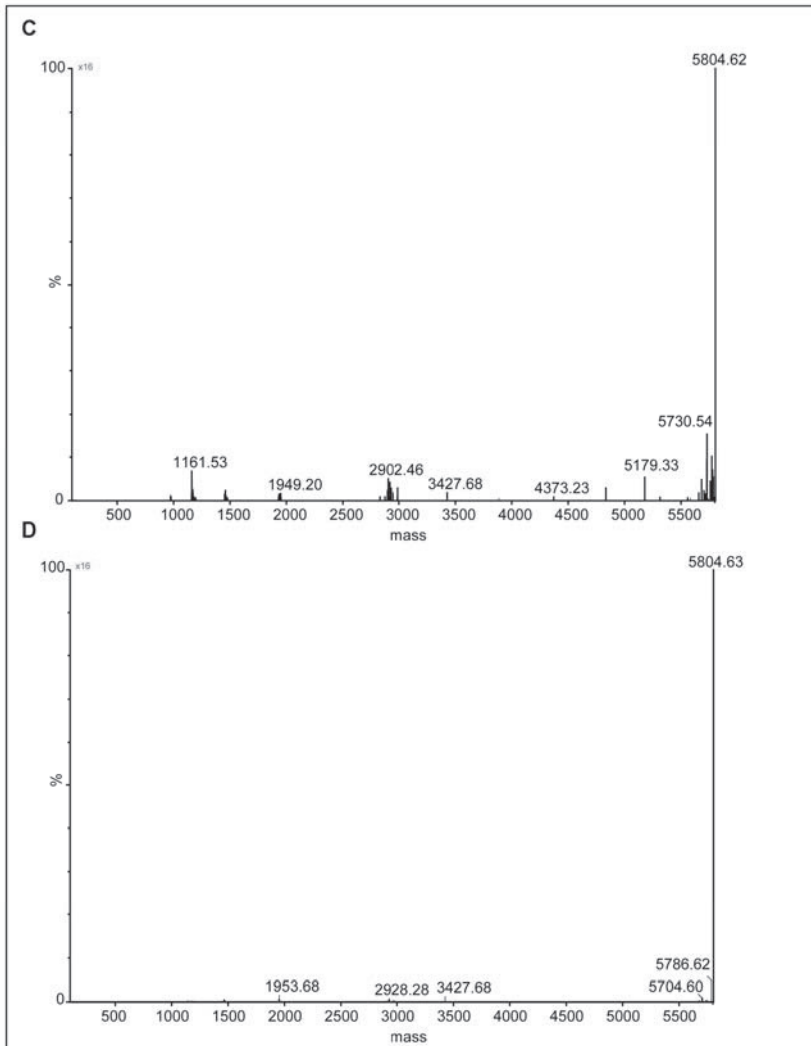

Figure 4. Mass spectrum (MS) of undigested insulin and undigested insulin oxidized for 3 hours at room temperature in 50 mM PB, pH 7.4. Spectra are shown for oxidized insulin (A) and native insulin (B) in the m/z range 5805-5961 to show the oxidized monomer. Spectra were obtained using the maximum entropy distribution algorithm implemented in the Masslynx MaxEnt software (Waters Corporation, Milford, MA) using an adduct of 1 proton. (Please note that 5804.63 represents the molecular mass of unprotonated insulin).

Figure 4. Mass spectrum (MS) of undigested insulin and undigested insulin oxidized for 3 hours at room temperature in 50 mM PB, pH 7.4. Spectra are shown for oxidized insulin (C) and native insulin (D) in the m/z range 100-5805 to show insulin fragments. Spectra were obtained using the maximum entropy distribution algorithm implemented in the Masslynx MaxEnt software (Waters Corporation, Milford, MA) using an adduct of 1 proton. (Please note that 5804.63 represents the molecular mass of unprotonated insulin).

The resulting intensity of oxidized monomer in insulin exposed to MCO was found to be ~300 fold higher than that in non-oxidized insulin (Figure 5), suggesting that the amount of insulin monomer containing oxygen had increased dramatically after MCO. These calculations are possible because after deconvolution of the mass-to-charge ratios, the absolute intensity of each ion is conserved. For better clarity, the oxidized monomer in insulin exposed to MCO and that of the non-oxidized insulin are presented in the mass range 5805-5961 (Figure 4, panel A and B). Figure 4, panel A shows that the intensity of the mass signal at 5820.62 Da (indicating incorporation of one oxygen atom) and the intensity of the mass signal at 5852.61 Da (indicating incorporation of three oxygen atoms) are much more intense after oxidation. Note that signals indicating incorporation of several other numbers of oxygen atoms were detected too, although with lower intensity. Although MS analysis cannot detail which amino acid is exactly oxidized, our calculation provides a first estimation for the extent of MCO in the insulin monomer.
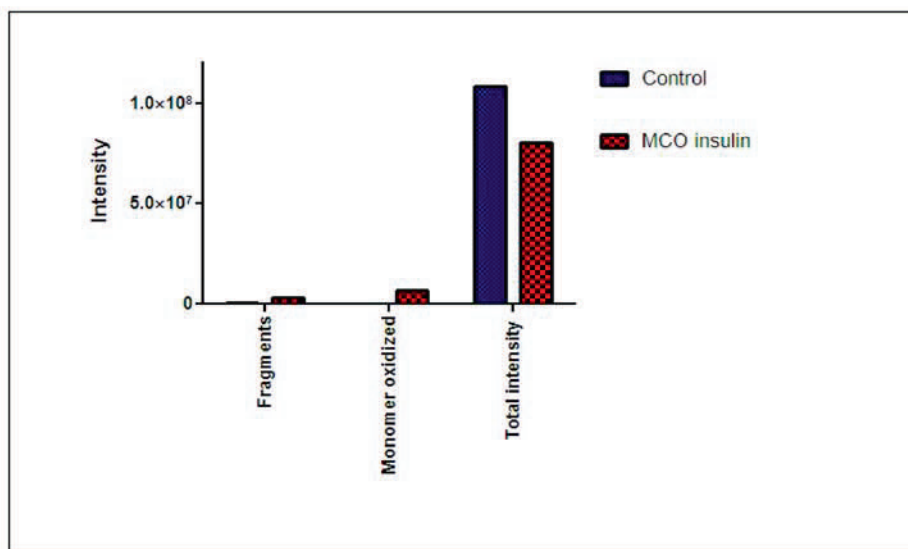
Figure 5. Plot representing the intensity of fragments and oxidized monomer relative to the total intensity of all detected peaks for native insulin (control, in blue) and insulin oxidized for 3 hours at room temperature in 50 mM PB, pH 7.4 (in red). Data are derived from the spectra reported in Figure 4.

Fragmentation

In order to detect and quantify the yield of insulin fragments relative to native insulin and to understand if fragmentation, measured by SEC, occurred during sample preparation for analysis (reduction, alkylation, ABS-derivatization and digestion) or prior to sample preparation, i.e. during the exposure of insulin to Cu^{2+} and ascorbate, MS analysis of native and oxidized insulin was performed prior to and after reduction, alkylation, ABS-derivatization and digestion. Panel C and D of Figure 4, represent a zoom (16X) into the mass region between 100 and 5805 Da: clearly the oxidized insulin contains new peptides of a lower molecular weight compared to insulin. We processed the MS spectra of oxidized and non-oxidized insulin as follows: 1) deconvolution using the maximum entropy distribution algorithm; 2) sum of the intensities of all the fragments with a molecular weight between that of glycine (the smallest amino acid in insulin) and the molecular weight measured for insulin (i.e. 5804.6 Da) minus the molecular weight of glycine, after hydrolysis. The procedure that was employed is summarized in formula 1:

$$I_{total} = \sum_{i=j}^{k} [Fragment]_i \begin{cases} j = Mw(Gly) = 75.03 \\ k = Mw(Insulin - Gly + H_2O) \\ k = 5804.63 - 75.03 + 18 \\ [Fragment]i = Mw \ of \ the \ i^{th} \ fragment \end{cases} \quad (1)$$

The MS spectra of the oxidized and native insulin that were used for this calculation are displayed in Figure 4, panel C and D, respectively. Figure 5 represents the histograms plotted using the intensity values of the same spectra. The total intensity of the control (i.e. native insulin), was calculated between masses of 0 Da and 36000 Da, i.e. until just beyond the theoretical molecular weight of the insulin hexamer. Based on this calculation, the total yield of fragments in the non-oxidized control was 0.3% compared to 3.1% in insulin exposed to Cu^{2+} and ascorbate (percentages have been calculated dividing the total intensity of the fragments over the total intensity of the native insulin used as a control). For a better view of the fragments, we provide a zoom of the range between 100-5805 Da of native insulin and oxidized insulin (Figure 4, panel C and D). Altogether these results suggest that MCO induces peptidic fragmentation. We did not explore in more detail

the mechanism(s) by which such fragmentation occurs, nor did we search for all the possible cross-links in which the new N-termini could be involved. However, we did note the generation of new cross-links between insulin and such fragments (see below), which contain new N-termini and C-terminal amino acids not generated by Glu-C digestion.

Identification of chemical modifications by MS/MS analysis of reduced, alkylated, ABS-derivatized and digested samples

The FT-ICR MS analysis of oxidized and native insulin (control) was performed after reduction, alkylation, ABS-derivatization and digestion with Glu-C, by injecting the samples into the mass spectrometer by liquid chromatography. The aim of this analysis was to map the chemical modifications in the primary structure of insulin, to identify the nonreducible cross-links involved in insulin aggregation and to identify the fragments.

Because different kind of oxidized, ABS-derivatized, and non-oxidized fragments were found, we hereafter define "fragments" as peptides resulting from at least one cleavage site that differs from the one expected from proteolytic digestion by Glu-C (i.e. after glutamic acid; Glu; E) and we define "Glu-C fragments" as peptides originating from the expected proteolytic cleavage sites of Glu-C.

Table II lists all the detected "Glu-C fragments" (oxidized and ABS-derivatized) and "fragments" (oxidized, ABS-derivatized and non-oxidized). Non-oxidized Glu-C fragments were measured for both oxidized and native insulin but are not listed in Table II, since their presence was expected and represents the majority of Glu-C fragments. MS/MS spectra of all fragments is available in the supplementary material, online on http://link.springer.com/article/10.1007%2Fs11095-012-0755-z).

Non-oxidized fragments are only briefly discussed in this manuscript, because the main focus of our work was to elucidate the chemical mechanisms of insulin aggregation during MCO. As displayed in Table II, the following section is divided into the following subsections: oxidized Glu-C fragments, ABS-derivatized Glu-C fragments, oxidized fragments, ABS-derivatized fragments, non-oxidized fragments. Regarding the description of these fragments, the prefixes and subscripts A and B refer to sequences from the A

and B chain, respectively, while b and y refer to the cleavage sites according to the nomenclature introduced by Roepstorff [34].

Table II. Fragments identified in reduced, alkylated, ABS-derivatized and digested (Glu-C) insulin and oxidized insulin, as determined by mass spectrometry.§

Samples	Glu-C fragments oxidized	Glu-C fragments ABS-derivatized	Fragments oxidized	Fragments ABS-derivatized	Fragments Non-oxidized
Oxidized insulin	R$_{B22}$GFF*Y*TPKT$_{B30}$ Fig. S1 A, B, C	R$_{B22}$GFFY*Y*TPKT$_{B30}$ Fig. S5	F*$_{B1}$VNQHLCG$_{B6}$ Fig. S7	A$_{B14}$LY*LVCG$_{B19}$ Fig. S10	L$_{A13}$YQLE$_{A17}$ Fig. S13
	A$_{B14}$LY*LVCGE$_{B21}$ Fig. S2	R$_{B22}$GFFY**TPKT$_{B30}$ Fig. S6	E$_{B21}$RGF**FYTPKT$_{B30}$ Fig. S8	S$_{A12}$LY*QLE$_{A17}$ Fig. S11	I$_{A10}$CSLYA$_{14}$ Fig. S14
	F*$_{B1}$VNQHLCGSHLVE$_{B13}$ Fig. S3		F*$_{B1}$VNQHLCGSHLVEAL$_{B15}$ Fig. S9	S$_{A12}$LY**QLE$_{A17}$ Fig. S12	S$_{A12}$LYQLE$_{A17}$ Fig. S15
	F**$_{B1}$VNQHLCGSHLVEALYLVCGE$_{B21}$ Fig. S4		H$_{B5}$LCGSH*LVEAL$_{B15}$ MS/MS not shown		S$_{A12}$LYQL$_{A16}$ Fig. S16
			F$_{B1}$VNQH*LCGS$_{B9}$ MS/MS not shown		Y$_{A14}$QLE$_{A17}$ Fig. S17
			F$_{B1}$VNQH*LCGSH L$_{B11}$ MS/MS not shown		G$_{B8}$SHLVE$_{B13}$ Fig. S18
					H$_{B10}$LVEAL$_{B15}$ Fig. S19
					F$_{B1}$VNQHLCGS$_{B9}$ Fig. S20
					Y$_{B16}$LVC$_{B19}$ Fig. S21
					G$_{B20}$ERGFFY$_{B26}$ Fig. S22
					G$_{A1}$IVEQC$_{A6}$ Fig. S23
					A$_{B14}$LYLVCG$_{B20}$ Fig. S24
					Q$_{A15}$LENYCN$_{A21}$ Fig. S25
					L$_{B6}$CGSHLVE$_{B13}$ Fig. S26
Insulin	R$_{B22}$GFFY*Y*TPKT$_{B30}$ Fig.S1 A, B, C				G$_{B23}$FFYTPKT$_{B30}$ Fig. S27
					L$_{A13}$YQLE$_{A17}$ Fig. S13

§ oxidation of insulin was performed by MCO for 3 hours at room temperature in 50 mM PB, pH 7.4.
* incorporation of one oxygen atom; ** incorporation of two oxygen atoms.
# incorporation of one ABS molecule; ## incorporation of two ABS molecules.

Oxidized Glu-C fragments

$R_{B22}GFF*Y*TPKT_{B30}$

The C-terminal region of chain B contains two Phe and one Tyr in positions B24, B25 and B26, respectively. DOPA and DOCH can arise from the oxidation of all these residues. Thus it becomes of primary importance to identify the ions, which allows us to discriminate between different oxidation products. The MS/MS spectra reported in supplementary Figure S1A provides evidence for the oxidation of Phe B25 to Tyr where * indicates the incorporation of one oxygen atom. The ion $b3^+$, despite its low intensity, indicates that Phe B24 is present in the native, non-oxidized state, where m/z 361.2 corresponds to the singly charged ion of the sequence RGF. Instead, the ion $b4^+$ provides evidence for oxidation of Phe B25. A careful analysis of the spectra (Figures S1B) reveals the contemporary presence of two different $y5^+$ ions, indicated as (*F4*)$y5^+$ with m/z 625.5 (which corresponds to a structure containing Tyr B26 oxidized) and, (*HO-F4*)$y5^+$ with m/z 609.4 (which corresponds to a structure containing Phe B25 oxidized). Panel C shows the $b4^+$ ions for these two coexisting structures, again representing oxidation of Phe B25 or Tyr B26, respectively. Hence, both Phe B25 and Tyr B26 are targets for the incorporation of one oxygen atom.

$A_{B14}LY*LVCGE_{B21}$

Supplementary Figure S2 displays the MS/MS data for the peptide ALY*LVCGE, which contains an expected Glu-C cleavage site, where Y* represents the incorporation of one oxygen into Tyr, i.e. the formation of DOPA (the Cys residue is alkylated with IAM). This product is confirmed through the presence of the ions $b3^+$-$b5^+$ (although $b3^+$ and $b4^+$ show low intensities) and $y5^+$. The singly charged ions $b3^+$, $b4^+$ and $b5^+$ indicate that the oxygen is in one of the following sequences: ALY, ALYL, or ALYLV. The singly charged ion $y5^+$ indicates that the sequence LVCGE is not oxidized. Therefore, oxygen incorporation must have occurred on one of the first three amino acids, ALY, where Tyr represents the most oxidation-sensitive target amino acid. Further evidence for Tyr B16 oxidation through ABS derivatization is given below.

F*$_{B1}$VNQHLCGSHLVE$_{B13}$

The N-terminus of chain B, Phe B1, displays a mass increase consistent with the oxidation of Phe B1 to cyclohexadienone. The MS/MS analysis (Figure S3) of the sequence F*VNQHLCGSHLVE indicates the formal addition of one oxygen atom and loss of 2 hydrogens from Phe B1, i.e. a mass increase of 14 Da through the appearance of the following ions: y9$^+$, which suggests that the sequence HLCGSHLVE is not oxidized, and b6$^+$, indicating the oxidation of the sequence FVNQHL. In this sequence, HL can be excluded as an oxidation target because of the nature of y9$^+$. Since in the sequence FVNQ, F is most sensitive to oxidation we conclude that oxidation targets Phe B1.

F**$_{B1}$VNQHLCGSHLVEALYLVCGE$_{B21}$

Phe B1 can be further oxidized to DOCH (Figure S4). The observed masses of the ions y17^{++}-H$_2$O and y17$^+$ exclude any oxidation of other amino acids sensitive to oxidation in F**$_{B1}$VNQHLCGSHLVEALYLVCGE$_{B21}$. The ion y17$^+$ with m/z 1899.82 shows an intensity which is about 80% of that of b16$^+$ with m/z 1898.75; thus, it should not be considered the first isotope peak of b16$^+$, which would be expected at m/z 1899.75.

ABS-derivatized Glu-C fragments

R$_{B22}$GFFY#TPKT$_{B30}$ and R$_{B22}$GFFY##TPKT$_{B30}$

Supplementary Figure S5 shows that Tyr B26 is converted to DOPA and/or DOCH, indicated by derivatization with one molecule of ABS (designated with the symbol #). The ions with m/z 361.28 and m/z 508.26, corresponding to b3$^+$ and b4$^+$, respectively, suggest that both Phe B24 and Phe B25 are not oxidized in this sequence. If for instance, Phe B24 (in the sequence RGF$_{B24}$FYTPKT) had been oxidized, the b3$^+$ ion would have been expected with m/z 377.28 (i.e. 361.28+16 Da). Instead, the ions b5$^+$ and b6$^+$ provide evidence that Tyr B26 is the target of oxidation. For additional evidence, supplementary Figure S6 displays the MS/MS spectrum of RGFFY##TPKT, derivatized with two molecules of ABS, as shown in the displayed structure. Here, the ions b3$^+$ and b4$^+$ are identical to the ones reported in Figure S5, although more intense. The ion with m/z 1037.29 corresponding to b5$^+$ and the ion y5$^+$, reported in the inset of Figure S6, confirm that the original Tyr

contains two ABS molecules. We had realized in the past [19-20], that derivatization with ABS can lead to the incorporation of one or two molecules of ABS into the final benzoxazole product (see scheme 1), depending on the availability of ammonia, resulting in competition of ammonia and ABS for the position in 6 of the benzoxazole. In addition to that, it seems that the competition between ammonia and ABS depends on the steric hindrance of the peptide which is derivatized: i.e., derivatization with two ABS molecules of sterically less accessible DOPA and DOCH can be kinetically less favorable than that of more exposed residues. It must be noticed that the sequence R_{B22}GFFYTPKT$_{B30}$, depicted in Figures S5 and S6, does not necessarily belong to the same B chain: in other words, the sequence derivatized with one ABS molecule could be involved in the formation of HMWO, while the doubly ABS-derivatized sequence can actually be present in the monomer as well, although oxidized, and subsequently be derivatized with two ABS molecules.

Oxidized fragments

F*$_{B1}$VNQHLCG$_{B8}$

Phe B1 is oxidized to hydroxylated Phe, indicated through the MS/MS data displayed in Figure S7: the ion y7$^+$ shows that none of the amino acids in the sequence C-terminal to Phe B1, VNQHLCG, is oxidized. Therefore, the ion b5$^+$ confirms oxidation of Phe B1. In fact, Phe hydroxylation can occur in positions ortho, meta and para; however, only the latter would lead to Tyr.

E$_{B21}$RGF**FYTPKT$_{B30}$

The MS/MS data displayed in Figure S8 are consistent with the oxidation of Phe B24 to DOCH. The ions b4$^+$ and y6$^+$ are fundamental to confirm the oxidation of Phe B24 since the first indicates the presence of the sequence ERGF**, and the second, suggests that the sequence FYTPKT is not oxidized. Further confirmation of the chemical structure in which Phe B24 is doubly oxidized arises from the detection of the internal fragment GF**FY with m/z 545.39, which can be produced during the analysis via specific pathways consistent with the mobile proton model [35].

F*$_{B1}$VNQHLCGSHLVEAL$_{B15}$

Phe B1 oxidation is also evident in the sequence F*VNQHLCGSHLVEAL, indicated by the MS/MS data displayed in Figure S9. Here it is sufficient to consider the ions y13+ and b14++. The ion y13+ indicates that the sequence NQHLCGSHLVEAL is not modified. On the other hand b14++ indicates oxidation in the sequence FVNQHLCGSHLVEA. By exclusion, this limits oxidation to the N-terminal subsequence FV, where F represents the most oxidation-sensitive amino acid.

ABS-derivatized fragments

A$_{B14}$LY#LVC$_{B19}$

We further corroborated the formation of DOPA through derivatization of the oxidized peptide ALY*LVC with ABS (Figure S10). The only possible site for derivatization in this peptide is an oxidized Tyr residue, since ABS derivatization requires the presence of DOPA (which during the derivatization is oxidized with $K_3Fe(CN)_6$ to DOCH).

S$_{A12}$LY#QLE$_{A17}$ and S$_{A12}$LY##QLE$_{A17}$

The MS spectra presented in Figures S11 and S12 provide evidence for the ABS derivatization of Tyr A14 within the sequence SLYQLE. Successful derivatization with one and two molecules of ABS indicates oxidation of Tyr A14 to either DOPA or DOCH. In both Figures the ions b3+ with m/z 560.11 and 730.07, respectively and, the ion y4+ with m/z 748.39 and 918.15, respectively, indicate derivatization of the original Tyr residue with one and two ABS molecules.

Non-oxidized fragments

Two types of non-oxidized fragments were detected in the control (Table II). They are likely generated during production or storage as a consequence of low traces of transition metals. Instead, 14 non-oxidized fragments were detected as a result of MCO, summarized in the last column of Table II. The MS/MS data of all these fragments are reported in the Supplementary Material (Figures S13-S27, available online).

Covenant cross-links

This section focuses on the identification of covalent cross-links by MS/MS analysis. Figure 6 summarizes all the covalent cross-links detected as a result of the MCO of insulin.
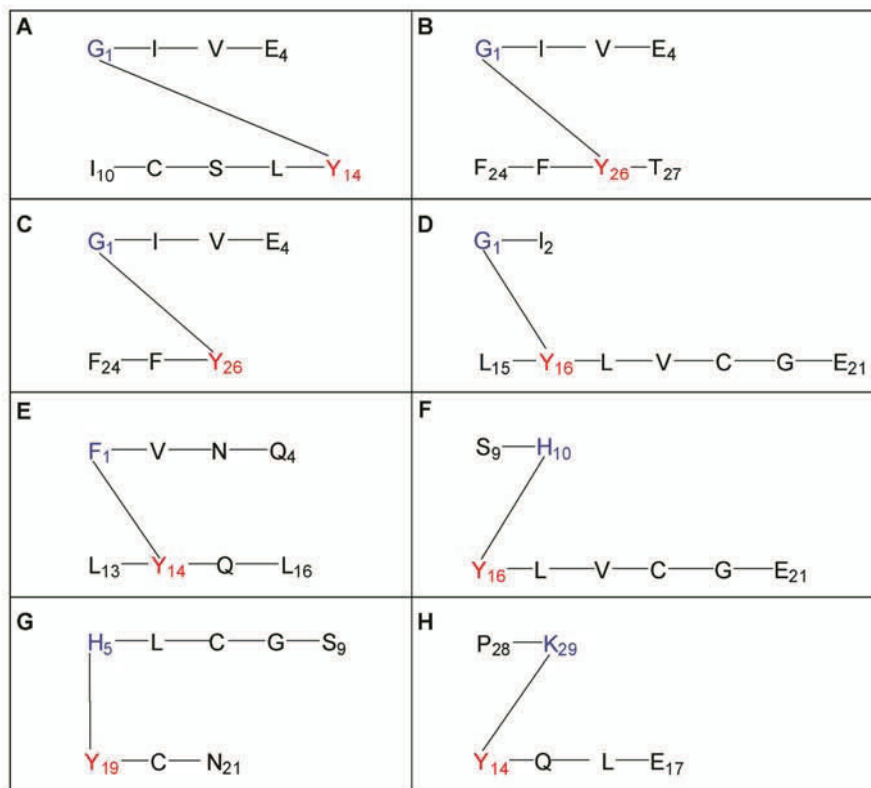

Figure 6. Identified inter-/intra-molecular cross-links measured after MCO of insulin, between (A) Gly A1-Tyr A14, (B and C) Gly A1-Tyr B26, (D) Gly A1-Tyr B16, (E) Phe B1-Tyr A14, (F) His B10-Tyr B16, (G) His B5-Tyr A19, (H) Lys B29-Tyr A14. In blue are shown the amino acids with nucleophilic properties and in red the amino acids oxidized to DOCH, which are Michael acceptor for nucleophilic addition. Further details are given in Figure 7 and in the main text in the section "***Covalent cross-links".***

Gly A1 –Tyr A14 cross-link

Figure 7 panel A displays the MS/MS data consistent with a cross-link between Gly A1 and Tyr A14. The ions Bb1+, By3+ and Ab3+ are the most relevant ones to confirm the cross-link between Gly A1 and Tyr A14.
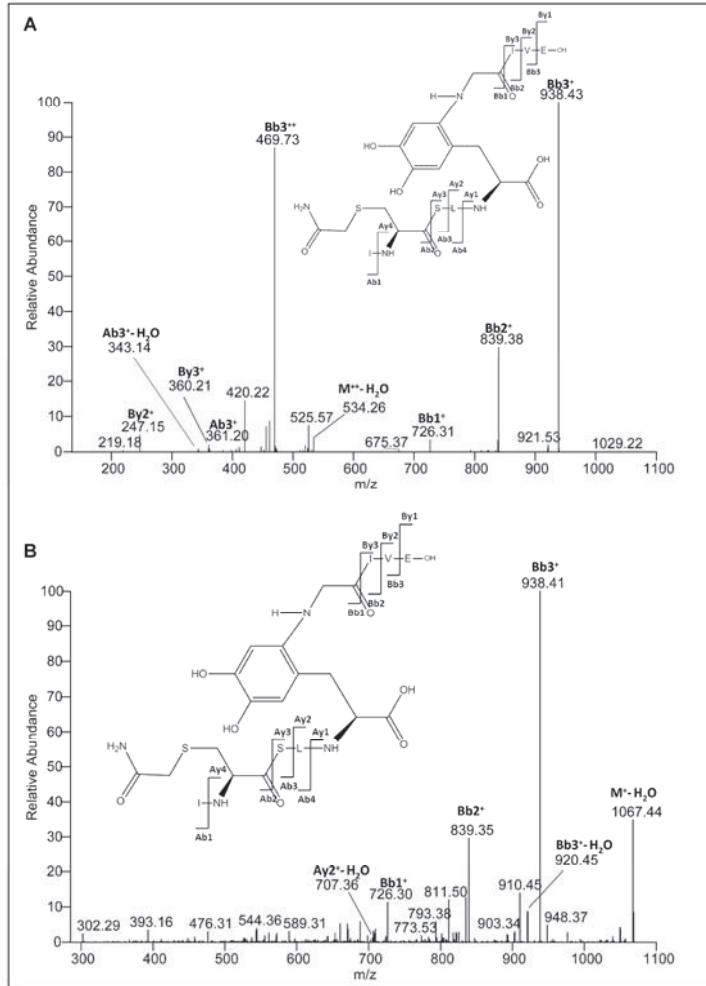
Figure 7. MS/MS of (A and B) sequence GIVE cross-linked through the glycine A1 to the sequence ICSLY. The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

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In particular Bb1+ shows that the sequence ICSLY is covalently attached to Gly. The ion By3+ indicates that the sequence IVE is not involved in the cross-link and, therefore, can be dissociated during the MS/MS analysis since it is connected to Gly A1 only through a peptide bond . Finally, the ion Ab3+ provides evidence that ICS is not involved in the cross-link. In the structure provided in Figure 7, panel A, the covalent attachment of Gly A1 to Tyr A14 is representatively shown for the position in 6 of the original DOPA (or DOCH) product. While, in principle, Michael addition is possible also at positions 1 and 2, position 6 is sterically less hindered, and that is why we selected to represent the adduct formation at position 6. However, we have currently no evidence for this regioselectivity. Further confirmation of the structure reported in Figure 7, panel A was obtained through the spectrum displayed in Figure 7, panel B, where the ion M+-H$_2$O was detected.

Gly A1 – Tyr B 26 cross-link

Figure 7, panel C represents the cross-link of Gly A1 of the sequence GIVE to Tyr B26. The mass spectra displayed in Figures 7, panel C, represents sodium adducts of the respective peptide fragment(s), likely due to incomplete removal of buffer during dialysis. Therefore, all the m/z of the ions presented in Figure 7, panel C and D, have an increased mass of 23 Da. The ion [By2+Na]+, representing the singly charged sequence VE, suggests that the amino acids Val and Glu are not involved in the new covalent bond. If this sequence had been involved in the new covalent bond with Tyr, it would not have been possible to detect the ion [By2+Na]+, since only dissociation of peptide bonds occurs during the low energy collision with the inert gas. [Bb2+Na]+ indicates that the sequence GI is covalently bound to the sequence FFY. We excluded the possibility of DOPA as the product of one of the Phe residues, since in such case the ion [Bb2+Na]+ would have a mass increase (relative to the expected mass of the fragment of unmodified insulin) larger than 16 Da (the oxidation of Phe to DOPA and DOCH requires two oxygen atoms); if one of the Phe residues were exclusively oxidized to Tyr, the ion [Bb2+Na]+ would feature the same molecular weight as the one detected in this MS/MS spectrum. In such case, however, it would not be possible to observe Michael addition, since the new Tyr residue (generated from the

mono-oxidation of Phe) is not a Michael acceptor unless it is further oxidized to a quinone.

In panel D of Figure 7 the original sequence FFY shows additional oxidation of both Phe residues (in contrast to panel C, which shows the native Phe residues in FFY): one of them to DOPA and the second one to a 6-amino substituted DOPA, likely originating from Michael addition of ammonia to a DOPA oxidation product.
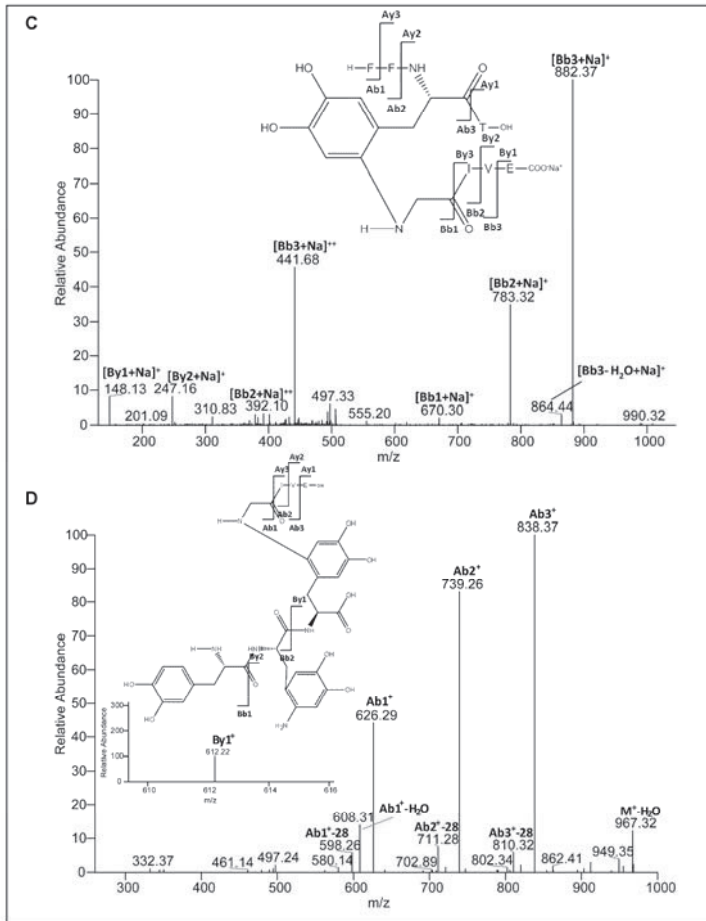

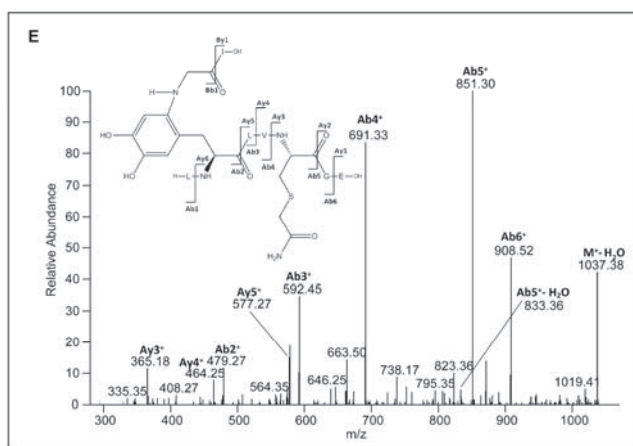**Figure 7.** MS/MS of (C) sequence GIVE cross-linked through the glycine A1 to the sequence FFYT, (D) sequence GIVE cross-linked through the glycine A1 to the sequence FFY. The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

The ions By1+, with m/z 612.22, displayed in the inset, and Ab1+, with m/z 626.29, unequivocally indicate that the covalent bond is between Gly and Tyr: By1+ represents the singly charged sequence Y-GIVE and Ab1+ corresponds to the singly charged sequence FFY-G. The combination of both demonstrates that the new covalent bond, not dissociable during MS/MS analysis, is located between Tyr and Gly. Importantly, our results show that predominantly Tyr oxidation products serve as Michael acceptors for cross-link formation during the MCO of insulin (see also below). There are several possible rationales for this behavior, including protein conformation and the respective yields of Tyr and Phe oxidation products: the oxidation of Phe to DOCH requires one additional oxidation step compared with that of Tyr to DOCH, suggesting that DOCH formation from Tyr is kinetically favorable.

Gly A1 – Tyr B16 cross-link

Figure 7 panel E displays MS/MS data consistent with a covalent cross-link between Gly A1 and Tyr B16. The most relevant ion in this figure is Ab2+ with m/z 479.27, which demonstrates that the sequence GI is covalently bound to LY. Since Ile and Leu do not contain functional groups amenable to Michael addition, we conclude that the covalent bond is formed between Gly and Tyr.
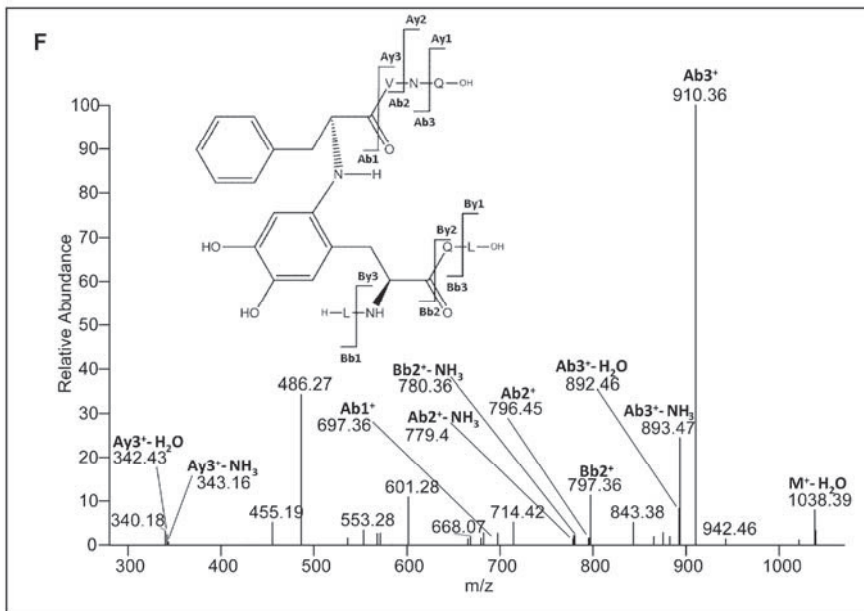

Figure 7. MS/MS of (E) sequence GI cross-linked through the glycine A1 to the sequence LYLVCGE The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

Phe B1 – Tyr A14 cross-link

Phe B1 is not only a possible target for oxidation; the free N-terminal amino group can react via Michael addition with DOCH. The spectrum depicted in panel F of Figure 7 shows the relevant ions consistent with a cross-link between Phe B1 and Tyr A14: Ab1+ with m/z 697.36 shows the existence of a peptide containing the following amino acids: Phe, Tyr, Gln and two Leu, plus the molecular weight of one oxygen atom. However considering only the ion Ab1+, it is not possible to localize the oxygen addition and to characterize the nature of the cross-link. The ion Bb2+, with m/z 797.36, indicates that the sequence FVNQ is connected to the sequence LY. The ion Ay3+ -NH₃, with m/z 343.16, excludes any covalent cross-link of the sequence VNQ. In the sequence LY only Tyr oxidation can lead to a Michael acceptor for the amino terminal of Phe B1, which suggests a cross-link such as depicted in Figure 7, panel F.

Figure 7. MS/MS of (F) sequence FVNQ cross-linked through the phenylalanine B1 to the sequence LYQL. The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

His B10 – Tyr B16 cross-link

Figure 7, panel G displays a cross-link between His B10 of the sequence SH and Tyr B16 of the sequence YLVCG. The relevant ions in this spectrum are Ab1+ and Ay4+. Ion Ab1+, with m/z 420.14, suggests that the sequence SH is covalently bound to the original Tyr residue (after oxidation of Tyr to DOCH). The deprotonated imidazole nitrogen of His is an appropriate nucleophile for Michael addition, demonstrated for example for His addition to dehydroalanine [36].
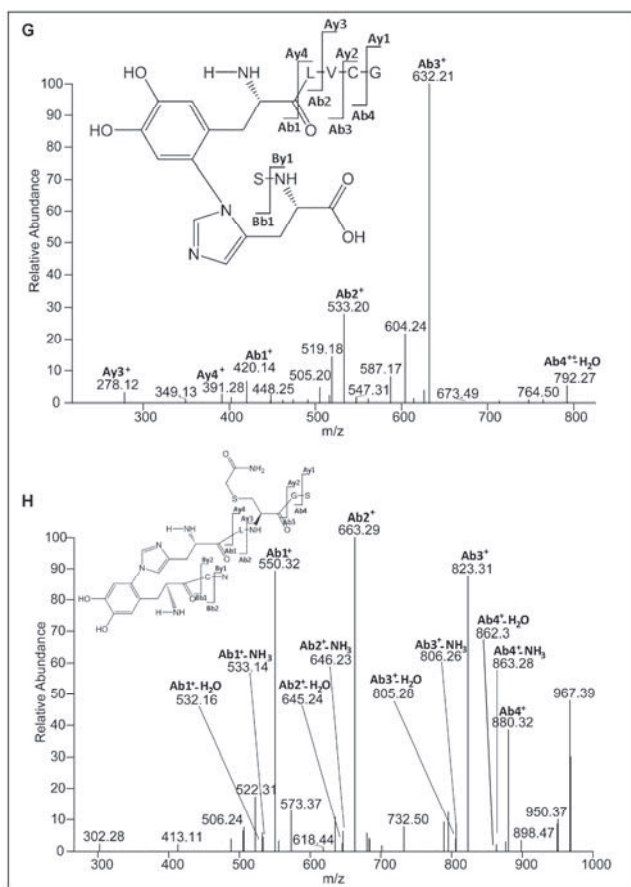

Figure 7. MS/MS of (G) sequence SH cross-linked through the histidine B10 to the sequence YLVCG, (H) sequence HLCGS cross-linked through the histidine B5 to the sequence YCN. The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

His B5 – Tyr A19 cross-link

Panel H of Figure 7 displays a cross-link between His B5 of the sequence HLCGS and Tyr A19 of the sequence YCN and the MS/MS data supporting this assignment. We note that the ion Ab1$^+$-NH$_3$ shows an intensity which is about 80% of that of Ab1$^+$-H$_2$O. Thus, the ion with m/z 533.14 should not be considered the second isotope of Ab1$^+$-H$_2$O, suggesting that the N-terminal amino group of the His is free and not involved in the new covalent bond.

Lys B29 – Tyr A14 cross-link

Figure 7, panel I represents the cross-link between Lys B29 and Tyr A14. The relevant ion in the spectra is Ab1$^+$, since it localizes the cross-link to Tyr and the amino acids Pro and Lys in the sequence PK. Because the MS/MS data in panel I shows no b ions, we cannot conclude whether the cross-link involves the side chain amino group of Lys or the amino group of Pro. The latter would require that non-oxidative fragmentation between Thr B27 and Pro B28 occurs prior to cross-linking; the side chain amino group of Lys B29 represents a good nucleophile and it can therefore be assumed that at least some of the cross-links are formed through the reaction of Tyr A14 with Lys B29.
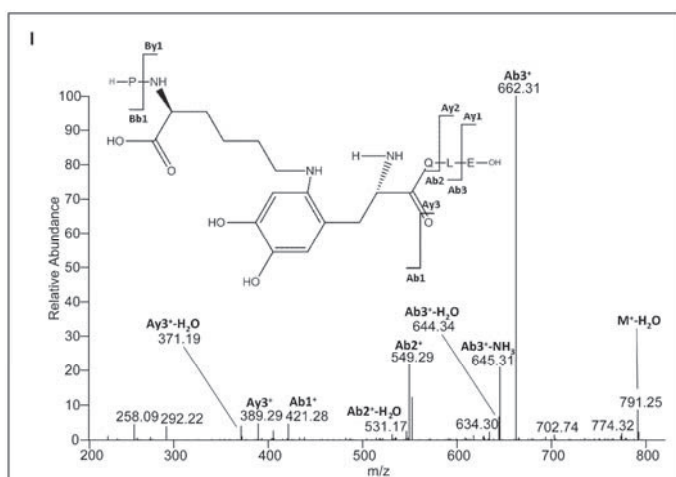

Figure 7. MS/MS of (I) sequence PK cross-linked through the lysine B29 to the sequence YQLE. The fragments have been obtained after reduction (DTT), alkylation (IAM), ABS-derivatization and digestion (Glu-C) of oxidized insulin. Further explanation can be found in the text.

Discussion

We performed a detailed mass spectrometric analysis of insulin in order to elucidate the mechanisms and amino acid residues involved in MCO and MCO-mediated aggregation. The oxidation of Phe and Tyr leads to the formation of catechol structures that can be further oxidized to DOCH. These oxidation products serve as Michael acceptors for cross-links with several nucleophiles present in the insulin sequence.

Formation of dityrosine, as a potential structure involved in the cross-linking of insulin, was not detected, neither with fluorescence measurement (see material and methods), nor with the help of MassMatrix software. This is not surprising since it has been reported that only the hydrogen peroxide/copper system is capable of inducing dityrosine formation [37].

Schiff base formation could potentially be in competition with Michael addition, nonetheless such cross-links can be reversed relatively easily and based on our results does not appear to be the main mechanism of insulin aggregation.

Changes in the primary structure, as reported in this paper, may alter the biological activity of insulin with severe consequences on the glycemic control.

Furthermore, changes in the secondary, tertiary and quaternary structure as a consequence of oxidation, as reported by us before [13], may generate new repetitive epitopes or open the access to hidden epitopes that could contribute to the formation of immunogenic products.

In addition to the characterization and localization of oxidation products, we provided quantitative data on the amount of fragments and oxidized monomers. An important feature of insulin exposed to MCO is the appearance of fragments, which increases the number of polypeptide nucleophiles available for Michael addition. At this point, we do not know whether the observed insulin fragmentation precedes or follows cross-link formation.

Furthermore, it is unknown whether Glu-C might have a different specificity towards oxidized protein. The actual mechanism of cleavage is currently unknown, but we can exclude the classical oxidative cleavage [38] since we did not detect the α,β-dicarbonyl products expected for such a mechanism. Instead, all non-specific cleavages appear to involve a hydrolytic cleavage of the respective peptide bonds. It may be possible that the addition of Cu^{2+} and

ascorbate promote the formation of peptide-metal complexes with hydrolytic activity, such as described for the cleavage of amides through Cu^{2+} [39].

Surprisingly, most of the non-specific peptide fragments we detected were already reported by other authors, e.g. after the oxidation of glycated insulin by Fenton chemistry [9, 33]; however, Guedes et al. [33] interpreted the cleavage mechanism as oxidative cleavage, which is *inconsistent* with the intact C- and N-termini of the detected peptide fragments.

Through MCO experiments at various pH, and in the presence of ammonia, we are able to confirm that Michael addition between insulin chains causes covalent aggregation. In the presence of ammonia, which competes for Michael addition to DOCH, aggregation was almost completely inhibited. Although such experimental conditions allow to prevent aggregation, we noted the introduction of an amino group on oxidized Tyr or Phe which might still have consequences for the immunogenicity of this small polypeptide hormone.

Several proteins have been shown to form aggregates after exposure to Cu^{2+}/ascorbate, such as recombinant SHa(29–231) prion protein [40], superoxide dismutase [41], monoclonal IgG2 [42], interferon alpha-2b [5-6] and interferon beta-1a [7]. The results presented in this paper may serve as a model to rationalize the chemical mechanisms of aggregation during MCO of proteins in general. Although insulin lacks Trp and Met, which are also prone to oxidation [43-44] and are present in most other proteins, DOPA and DOCH should not be formed from oxidation of Trp or Met, thus they cannot act as electron acceptor for Michael addition.

Since MCO was performed at pH 7.4, where insulin is present mainly in the hexameric state [45], the cross-links we measured are likely to be *inter*molecular cross-links, i.e. formed between different insulin molecules. As an example we calculated the distance of Gly A1 and Tyr A14 and Gly A1 and Tyr A19 within the same A-chain (using Swiss pdb viewer [46-47] and T6 human insulin at 1.0 Å resolution, 1MSO pdb file). An average of 18.5 Å was measured for a theoretical covalent bond between the amino group of Gly A1 and the carbon in ortho of the aromatic ring of Tyr A14, and an average of 8.3 Å for the amino group of Gly A1 and the carbon in ortho of the aromatic ring of Tyr A19, which seems to be too far for an *intra*molecular reaction (e.g., the length of the peptidic bond between Gly A1 and Ile A2 is 1.3 Å).

We also noticed that Phe residues, oxidized to DOCH, were not involved in cross-links, nor were they derivatized with ABS. In both cases this could be due to low accessibility of Phe residues which might belong to chains B involved in the formation of HMWO, in which only DOCH originated from Tyr is accessible for ABS derivatization. Moreover, in the case of cross-links it could be speculated that the oxidation of Tyr to DOCH (which requires the addition of only one oxygen atom), is kinetically favorable over the oxidation of Phe to DOCH (which requires two oxidation steps). This can potentially lead to a depletion of the nucleophiles available in the insulin molecule.

Conclusion

Oxidation is one important degradation process that proteins can undergo. In this work we highlighted the potential consequences of DOPA and DOCH formation and also illustrated how the knowledge of oxidation mechanisms can be utilized to investigate the mechanism of covalent aggregation. To this end, recombinant human insulin was used as a model: oxidation of aromatic amino acids residues, besides others, leads to α,β unsaturated carbonyl compounds, which are electron acceptors for Michael addition. These reactive groups, resulting from oxidation, not only can lead to covalent protein aggregation, as shown for insulin in this paper, but also may lead to cross-links between protein molecules and amino acids, which are typically present during cell culture and are often used as formulation excipients. This must be taken into consideration during production and formulation development of therapeutic proteins.

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Chapter 5

Triethylenetetramine prevents insulin aggregation and fragmentation during copper catalyzed oxidation

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Abstract

Metal catalyzed oxidation via the oxidative system Cu^{2+}/ascorbate is known to induce aggregation of therapeutic proteins, resulting in enhanced immunogenicity. Hence, inclusion of anti-oxidants in protein formulations is of great interest. In this study, using recombinant human insulin (insulin) as a model, we investigated the ability of several excipients, in particular triethylenetetramine (TETA), reduced glutathione (GSH) and ethylenediamine tetraacetic acid (EDTA), for their ability to prevent protein oxidation, aggregation and fragmentation. Insulin (1 mg/ml) was oxidized with 40 μM Cu^{2+} and 4 mM ascorbate in absence or presence of excipients. Among the excipients studied, 1 mM of TETA, EDTA or GSH prevented insulin aggregation upon metal catalyzed oxidation (MCO) for 3 hours at room temperature, based on size exclusion chromatography (SEC). At lower concentration (100 μM), for 72 hours at 4 °C, TETA was the only one to inhibit almost completely oxidation-induced insulin aggregation, fragmentation and structural changes, as indicated by SEC, nanoparticle tracking analysis, light obscuration particle counting, intrinsic/extrinsic fluorescence, circular dichroism and chemical derivatization. In contrast, GSH had a slight pro-oxidant effect, as demonstrated by the higher percentage of aggregates and a more severe structural damage, whereas EDTA offered substantially less protection. TETA also protected a monoclonal IgG1 against MCO-induced aggregation, suggesting its general applicability. In conclusion, TETA is a potential candidate excipient for inclusion in formulations of oxidation-sensitive proteins.

Introduction

Protein oxidation in liquid and solid formulations is a major concern in biotherapeutics development, as it can modify protein's structure, function and safety [1-2].

Bivalent copper ion (Cu^{2+}) in particular is an atmospheric pollutant that easily contaminates surfaces and experimental buffers and at sub-micromolar concentration (≤ 0.8 μM) initiates the auto-aggregation and oligomerization of Alzheimer's Aβ peptides [3].

Further, metal catalyzed oxidation (MCO) via the oxidative system Cu^{2+}/ascorbate [4] induces aggregation and alterations in protein structure [2, 5-6], and has been shown in preclinical models to increase the immunogenicity of therapeutic proteins [7-10].

Additionally, we recently showed that human insulin, exposed to the same oxidative system, forms irreversible covalent aggregates via Michael addition to 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH), formed as a consequence of phenylalanine and tyrosine oxidation [11]. Hence, molecules able to inhibit the dramatic effect of copper ions and its catalyzed oxidation of protein are of great importance in protein formulation. Since the year 2000, 48 parenteral drugs containing EDTA in various salt forms have been approved [12]. Although several classes of compounds including polyphenols [13], sugars [14], amino acids [15], thiols [16-17] and chelating agents [18] have been reported to have antioxidant properties, an effective anti-oxidant protecting proteins against copper mediated MCO has not yet been identified.

The aim of this work was to protect insulin from aggregation, fragmentation and oxidative modification upon oxidation induced via Cu^{2+}/ascorbate. With the use of complementary analytical techniques, including size exclusion chromatography (SEC), nanoparticle tracking analysis (NTA), light obscuration particle counting (LOPC), chemical derivatization with benzylamine and 2,4-dinitrophenylhydrazine (DNPH), circular dichroism, and intrinsic and extrinsic fluorescence, we show that 100 μM triethylenetetramine (TETA) [19], unlike reduced glutathione (GSH) [16-17] and ethylenediamine tetraacetic acid (EDTA) [20], is capable of inhibiting aggregation and oxidation of recombinant human insulin formulated in 50 mM phosphate buffer, pH 7.4. Moreover, we show that the anti-oxidant properties

of TETA are applicable to a monoclonal IgG1 as well. Based on our results, we believe that this chelating agent, already approved in 1985 by FDA for the treatment of Wilson's disease [21] and extensively investigated for inhibiting the oxidative stress in diabetic patients [22-32], is an excellent candidate for oxidation-sensitive protein formulations.

Materials and Methods

Materials

Recombinant human insulin (further referred to as insulin) containing 0.4% (w/w) zinc ions was provided by Merck, Oss, the Netherlands. A human monoclonal antibody of the IgG1 subclass (further referred to as IgG) was kindly provided by Dr. Vasco Filipe and described in earlier studies [33]. Disodium hydrogen phosphate, sodium sulfate, trisodium citrate, sodium azide, sucrose, DNPH, benzylamine, potassium ferricyanide ($K_3Fe(CN)_6$), TETA, EDTA, GSH, chlorogenic acid, DL-methionine, melatonine, L-histidine, (±)-α-tocopherol, L-tyrosine, L-carnosine, L-carnitine hydrochloride, copper (I) chloride, copper (II) chloride, ascorbic acid were purchased in the highest purity available from Sigma–Aldrich, Schnelldorf, Germany. Dialysis cassettes (Slide-A-Lyzer 0.5 to 3 mL, 2 kDa cutoff) and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Thermo Fisher Scientific, Breda, The Netherlands. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use.

Insulin formulations

Insulin formulations were prepared using a stock solution of 1.14 mg/mL insulin (concentration determined by UV spectroscopy [34], prepared by dissolving 11.4 mg insulin in 0.5 mL 0.1 M HCl, followed by addition of 9.5 mL 50 mM sodium phosphate buffer, pH 7.4 (PB). All the formulations contained a final concentration of 1 mg/mL insulin in PB and, when required, 40 µM Cu^{2+} and 4 mM ascorbate (positive control, excipient free MCO insulin) and 100 µM excipients (MCO insulin containing excipients).

The negative control was prepared by adding 120 µL PB to 880 µL insulin stock solution. The positive control was obtained by adding to 880 µL insulin stock solution, 10 µL PB, followed by 100 µL $CuCl_2$ solution (10x stock in PB)

and, after 10 minutes, 10μL ascorbate solution (100x stock in PB). The same procedure was followed to prepare MCO insulin containing excipients, by adding instead of PB, 10 μL excipient solution (TETA or GSH or EDTA, 100x stock in PB).

Metal catalyzed oxidation

Insulin samples (1 mg/mL) were oxidized for 24, 48 and 72 hours at 4 °C. Pilot studies conducted with several excipients (Table I) were performed using a final excipient concentration of 1 mM (previously prepared as 10 mM stock solutions in PB). MCO was performed for 3 hours at room temperature, using the same Cu^{2+} and ascorbate concentrations as indicated above. Note that the pH of L-tyrosine stock solution was adjusted to about 10 to increase its solubility, however this did not affect the pH of the final insulin formulation. Vitamin E was prepared by previous dissolution of the oil (4.0 μL, equivalent to 4.3 mg) in 10 μL of analytical grade acetone, followed by dissolution in 990 μL PB, to obtain a stock solution of 10 mM, which was then further diluted 10 fold in the final insulin formulation. All the other excipients tested were soluble at 10 mM concentration in PB.

SEC was performed right after the desired time of incubation, without dialysis of the samples. For all other analyses described in this work (see below), samples were analyzed after 72 hours of MCO at 4 °C and extensive dialysis against PB.

The antioxidant properties of TETA were further studied with IgG, prepared at a concentration of 0.5 mg/mL in 10 mM sodium citrate buffer, 5% (w/v) sucrose, pH 6.0 (CSB). MCO was performed with Cu^{2+} and ascorbate (prepared as 10x and 100x concentrated stock solution in CSB), and used at a final concentration of 40 μM and 4 mM, respectively. TETA was prepared as 10 mM stock in the same buffer and used at a final concentration of 100 μM. MCO was conducted for 3 hours at room temperature. SEC was performed immediately after MCO without dialysis of the samples and Bis-ANS extrinsic fluorescence measurements were performed after extensive dialysis against CSB, as described below.

Size Exclusion Chromatography

Insulin and IgG were analyzed by SEC as previously reported [5, 10]. Briefly an Insulin HMWP Column, 7.8 × 300 mm (Waters, Milford, MA, US) and an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Palo Alto, California, US) coupled to an ultraviolet (UV) detector set at 276 nm was employed for insulin. The mobile phase was composed of a mixture of 1 g/L L arginine aqueous solution/acetonitrile/glacial acetic acid 65:20:15 (v/v/v). A TSK Gel 4000 SW_{XL} column (300 mm x 7.8 mm) with a TSK Gel 4000 SW_{XL} pre-column (Tosoh Bioscience, Montgomeryville, PA, USA) was used for IgG, employing the same system and UV detector, set at 280 nm. The mobile phase was composed of 100 mM sodium phosphate, 100 mM sodium sulfate, 0.05 % (w/v) sodium azide, pH 7.1. A flow rate of 0.5 ml/min was applied for both analyses.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) measurements were performed at room temperature with a NanoSight LM20 (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK), equipped with a sample chamber with a 640 nm laser. Sample, at a concentration of approximately 0.5 mg/mL, were injected into the chamber automatically using sterile syringes (BD Discardit II, New Jersey) connected to an automatic pump (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK). The software used for capturing and analyzing the data was the NTA 2.0 Build 127 (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK). Samples were measured for 90 s with manual shutter and gain adjustments. The "single shutter and gain mode" was used to capture the protein aggregates. The mean arithmetic size was calculated by using the NTA software. Each sample was measured in duplicate. Placebo formulations showed a particle concentration that was too low to allow accurate measurements, hence subtraction of the buffer solution counts from the sample counts was not performed.

Light obscuration particle counting

Light obscuration analysis was performed using a PAMAS SVSS-C (PAMAS GmbH, Bad Salzuflen, Germany). The pre-run volume was 0.3 mL and for each

sample, three measurements were performed using a volume of 0.2 mL per measurement. Between each measurement, the instrument was washed with Millipore Q water, followed by 30% (v/v) ethanol in water, if necessary, until the background signal was less than 10 particles/mL. Placebo formulation signals were subtracted from the corresponding protein sample signals.

Intrinsic and Bis-ANS extrinsic steady state fluorescence spectroscopy

To measure the intrinsic tyrosine fluorescence, dialyzed insulin samples were diluted in PB to 0.1 mg/mL. Duplicates of 200 μL of each sample, pipette into wells of a flat black 96 well plate (Greiner Bio-One B.V., Alphen a/d Rijn, NL) were measured with a Tecan infinite M1000 fluorometer (Tecan group Ltd., Männedorf, Switzerland). The excitation wavelength was set at 275 nm and the emission spectrum was recorded between 290-400 nm.

Extrinsic Bis-ANS fluorescence was used to monitor the formation of exposed hydrophobic regions. To 198 μL (n=2) of 0.1 mg/mL dialyzed insulin or IgG sample, 2 μL Bis-ANS (stock 100 μM in water), was added to a final concentration of 1 μM. Samples were excited at 385 nm using the instrument described above and the emission spectra were recorded from 400 nm to 600 nm. A step size of 2 nm, 50 flashes with a frequency of 400 Hz, a gain of 189 for IgG and 150 for insulin and, a Z-position of 21.5 mm for IgG and 20.0 mm for insulin, were used.

Benzylamine derivatization

Benzylamine derivatization was performed to determine the relative amount of DOPA (3,4-dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid) in each insulin sample [35]. The dialyzed samples in 50 mM ammonium bicarbonate (ABI) pH 9.0 were diluted with the same buffer to 0.1 mg/mL. To 500 μL of these solutions, 1 μL of pure benzylamine was added to a final concentration of 20 mM. Next, 5 μL of 50 mM $K_3Fe(CN)_6$ dissolved in ABI pH 9.0, were added to a final concentration of 0.5 mM. The reaction was conducted for 2h at room temperature before performing a fluorescence emission scan with an excitation wavelength of 360 nm and an emission range of 400-550 nm. Controls for non-specific

fluorescence included derivatization reagents (without protein) and non-derivatized protein incubated under the same conditions.

Carbonyl analysis with DNPH

Free carbonyl groups, which can arise from oxidation of lysine, proline and isoleucine, were measured using a sensitive ELISA method described by Buss et al. [36], which requires less protein than the conventional colorimetric carbonyl assay [37], besides being more accurate [38]. Briefly, 45 µL of dialyzed sample in PB were mixed with 45 µL 10 mM DNPH in 2 M HCl to allow DNPH to react (via Schiff base formation) with potential carbonyl groups generated on the insulin molecule during MCO. After 45 min. of incubation at room temperature, 10 µL of this solution were added to 1 mL PB. Next, 200 µL aliquots were added in triplicate into wells of a Greiner Microlon 96 well ELISA plate and incubated overnight. Subsequently the wells were washed 5 times with 300 µL washing buffer (PB with 0.05 % (w/v) Tween 20) and blocked for 2 hours at room temperature with 300 µL blocking buffer (0.1% w/v BSA, 0.1% Tween 20 in PB). Next, plates were washed three times with washing buffer and 200 µL of antibody solution (anti-DNP-antibody 1:1000, 0.1% BSA, 0.1% Tween 20 in PB) were added to the wells for 1 h at 37 °C. After a final washing step, the ELISA was developed by the addition of 100 µL TMB. Plates were incubated until a blue color became clearly visible (~10 min) and the colorimetric reaction was then stopped by the addition of 100 µL 0.18 M sulfuric acid. Absorbance at 450 nm was measured using Tecan's infinite M1000 plate reader controlled by the i-control 1.5 software (Tecan group Ltd., Männedorf, Switzerland). In order to roughly estimate the nmol of carbonyl groups formed per mg of insulin, MCO insulin excipients free and non oxidized insulin, were analyzed via the classical colorimetric assay, as previously described [37].

Circular dichroism spectroscopy

Near- and far-UV circular dichroism (CD) measurements were performed on a J 815 CD spectrometer (Jasco International, Tokyo, Japan) connected to a Jasco PTC 423S temperature controller set to 25 °C. For near-UV CD analysis, 800 µL sample diluted to 0.8 mg/mL in PB was measured in a 1-cm path

length quartz cuvette while for far-UV CD, 300 μL sample diluted to a concentration of 0.2 mg/mL in PB was measured in a 1-mm path length quartz cuvette. Spectra were recorded from 250-320 nm and 190-250 nm for near- and far-UV CD measurements, respectively, at a scan rate of 100 nm/min, a bandwidth of 1 nm and a response time of 2 s. CD spectra of six sequential measurements were averaged and corrected for the blank (PB). The CD signals were converted to molar ellipticity per amino acid residue ($[\theta]$). Alpha helix content was estimated based on $[\theta]$ at 223 nm ($[\theta]_{223}$), according to Pocker et al [39].

Results

Aggregate and particle content: SEC, NTA, LOPC

At the beginning of this work, extensive pilot studies were conducted to test the potential antioxidant activity of several excipients at 1 mM concentration: L-carnitine, L-carnosine, chlorogenic acid, etylendiaminotetraacetic aicd (EDTA), melatonine, DL-methionine, reduced L-glutathione (GSH), triethylenetetramine (TETA), histidine, (±)-α-tocopherol (vitamin E) and L-tyrosine. After confirming via SEC that none of the excipient employed affected insulin's aggregation state after 3 hours of incubation at room temperature (data not shown), oxidative studies were performed as previously reported [5]. Results (Table I) indicate that insulin oxidized in presence of 1 mM of one of the excipients reported above, shows similar or even higher amounts of aggregates compared with insulin oxidized in absence of excipients, except for TETA, GSH and EDTA, where no differences with the negative control were detected (Table I). Based on the results of the initial screening, TETA, GSH and EDTA were selected to be studied in more detail for their putative protective action against MCO of insulin.

Table I. Aggregate content in MCO insulin samples from a screening study.

MCO [a]	Excipient (1 mM)	Aggregate content (%) [b]
No	None (negative control)	0.5 ± 0.2
Yes	None (positive control)	21.5 ± 4.2
Yes	L-carnitine	24.2 ± 5.4
Yes	L-carnosine	22.7 ± 0.2
Yes	Chlorogenic acid	39.3 ± 1.2
Yes	Melatonine	24.1 ± 6.9
Yes	DL-methionine	41.6 ± 7.1
Yes	L-histidine	29.2 ± 6.1
Yes	(±)-α-Tocopherol (vitamin E)	32.9 ± 4.0
Yes	L-tyrosine	25.8 ± 1.3
Yes	TETA	0.7 ± 0.2
Yes	GSH	0.8 ± 0.2
Yes	EDTA	0.6 ± 0.1

[a] MCO was performed by incubation with 40 µM Cu^{2+} and 4 mM ascorbate for 3 hours at room temperature.

[b] Calculated from SEC data as previously reported [8].

The experimental procedure to test TETA, GSH and EDTA, was established based on the typical storage temperature of liquid protein formulations and on using a minimal required amount of excipients based on previous work on the anti-oxidant activity of EDTA [20]. Additionally, at 1 mM concentration, GSH still presents some reducing activity with respect to insulin disulfide bridges, while EDTA and, to a lower extent TETA, appear to sequester zinc ions, as indicated by near-UV CD which showed a decrease in [θ] at 273 nm ([θ]$_{273}$; data not shown). MCO studies were therefore conducted with 100 µM excipient, at 4 °C for 72 hours.

SEC was used to demonstrate that none of the excipients used affected insulin's aggregation state during the applied incubation time (data not shown). Following MCO, the chromatographic behavior of each sample was investigated (Figure 1A). Native insulin shows a single peak with a retention time of 17.3 minutes, representing monomeric insulin. Note that the mobile phase employed contains acetonitrile and acetic acid, which dissociate native non-covalent oligomeric insulin species (i.e. dimer, tetramer, hexamer) normally present in PB at pH 7.4 in presence of zinc ions [44], while it does not dissociate the covalent aggregates obtained by MCO [11]. The elution behavior of MCO insulin, formulated without excipients, presents several peaks eluted earlier than 17.3 minutes as well as a broad main peak eluting at a slightly longer retention time. This indicates that MCO induced extensive aggregation and fragmentation. The amount of insulin aggregates was calculated as previously reported [5], using the area under the curve (AUC) obtained by SEC (i.e. $AUC_{aggregate\ peak}/AUC_{total\ native}$ x 100%) (Figure 1A).
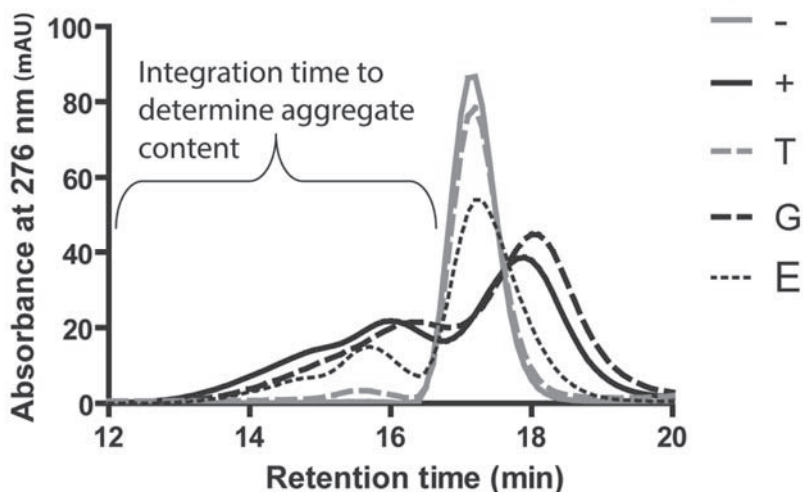
Figure 1A. SEC profiles of insulin samples incubated for 72 h. Brackets delineate the time region for AUC integration used for calculation of the aggregate content. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 µM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of (TETA (T), GSH (G), EDTA (E)) 100 µM excipients as indicated in the graph.

The chromatogram of MCO insulin formulated with 100 µM TETA almost completely overlapped with that of native insulin, suggesting TETA's protective effect towards aggregation and fragmentation. GSH (100 µM) formulated MCO insulin showed a similar behavior as excipient free MCO insulin, indicating absence of any beneficial activity of GSH at this concentration. Finally, in EDTA (100 µM) formulated MCO insulin high amounts of aggregates were measured, but fragmentation seemed to be partially inhibited, as indicated by the absence of species eluting after the monomeric peak. Figure 1B illustrates the strong time-dependent increase in aggregate content of MCO insulin formulated without excipients or with GSH or EDTA. In contrast, after 24 and 48 hours the aggregate content of MCO insulin formulated with TETA was almost as low as that in unmodified insulin (negative control), and only after 72 hours the amount of aggregates calculated had increased to a few percent. In conclusion, 100 µM TETA is highly effective in preventing MCO induced insulin aggregation and fragmentation while the other two candidates had hardly any protective effect under our experimental conditions.
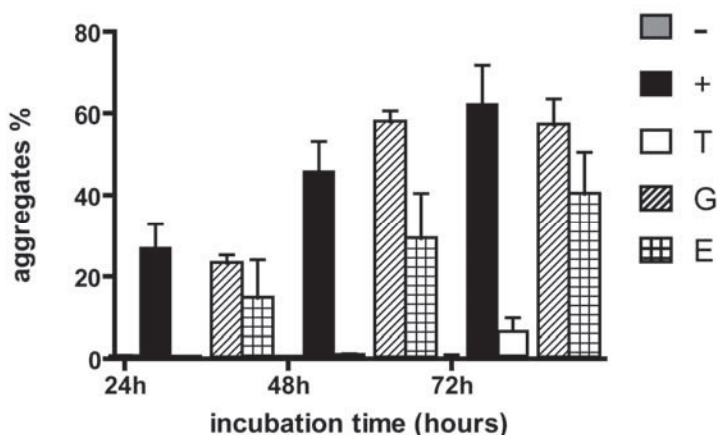
Figure 1B. Percentages of aggregated insulin calculated from SEC profiles after 24 h, 48 h and 72 h of incubation time at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 µM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of (TETA (T), GSH (G), EDTA (E)) 100 µM excipients as indicated in the graph. Error bars represent the deviation from the average of two batches.

Interestingly, the same concentration of TETA appeared to be a valuable antioxidant also in presence of monovalent copper ions (40 μM Cu$^+$) as indicated by the absence of aggregates (based on SEC, data not shown) during MCO performed for 3 hours at room temperature using the oxidative system Cu$^+$/ascorbate.

NTA was used to monitor the formation of submicron-sized particles during MCO and the results are presented in Figure 2 and Table II. For native insulin relatively low particle counts were obtained. For the positive control, an approximately seven fold higher particle concentration was measured. MCO insulin formulated with TETA showed low particle counts comparable to those in the negative control, demonstrating a protective effect of TETA, whereas samples containing GSH or EDTA contained substantially higher particle concentrations.
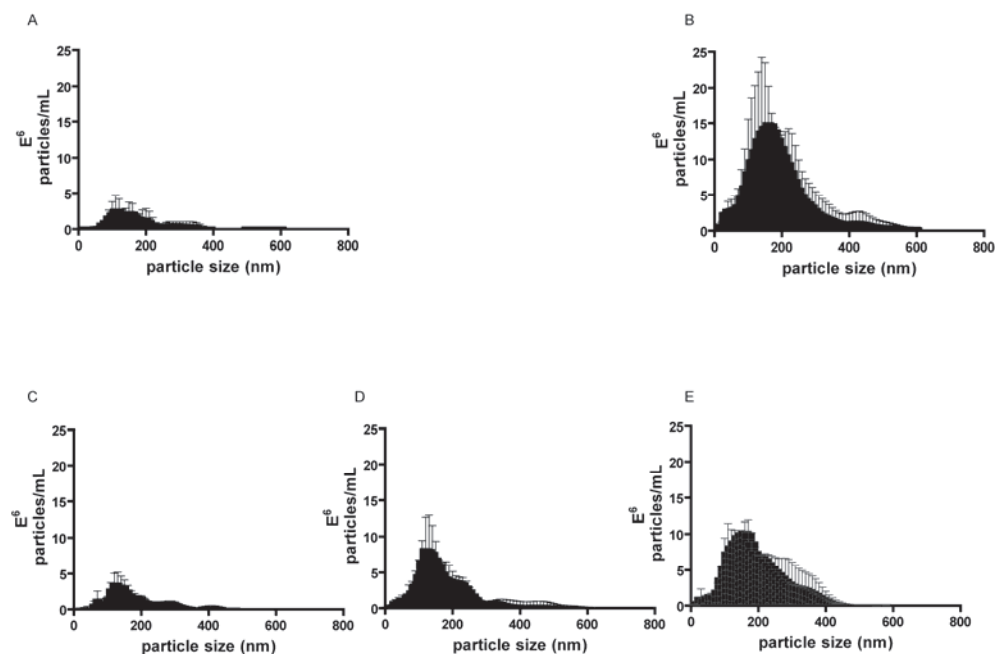
Figure 2. NTA analysis of insulin samples incubated for 72 h: Negative control (insulin incubated in PB) (**A**), other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence of excipient (**B**) (positive control) or in presence of 100 μM TETA (**C**), GSH (**D**), or EDTA (**E**). Error bars represent the deviation from the average of two batches.

LOPC was employed to estimate micron-sized aggregates. Non oxidized insulin contained a low background of $0.79 \pm 0.27*10^3$ particles/mL. In the positive control, the particle concentration in the range 1-10 µm was about ten fold higher than in the negative control (see Table II). MCO of insulin formulated with GSH presented a two fold increase in particle concentration when compared with native insulin, while the TETA- and EDTA-containing formulations showed an approximately three fold increase in particle concentration (in the range 1-10 µm), suggesting a similar inhibiting effect of the excipients on micron-sized particle formation. Micron particles in the larger size range (>10 µm) were barely detected in most of the samples, although the level for the EDTA-containing sample was slightly elevated (Table II).

Table II. Particles concentration and spectroscopic features of insulin samples formulated with and without excipients.

Sample[a]	NTA[b]		LOPC particles concentration (10³/mL)[c]			CD	Benzoxazole fluorescence
	Mean size (nm)	Particles concentration (10⁸/mL)	1-10 μm	10-25 μm	>25 μm	% alpha helix[d]	Intensity (a.u.)[e]
-	193.7 ± 3.8	0.38 ± 0.01	0.79 ± 0.27	0.06 ± 0.05	0.02 ± 0.02	44.9 ± 1.9	5.2 ± 0.3
+	216.9 ± 27.7	2.60 ± 0.05	7.65 ± 3.31	0.08 ± 0.05	0.02 ± 0.01	34.3 ± 2.9	16.5 ± 0.7
T	182.1 ± 12.2	0.69 ± 0.10	2.44 ± 0.40	0.05 ± 0.01	0.02 ± 0.007	39.6 ± 0.9	6.3 ± 0.4
G	193.2 ± 20.4	1.14 ± 0.04	1.71 ± 0.21	0.02 ± 0.02	0.01 ± 0.005	26.9 ± 0.5	15.8 ± 2.5
E	200.1 ± 22.4	1.80 ± 0.25	2.85 ± 0.92	0.2 ± 0.1	0.1 ± 0.09	38.5 ± 0.7	15.0 ± 1.7

[a] Negative control (-), positive control (excipient free MCO insulin,+), MCO insulin formulated with 100 μM TETA (T), GSH (G), and EDTA (E).

[b] Placebo formulations showed negligible particle counts in NTA.

[c] For LOPC analysis, particle counts of placebo formulations were subtracted from the corresponding protein sample. Typically, the particle concentration of placebo formulations was between 0.19-0.40*10³ particles/mL, between 0.01*-0.05*10³ particles/mL and negligible in the size ranges 1-10 μm, 10-25 μm and >25 μm, respectively.

[d] Calculated according to Pocker et al [39].

[e] Calculated based on the fluorescence at the emission maximum (460 nm).

Chemical modifications: DOPA, DOCH and carbonyl content

As previously reported by Sharov et al. [40-41], benzylamine and its derivatives can selectively form a benzoxazole with characteristic fluorescence, after reaction with phenylalanine and tyrosine oxidation products like DOPA (3,4 dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid), providing a useful qualitative tool to identify the presence of specific oxidative chemical modifications. Results (Table II) clearly indicate that oxidized phenylalanine and tyrosine are barely detectable in non oxidized insulin, while in the positive control (excipient free MCO insulin), a three fold higher fluorescence was measured, indicating chemical modification of these aromatic amino acid residues. TETA formulated MCO insulin appeared not to contain these oxidized species, as revealed by its benzoxazole fluorescence spectrum, the intensity of which was comparable to that of plain (non oxidized) insulin treated with benzylamine. By contrast, oxidized insulin formulated with GSH and EDTA showed increased benzoxazole fluorescence intensity comparable to that of the positive control, pointing to the generation of DOPA and DOCH during MCO (Table II).

Other widely used markers of oxidative stress are carbonyl groups, which can be formed during reaction of lysine, proline and isoleucine with reactive oxygen species [42-43]. Figure 3A shows results of the carbonyl content measured for all samples via a sensitive ELISA method. Native insulin gave an OD 450 nm signal comparable to that of the blank (reagent incubated in buffer). The positive control, presented a much higher absorbance, caused by the hydrazone formed between DNPH and new carbonyl groups on the insulin molecule. According to a direct spectroscopic measurement of DNPH-modified MCO insulin, the carbonyl content in the positive control was estimated to be 24.8±7.9 nmol/mg of insulin, which is in the same order of magnitude as the value reported by Montes-Cortes et al. for insulin exposed to plasma from diabetic patient containing reactive oxygen species (ROS), for 4 hours at 37 °C [42]. The relatively small increase in the carbonyl content observed for TETA-containing insulin formulation exposed to MCO, relative to that of the positive control, suggests that TETA almost totally inhibits MCO induced insulin oxidation. In contrast, GSH and EDTA did not have any protecting effect (Figure 3A).
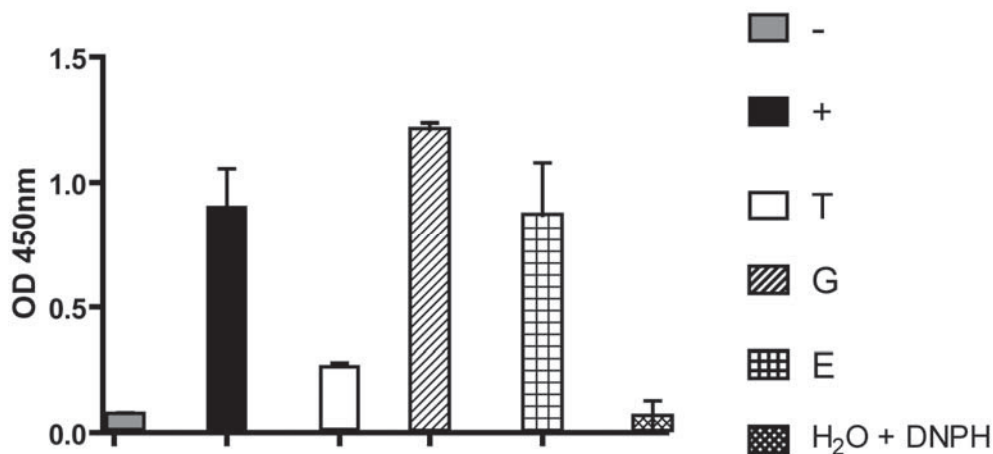
Figure 3A. Carbonyl content of insulin samples incubated for 72 h at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), GSH (G), or EDTA (E), as indicated in the graph. Error bars represent the deviation from the average of two batches.

Secondary and tertiary structure: far-UV CD, intrinsic and extrinsic steady state fluorescence

Spectroscopic techniques can provide useful information about the higher order structural features of a protein. To investigate whether the excipients used would disturb insulin's quaternary structure, near-UV CD measurements were performed after incubating the formulations for 72 hours at 4 °C (without MCO). The negative magnitude of the near-UV CD signal at 273 nm ($[\theta]_{273}$) is directly proportional to the native noncovalent association state of insulin (i.e. monomer < dimer < hexamer) [44]. Our results suggest that with 100 μM of excipient, insulin remained in its tetrameric-hexameric state when formulated with TETA or GSH in PB ($[\theta]_{273}$ 289.4±0.2 and 312.5±1.5 deg cm^2 dmol^{-1}, respectively). However, in the EDTA-containing formulation insulin's quaternary structure was partially dissociated towards a dimeric-tetrameric equilibrium, as indicated by a reduced $[\theta]_{273}$ (227.1±3 deg cm^2 dmol^{-1}). This was expected because EDTA in this concentration is known to deplete zinc from insulin, resulting in a drop of the negative CD signal [44]. Therefore, near-UV CD was not further performed in the following studies on the antioxidant activity of each excipient.

For insulin, far-UV CD has been widely used to estimate the alpha helix content [39, 45-46]. Table II shows that the content in alpha helix in the negative control is about 45%, which is in line with the literature [39]. MCO of excipient free insulin induced approximately 10% loss in alpha helix content as expected based on our previous studies [39]. TETA insulin showed a minimal loss of 5%, when compared to negative control, while insulin's secondary structure in MCO insulin formulated with GSH, appeared to be seriously perturbed, leading to approximately 20% loss in alpha helix. This is likely due a pro-oxidant activity of the excipient, which in fact can reduce Cu^{2+} into Cu^+, which eventually generates hydroxyl radical species in aerobic environment [1]. EDTA formulated MCO insulin showed a slight decrease in alpha helix content roughly comparable to that of TETA formulated MCO insulin.

Fluorescence spectroscopy performed with an excitation wavelength of 275 nm gives information about the environment of the four tyrosine residues in insulin at positions A14, A19, B16 and B26. Non oxidized insulin showed an emission maximum at 299±1 nm (Figure 3B). MCO insulin showed a substantial decrease in the fluorescence intensity but no shift in the emission maximum, suggesting a change in tertiary structure and/or chemical modification of tyrosine residues. The fluorescence emission spectrum of MCO insulin containing TETA almost completely overlapped with that of non oxidized insulin, confirming the protective effect of TETA against MCO. GSH formulated MCO insulin showed a spectrum comparable to that of excipient free MCO insulin, while MCO of insulin in presence of EDTA appeared to result in partial loss of its structural features.

Extrinsic Bis-ANS fluorescence was used as a sensitive probe for the formation of new hydrophobic surfaces in insulin exposed to MCO. A moderate increase in Bis-ANS fluorescence of the MCO (excipient free) sample was observed, as previously reported [5], while all other formulations tested showed Bis-ANS fluorescence spectra comparable to that of the negative control (data not shown). This indicates that all the excipients used inhibit MCO-induced formation of new hydrophobic surfaces.
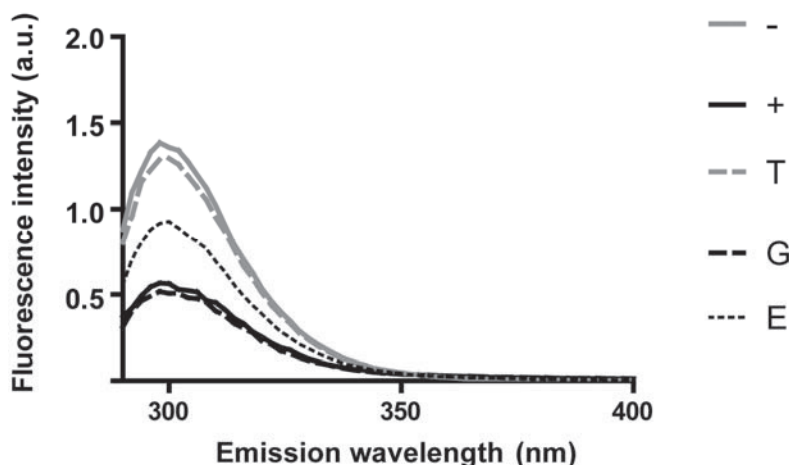
Figure 3B. Intrinsic steady-state fluorescence of insulin samples incubated for 72 h at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), GSH (G), or EDTA (E), as indicated in the graph. Intrinsic steady-state fluorescence data were obtained averaging the spectra of two batches.

Inhibition by TETA of aggregation and fragmentation of a monoclonal antibody during MCO

To confirm the protective properties of TETA using another therapeutic protein, MCO was applied to IgG and the products were analyzed by SEC and extrinsic Bis-ANS fluorescence, which were previously shown to be suitable techniques to pick up oligomers, fragments and structural modification of this IgG exposed to MCO [10]. Figure 4A shows the SEC profiles of untreated IgG and MCO IgG in presence and absence of 100 μM TETA and Figure 4B the monomer, aggregate and fragment contents derived from the SEC analysis.

The results clearly illustrate the protection by TETA against aggregation and fragmentation of MCO IgG.

Moreover, the fluorescence of Bis-ANS, a dye sensitive to hydrophobic regions in proteins, indicated that TETA prevents the generation of aggregates containing new hydrophobic regions during MCO of IgG (Figure 5).
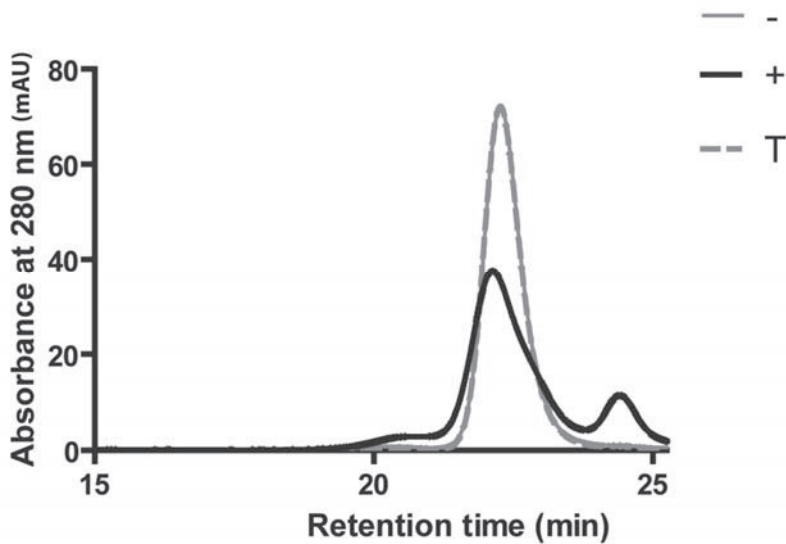
Figure 4A. SEC profiles of IgG samples. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph.
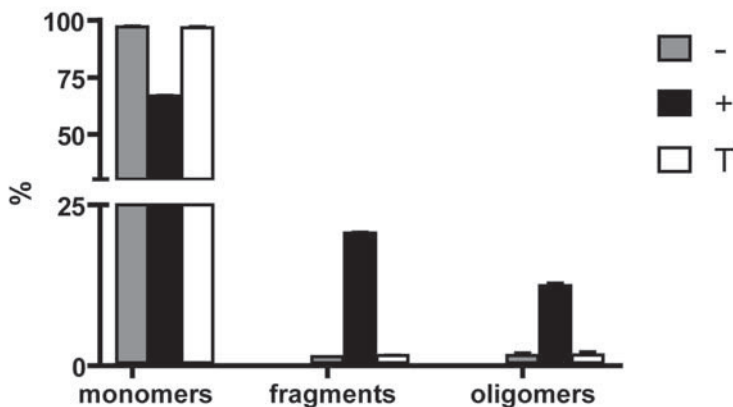

Figure 4B. Percentages of aggregated and fragmentated IgG calculated from SEC profiles after 3 h of incubation time at room temperature. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph. Error bars represent the deviation from the average of two batches.
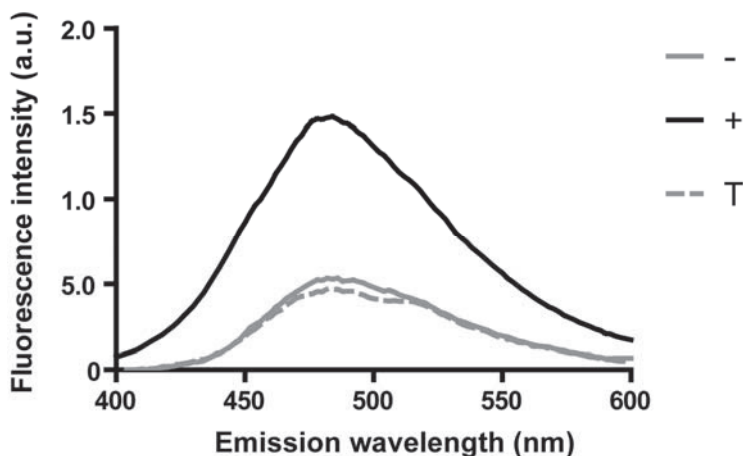
Figure 5. Extrinsic Bis-ANS fluorescence of IgG samples after 3 h incubation at room temperature. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph. Spectra represent the average obtained from two batches.

Discussion

MCO represents a common degradation pathway of protein and peptide [47] therapeutics and justifies the search for formulation strategies for its inhibition. Classic inhibitors of MCO are chelating agents such as EDTA. Depending on the stability constant of the coordination complex that each chelating agent can form with different metals, the choice of the chelator can depend on the metal involved in the catalyzed oxidation. Excess of EDTA, relative to Cu^{2+}, is often used to quench MCO involving Cu^{2+} but several studies involving Fe^{2+} have suggested its pro-oxidant activity [20, 48], thus favoring protein degradation.

In this work it is shown that the chelating agent TETA protects insulin and IgG from aggregation and fragmentation during MCO via Cu^{2+}/ascorbate. TETA is an FDA approved drug [21] and it has been recently widely investigated in the treatment of diabetes mellitus for its selective copper ion-chelating properties [23], therefore it was selected as antioxidant in the present studies. GSH was included in our study for its radical scavenging activity [49] and because it was reported to inhibit copper catalyzed oxidation in presence of a reducing agent such as ascorbic acid [16], besides being an excellent antioxidant in the human body [17]. EDTA is broadly used as chelating agent in pharmaceutical

formulations [20] and has been previously used to quench metal catalyzed oxidation involving copper ions [2, 4, 7, 8].

In this work we showed that TETA, among the excipients employed (100 μM, in a molar excipient:Cu^{2+} ratio of 2.5:1) almost totally prevented insulin aggregation and fragmentation, as monitored by SEC, NTA and LOPC analysis. TETA still prevented insulin aggregation at 50 μM, but to a lesser extent than at 100 μM (data not shown).

Other structural features of insulin were shown to be largely preserved in presence of 100 μM TETA, as demonstrated by far-UV CD and fluorescence studies. Chemical modifications responsible for insulin aggregation (during MCO under our applied conditions), namely DOPA and DOCH [11], appeared to be inhibited by TETA, based on a chemical derivatization assay with benzylamine. These observations, together with TETA's observed protective effect against MCO-induced aggregation of a monoclonal antibody, suggest that it is a promising candidate to be considered for oxidation-sensitive protein formulations. However, follow-up studies are needed to elucidate whether TETA also inhibits oxidation of amino acid residues like methionine and tryptophan (which are not present in insulin), the oxidation of which can have undesirable pharmacological consequences [50].

The beneficial properties of TETA are probably due the high affinity constant for the complex TETA:Cu^{2+} (log K ≈ 15.0 at pH 7.0) and the square-planar geometry in which Cu^{2+} is most stable [19]. GSH instead does not inhibit MCO, probably because at the concentration used, the amount of reduced glutathione (GSH) responsible for the antioxidant activity [16] is not enough to sequester copper ions, as part of GSH reduces Cu^{2+} into Cu^+, yielding GSSG which is unable to prevent MCO [16].

Increasing the concentration of GSH might better chelate copper ions, however due to its reducing properties, GSH is deleterious for the integrity of disulfide bridges, as indicated by the reduction of insulin's disulfide bridge at 5 and 1 mM within 3 hours at room temperature (data not shown).

Interestingly, EDTA can form a stable complex with Cu^{2+}, with a slightly higher stability constant than TETA (log K = 15.9 at pH 7.4) [51], but the geometry of this stable complex can be lost after interactions with buffer salts [52-53], rendering the metal ion available for catalyzing protein oxidation. Higher concentrations of EDTA might however present beneficial effects for

proteins where metal coordination is not crucial for maintaining native structural features.

Conclusion

We described that micromolar concentrations of TETA, in comparison with other potential anti-oxidants, effectively inhibits copper catalyzed protein oxidation, aggregation and fragmentation. Since metal ions are ubiquitous and often contaminate therapeutic protein formulations inducing oxidative chemical modifications, which eventually can produce inactive and immunogenic products, TETA should be taken into serious consideration as a protein formulation excipient. Follow-up studies are warranted to fine-tune the optimal protective concentration of TETA, according the protein under investigation.

Acknowledgements

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Chapter 6

Identification of oxidation sites and covalent cross-links in metal catalyzed oxidized interferon beta-1a: potential implications for protein aggregation and immunogenicity

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Abstract

Oxidation via Cu^{2+}/ascorbate of recombinant human interferon beta-1a (IFNβ-1a) leads to highly immunogenic aggregates, however it is unknown which amino acids are modified and how covalent aggregates are formed. In the present work we mapped oxidized and cross-linked amino acid residues in aggregated IFNβ-1a, formed via Cu^{2+}/ascorbate catalyzed oxidation. Size exclusion chromatography (SEC) was used to confirm extensive aggregation of oxidized IFNβ-1a. Circular dichroism and intrinsic fluorescence spectroscopy indicated substantial loss of secondary and tertiary structure, respectively. Derivatization with 4-(aminomethyl) benzenesulfonic acid was used to demonstrate, by fluorescence in combination with SEC, the presence of tyrosine (Tyr) oxidation products. High performance liquid chromatography coupled to electrospray ionization mass spectrometry of reduced, alkylated and digested protein was employed to localize chemical degradation products. Oxidation products of methionine, histidine, phenylalanine (Phe), tryptophan and Tyr residues were identified throughout the primary sequence. Covalent cross-links via 1,4- or 1,6-type addition between primary amines and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid, an oxidation product of Phe and Tyr) were detected. There was no evidence of disulfide bridge, Schiff base, or dityrosine formation. The chemical cross-links identified in this work are most likely responsible for the formation of covalent aggregates of IFNβ-1a induced by oxidation, which have previously been shown to be highly immunogenic.

Introduction

Recombinant human interferon beta (IFNβ), a cytokine with anti-inflammatory and tumor-suppressor functions [1] is considered as the first choice treatment of multiple sclerosis, a severe neurodegenerative autoimmune disease [2]. Interferon beta-1a (IFNβ-1a), in contrast to interferon beta-1b (IFNβ-1b) [3], is glycosylated at asparagine 80 [4] carrying mainly biantennary and triantennary glycan structures [5-6]. IFNβ-1a has an average molecular weight of about 22.5 kDa, contains 166 amino acid residues and is produced in Chinese hamster ovarian (CHO) cells [6]. As described by Karpusas et al. [7], the protein contains five alpha helices, an intramolecular disulfide bridge between cysteines in positions 31 and 141, and a free cysteine (Cys) in position 17.

Like many other protein therapeutics, IFNβ is prone to aggregation, which has been correlated with enhanced immunogenicity [8]. Furthermore, it has been demonstrated that the removal of protein aggregates reduces the immunogenicity of IFNβ formulations [9-11]. In addition, IFNβ-1b (Betaferon®), which contains more aggregates than IFNβ-1a products (Avonex®, Rebif®), has been found to be the most immunogenic IFNβ product in several preclinical and clinical studies [12-13].

Protein aggregates induced by metal-catalyzed oxidation (MCO) have been found to be particularly immunogenic in preclinical models. For instance, Hermeling et al. showed that recombinant human interferon alpha-2b (IFNα-2b) forms immunogenic aggregates upon oxidative stress induced by Cu^{2+}/ascorbate catalyzed oxidation, using a transgenic mouse model immune tolerant to the human protein [14-15]. Similarly, IFNβ-1a aggregates and monoclonal antibody aggregates, both produced via the same oxidative system, were shown to be highly immunogenic in transgenic immune tolerant mouse models [8, 16]. We therefore believe that characterization of chemical modifications and cross-links caused by Cu^{2+}/ascorbate catalyzed oxidation is of high importance, since these data may shed light on structure-immunogenicity relationships for IFNβ-1a and potentially other therapeutic proteins.

The scope of the present work was to identify the chemical modifications in Cu^{2+}/ascorbate oxidized IFNβ-1a, including covalent cross-links that may be involved in aggregation. Here we report a comprehensive characterization of

oxidative modifications in IFNβ-1a following MCO and discuss putative mechanisms of MCO-induced IFNβ-1a aggregation. Our data strongly support the formation of electrophilic oxidation products, which can be further involved in aggregate formation via 1,4- or 1,6-type addition to pre-existing protein nucleophiles, such as primary amine groups, in agreement with our recent mechanistic studies on insulin as a model protein [17].

Materials and Methods

Materials

IFNβ-1a (0.26 mg/mL protein in 20 mM sodium acetate buffer, pH 4.8, containing 154 mM arginine) was kindly provided by Biogen Idec Inc. (Cambridge, MA, USA). PNGase F, L-ascorbic acid, ethylenediamine tetraacetic acid (EDTA), copper dichloride, arginine, monobasic and dibasic sodium hydrogen phosphate, ammonium bicarbonate (ABI), sodium chloride, sodium azide, SDS, dithiothreitol (DTT), iodoacetamide, glacial acetic acid, and acetonitrile were purchased from Sigma–Aldrich (St.Louis, MO, USA). Milli-Q water was used for the preparation of all the formulations. All chemicals were of analytical grade and used without further purification. 4-(aminomethyl) benzenesulfonic acid (ABS) was synthesized according to a published procedure [18]. Endoproteinases Glu-C and trypsin were purchased from Promega (Madison, WI, USA).

Preparation of untreated and oxidized IFNβ1-a formulations

IFNβ-1a was prepared and treated as reported by van Beers et al [8]. Briefly, the protein solution was dialyzed against 100 mM sodium phosphate buffer and 200 mM sodium chloride, pH 7.2 (PBS). This dialyzed solution is referred to as untreated IFNβ-1a. To obtain oxidized IFNβ-1a, untreated IFNβ-1a, diluted to 200 μg/mL with PBS, was incubated with 4 mM ascorbate and 40 μM $CuCl_2$ for 3 hours at room temperature. The oxidation reaction was stopped by adding 100 mM EDTA to a final concentration of 1 mM as previously reported [17].

ABS derivatization

Untreated and oxidized protein (0.2 mg/mL) were dialyzed against 50 mM ABI, pH 8.0, using a 3.5-kDa MWCO Slide-A-Lyzer Cassette (Asheville, NC, USA), before derivatization with ABS. To 250 µL of dialyzed samples 7.5 µL of 0.1 M sodium hydroxide were added to adjust the pH to 9.5. Then 100 mM ABS stock solution was added to a final concentration of 10 mM. Subsequently, 5 mM $K_3Fe(CN)_6$ stock solution in Milli-Q water was added to a final concentration of 0.5 mM. The reaction was conducted for 1 hour at room temperature, before performing SEC analysis or fluorescence measurement (as reported below in the section Size exclusion chromatography and Fluorescence spectroscopy, respectively). Controls for non-specific fluorescence included (i) oxidized and non-oxidized protein prior to derivatization and (ii) reagents alone (i.e. ABS/$K_3Fe(CN)_6$) incubated under the same conditions.

Size exclusion chromatography

To determine the aggregate content and to investigate the effect of reducing agents on oxidized and untreated IFNβ-1a, SEC was performed by using a TSKgel Super SW2000 column protected by a Super SW guard column (Sigma Aldrich, St. Louis, MO, USA). An SPD-6AV UV and fluorescence detector (Shimadzu, Columbia, MD, USA) was used to record the chromatograms at a wavelength of 280 nm and at excitation/emission wavelength combination of 295/350nm, respectively. A flow rate of 0.35 mL/min was applied using a 515 HPLC pump and 717 Plus autosampler (Waters, Milford, MA, USA). The mobile phase consisted of 200 mM sodium chloride, 0.05% (w/v) sodium azide and 0.1% (w/v) SDS in 100 mM sodium phosphate, pH 7.2, which was filtered through a 0.2-µm filter prior to use.

To detect fluorescent benzoxazole products in the ABS-derivatized samples, we used an Insulin HMWP Column, 7.8 × 300 mm (Waters) connected to a HPLC system (Shimadzu, Columbia, MD, USA) coupled with a Shimadzu RF-20A fluorescence detector. Excitation and emission wavelengths were set at 360 and 490 nm, respectively. The mobile phase composition and flow rate were as previously reported [19].

Fluorescence spectroscopy

The emission spectra of the ABS-derivatized samples, diluted two fold in ABI, were measured in a 0.5-mL quartz fluorescence cuvette with an RF-5000U fluorescence spectrophotometer (Shimadzu) with excitation and emission wavelengths range set at 360 and 400-600 nm, respectively, and bandwidths set at 5 nm.

To test the potential presence of dityrosine, fluorescence of the oxidized IFNβ-1a was measured in triplicate, using excitation and emission wavelengths set at 315 and 420 nm, respectively, as described previously [20]. Tryptophan (Trp) intrinsic fluorescence was measured with a Tecan infinite M1000 fluorometer (Tecan group Ltd., Männedorf, Switzerland) upon excitation at 295 nm and emission recorded from 310-500 nm with a step size of 2 nm, 50 flashes (frequency 400 Hz), a gain of 150 and a Z-position of 20.0 mm. Duplicates of 200 μL of each sample were analyzed in black polystyrene 96-well plates (Greiner Bio-One, Frickenhausen, Germany).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of untreated and oxidized IFNβ-1a (0.1 mg/mL) were recorded from 190 to 250 nm, using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-mm (far-UV CD) path length quartz cuvette at 25 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of six repeated scans and background was corrected with the corresponding buffer spectrum. The CD signals were converted to mean residue ellipticity $[\theta]_{mrw, \lambda}$, using a mean residue weight (MRW, calculated as MRW=M/(N-1), where M is the average molecular weight of the untreated (glycan-free) protein (i.e. 20027 Da, based on the protein's chemical formula $C_{908}H_{1408}N_{246}O_{252}S_7$) and N is the number of amino acid residues in the chain).

Reduction, alkylation and digestion

Untreated and oxidized protein (0.2 mg/mL) were dialyzed against 50 mM ABI, pH 8.0, using a 3.5-kDa MWCO Slide-A-Lyzer Cassette (Asheville, NC, USA), and reduced by addition of 50 mM DTT, freshly prepared in 50 mM ABI,

pH 8.0, to a final concentration of 5 mM. The samples were incubated for 45 minutes at 45 °C using a Thermo NES heating bath (Thermo Scientific, NC, USA). Subsequently, 200 mM iodoacetamide, freshly prepared in 50 mM ABI, pH 8.0, was added to a final concentration of 20 mM. The digestion was performed as follows: IFNβ-1a was incubated with Glu-C endoproteinase at a IFNβ-1a/Glu-C ratio of 10:1 (w/w), for 1 hour at 37 °C. Next, trypsin was added (IFNβ-1a/trypsin ratio 20:1 (w/w)) and the mixture was incubated for an additional hour at the same temperature. Finally, 4 μL of a solution of PNGase F, dissolved according to the manufacturer's protocol, was added to 76 μL of the protein mixture, which was then incubated overnight at 37 °C.

MS/MS analysis

Digested and non-digested samples were analyzed by means of an LTQ-FT hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo-Finnigan, Bremen, Germany) [21]. The MS/MS spectra were analyzed with the web-based software MassMatrix [22-25], which was used to simulate mass spectra and the theoretical fragment tables of the b- and y-ions [26] for the native, oxidized and cross-linked peptide products. The simulated spectra were compared to the experimental MS/MS spectra in order to validate the proposed product and cross-link structures. A mass accuracy sensitive probability-based scoring algorithm for database searching of tandem mass spectrometry data by MassMatrix software was employed for peptide identification; the manual filter was set at mass/charge ratio (m/z) accuracy <0.1 amu for both parent peptide and fragment ions.

Results

Comparison of untreated and oxidized IFNβ-1a by SEC and spectroscopic methods

SEC analysis was used to verify whether the aggregation profile of oxidized IFNβ-1a was similar to that observed earlier [8] and to study a potential involvement of disulfide bridging in aggregate formation. Comparison of the chromatograms of untreated and oxidized IFNβ-1a (Figure 1A) shows extensive aggregation of the oxidized product and both chromatograms are in close agreement with the results published by van Beers et al [8].

Furthermore, the treatment of oxidized IFNβ-1a with DDT did not have a major impact on the elution behavior, indicating that the majority of the aggregates were formed by non-reducible covalent bonds. In contrast, we observed that for untreated IFNβ-1a, the reduction with DDT resulted in a decrease in dimer content by approximately 60% (Figure 1A), indicating that most of the dimers in the untreated IFNβ-1a were formed through disulfide bridging.
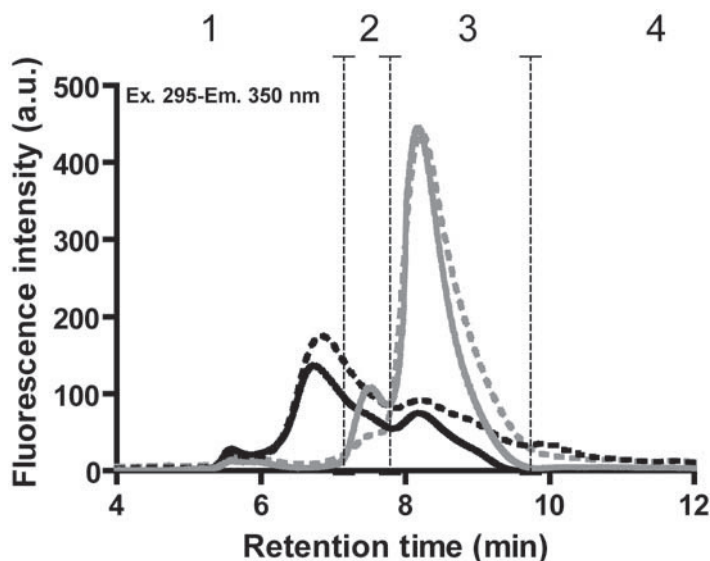

Figure 1A. SEC (TSKgel Super SW2000 column) with intrinsic tryptophan fluorescence detection of untreated IFNβ-1a before (solid grey chromatogram) and after reduction and alkylation (dashed grey) and oxidized IFNβ-1a before (solid black) and after reduction and alkylation (dashed black). The vertical dashed lines show the range of each peak: (1) mainly representing oligomers, (2) dimers, (3) monomers, (4) fragments.

SEC with fluorescence detection of ABS-tagged proteins was used to study whether the monomers or aggregates contain DOPA (3,4-dihydroxyphenylalanine) and/or DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid), produced by oxidation of tyrosine (Tyr) and/or phenylalanine (Phe) residues and subject to fluorogenic derivatization with ABS [17] and/or 5-hydroxytryptophan, a Trp oxidation product which can also form fluorescent benzoxazole products upon ABS derivatization [27-28]. Our data show that after derivatization of the oxidized and dialyzed protein with

ABS, the total SEC fluorescence peak area (i.e. the sum of all the peak areas under the curve) for the ABS-derivatized oxidized IFNβ-1a is about six fold higher than that of the ABS-derivatized untreated protein (Figure 1B), indicating that MCO of IFNβ-1a leads to formation of DOPA/DOCH and/or 5-hydroxytryptophan residues in the oxidized protein.

ABS derivatization of non-oxidized IFNβ-1a also produces a weak fluorescent signal mainly associated with protein monomer (Figure 1B), suggesting a background oxidation in the untreated protein.
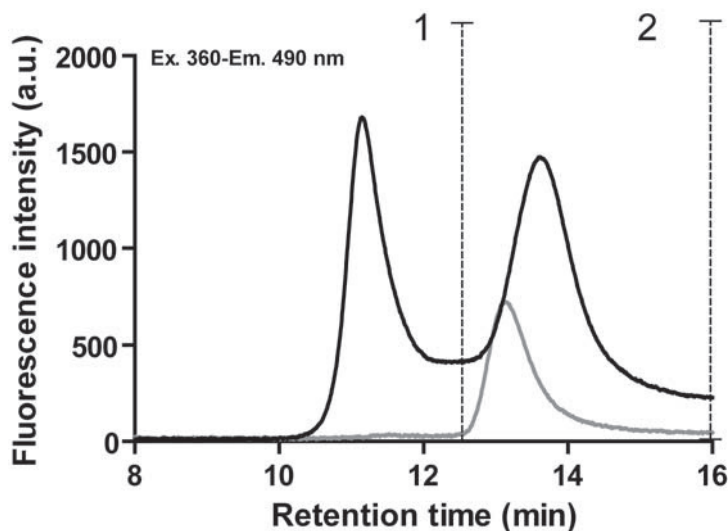
Figure 1B. SEC (Insulin HMWP Column) with ABS fluorescence detection of ABS-derivatized untreated IFNβ-1a (grey) and ABS-derivatized oxidized IFNβ-1a (black). The vertical dashed lines show the range of each peak: (1) mainly representing oligomers, (2) dimers and monomers (with this column it is not possible to discriminate dimer from monomer).

The ABS fluorescence of ABS derivatized oxidized and untreated IFNβ-1a was also measured by steady-state fluorescence. The results show a seven fold increase in fluorescence intensity for the oxidized protein (Figure 2), which is consistent with the SEC data.
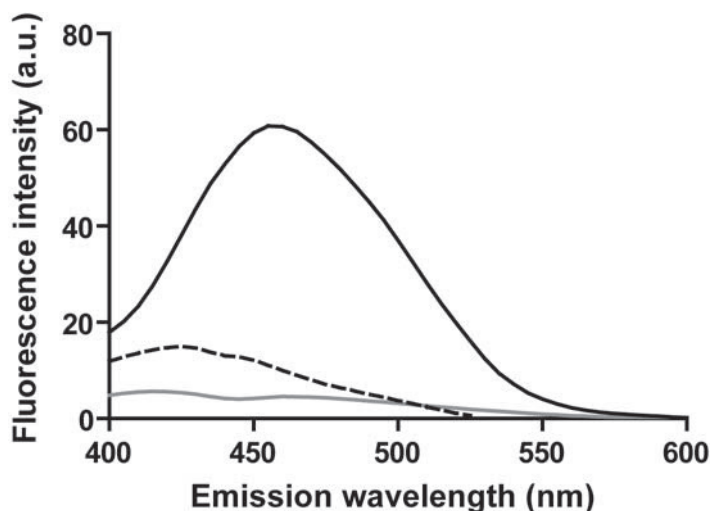
Figure 2. Extrinsic fluorescence emission spectra of ABS-derivatized untreated IFNβ-1a (grey), ABS-derivatized oxidized IFNβ-1a (black), and oxidized IFNβ-1a (dashed black). Spectra represent the average of two batches.

Further structural characterization was performed by CD (Figure 3) and intrinsic steady-state fluorescence measurements (Figure 4). Likely as a result of oxidation and cross-link formation, it was found that the content of alpha helix in oxidized IFNβ-1a had decreased, as indicated by the drop in negative CD signal, with a concomitant increase in random coil structure, as indicated by the higher 208/222 nm ratio measured for the oxidized protein (1.041 ± 0.024) as compared with its native counterpart (0.729 ± 0.004) [29-30].
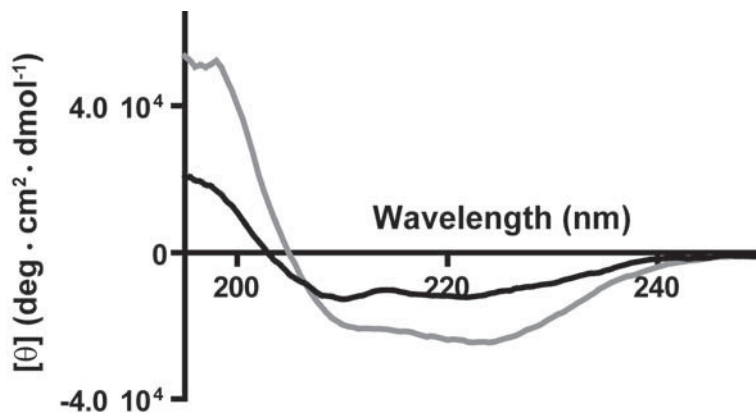
Figure 3. Far-UV CD spectra of untreated IFNβ-1a (grey) and oxidized IFNβ-1a (black). Spectra represent the average of two batches.

Moreover, a substantial decrease of Trp fluorescence was observed, pointing to chemical modification of Trp residues and/or loss of tertiary structure in general. No shift in the emission maximum was however observed.
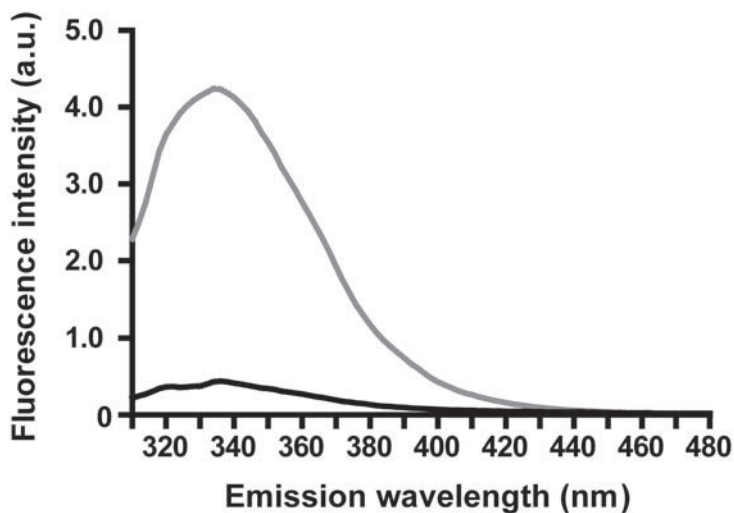

Figure 4. Intrinsic steady-state fluorescence of untreated IFNβ-1a (grey) and oxidized IFNβ-1a (black). Spectra represent the average of two batches.

Mass spectrometry analysis

Undigested IFNβ-1a

The data of liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) analysis of undigested untreated IFNβ-1a (Figure 5A) are in agreement with previously reported results [31]: the spectrum of the protein represents a mixture of multiple glycoforms (mainly fucosylated and sialylated protein) where the most abundant isoform contains one mono-fucosylated, biantennary structure carrying two sialic acid residues, featuring a total molecular weight of 22376 Da. The spectrum of the oxidized protein represents a single broad peak with a maximum at 22460.5 Da resulting from an overlay of multiple heterogeneously oxidized protein isoforms (Figure 5B), similar to results published for another oxidized protein [32].
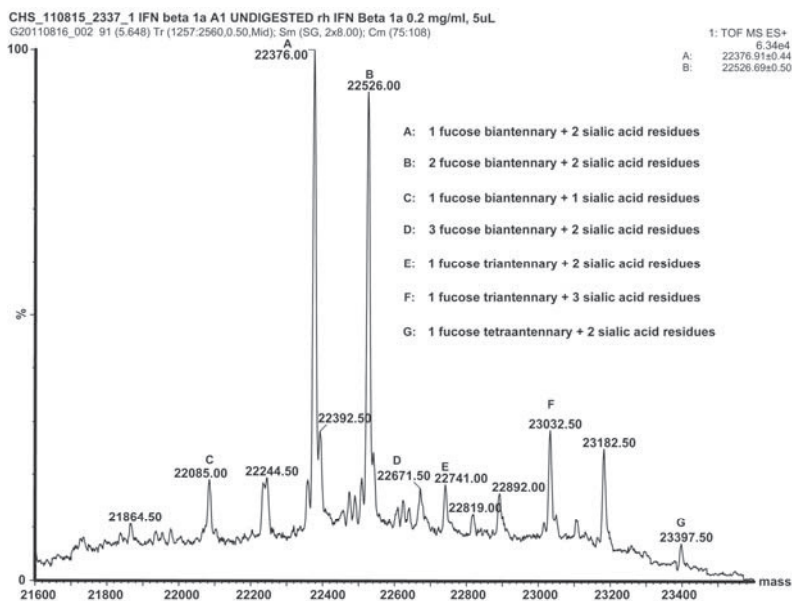

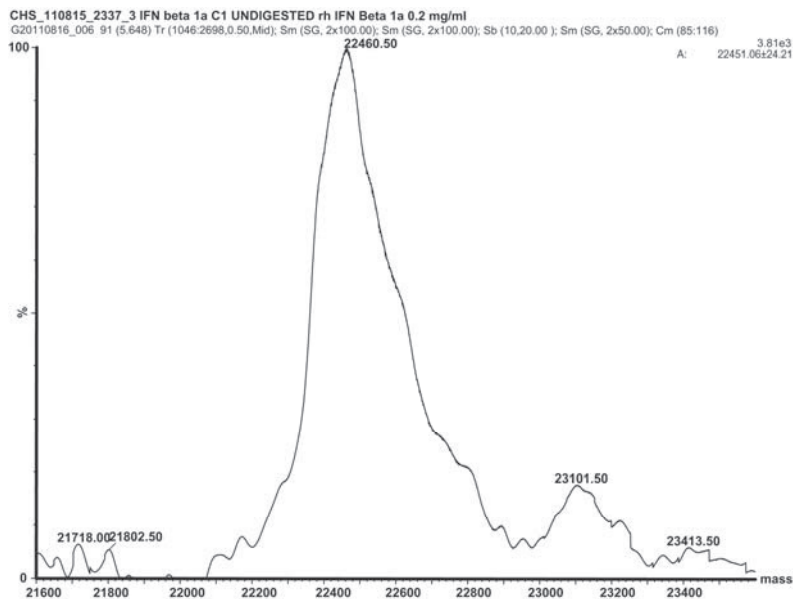**Figure 5A.** Deconvoluted mass spectrum of undigested untreated IFNβ-1a

Figure 5B. Deconvoluted mass spectrum of undigested oxidized IFNβ-1a

Sequence-specific analysis of IFNβ-1a oxidative modifications

This section focuses on the identification of amino acid sequence-specific oxidative protein modifications through MS/MS analysis of IFNβ-1a digests and the results will be presented in the following order: Methionine (Met), Phe, Histidine (His), Tyr and Trp oxidation. It is noteworthy that some peptide sequences potentially contain more than one modification, and that some MS/MS spectra may represent a mixture of peptide isoforms with the same m/z but different locations of oxidative modifications within the sequence. Table I lists all the chemically modified peptides detected. Although a few modifications were observed also in peptides derived from the untreated protein (Table I, last column), most modifications were observed in peptides from oxidized IFNβ-1a and involved Met, Phe, His, Tyr and Trp residues.

Table II shows all the potential targets for MCO, including the ones found oxidized in the present study.

Table I. Chemical modifications measured by LC-ESI-MS/MS in peptides from oxidized and untreated IFNβ1a obtained after reduction, alkylation and enzymic digestion.

Chemical modifications and reference to corresponding mass spectrum	Peptide	Experimental m/z	Charge	Theoretical mass	Δm	Oxidized IFNβ1a*	Un-treated IFNβ1a*
$M_1(O)$ Figure S1	$M_1SYNLLGFLQR_{11}$	679.35	2	1356.70	0.00	+	+
$M_1(O), Y_3(O)$ Figure S2	$M_1SYNLLGFLQR_{11}$	687.35	2	1372.69	+0.01	+	-
$Y_3(DOCH)$ Figure S3	$M_1SYNLLGFLQR_{11}$	678.34	2	1354.68	0.00	+	-
$M_1(loss\ CH_3S)$ Figure S4	$M_1SY\ NLLGFLQR_{11}$	647.35	2	1292.68	+0.02	+	+
$F_{38}(O)$ Figure S5 (a-c)	$M_{36}NFDIPEEIK_{45}$	626.30	2	1250.59	+0.01	+	-

$M_{36}(O)$ Figure S6(a-c)	M_{36}NFDIPEEIK$_{45}$	626.30	2	1250.59	+0.01	+	+
$M_{62}(O)$ Figure S7	M_{62}LQNIFAIFR$_{71}$	634.84	2	1267.68	0.00	+	-
M_{62}(loss CH$_3$S) Figure S8	M_{62}LQNIFAIFR$_{71}$	402.23	3	1203.68	+0.01	+	-
$M_{117}(O)$, $H_{121}(O)$ Figure S9	L_{116}MSSLHLK$_{123}$	480.76	2	959.52	0.00	+	-
$F_{50}(O)$, Q(deamidation in several positions) Figure S10	Q_{46}LQQFQK$_{52}$	468.76	2	935.48	+0.04	+	-
$H_{93}(O)$ Figure S11	N_{86}LLANVYHQINHLK$_{99}$	846.96	2	1691.92	0.00	+	-
$H_{97}(O)$ Figure S12	N_{86}LLANVYHQINHLK$_{99}$	846.96	2	1691.92	0.00	+	-
$H_{93}(O)$, $H_{97}(O)$ Figure S13	N_{86}LLANVYHQINHLK$_{99}$	854.96	2	1707.91	+0.01	+	-

Modification	Peptide						
H_{93}(asn) Figure S14(a-b)	N_{86}LLANVYHQINHLK$_{99}$	827.46	2	1652.91	+0.01	+	-
H_{97}(asn) Figure S15(a-b)	N_{86}LLANVYHQINHLK$_{99}$	827.46	2	1652.91	+0.01	+	-
H_{131}(O), Y_{132}(O) Figure S16(a-c)	I_{129}LHYLK$_{134}$	401.74	2	801.48	0.00	+	-
Y_{138}(DOCH), H_{140}(O) Figure S17	E_{137}YSHCAWTIVR$_{147}$	697.81	2	1393.62	0.00	+	-
H_{140}(O), C_{141}(IAM)# Figure S18	Y_{138}SHCAWTIVR$_{147}$	654.81	2	1307.62	0.00	+	-
W_{22}(O), N_{25}(deamidation) Figure S19	L_{20}LWQLNGR$_{27}$	508.78	2	1015.55	+0.01	+	+
W_{22}(N-fkyn), N_{25}(deamidation) Figure S20	L_{20}LWQLNGR$_{27}$	516.78	2	1031.55	+0.01	+	+

* + detected; - not detected

#(IAM): alkylation with iodoacetamide

Table II. Oxidation prone amino acid residues and oxidized amino acids detected in peptides derived from oxidized IFNβ-1a.[1]

Methionine	Phenylalanine	Tyrosine	Tryptophan	Histidine
1	*8*	**3**	**22**	**93***
36	*15*	*30*	*79*[#]	**97***
62	**<u>38</u>**	*60*	*143*[#]	**<u>121</u>***
117	**50**	*92*		**131**
	67	<u>*125*</u>		**140**
	70[#]	<u>*126*</u>		
	111	**<u>132</u>**		
	154[#]	**138**		
	156	*155*		
		163		

[1] Bold numbers: oxidized residues; bold underlined numbers: oxidized residues involved in H-bond critical for the correct folding of IFNβ-1a; underlined numbers: residues involved in H-bond critical for the correct folding of IFNβ-1a (Karpusas et al. [7]); plain italic numbers: non-oxidized residues.

[#] Hydrophobic residues that stabilize the core of the molecule.

* Residues that coordinate a zinc ion, responsible for dimer formation.

A supplementary Table S1 (available online on Molecular Pharmaceutics) shows all the potential amino acid modifications included in the settings of a database search for oxidized, reduced, alkylated and digested IFNβ-1a samples. All annotated MS/MS spectra for oxidative protein modifications along with suggested peptide structures presented as inserts are shown in the supplementary Figures S1-S20 (available on line on Molecular Pharmaceutics). Note that in the supplementary figures, the peptide structures of the non-modified amino acid residues are displayed in a one-letter code, while the respective full structures are drawn for the suggested oxidatively modified and alkylated/deamidated residues.

Methionine oxidation

Met1 has been found susceptible to oxidation already by other authors [7, 31]. The b_2^+ ion in Figure S1, where the peptide M_1SYNLLGFLQR$_{11}$ is displayed, shows the incorporation of one oxygen atom (+16 Da) into the sequence

Met1Ser2: here, Ser is less prone to oxidation than Met, hence the target of oxidation is likely Met1. Figure S2 shows the same peptide with two oxygen atoms incorporated (+32 Da), which may represent a mixture of two sequences where either Met1 is oxidized to its sulfone, or DOPA (formed from Tyr3) and Met sulfoxide are present together. This is illustrated in the inserts to Figure S2, showing two potential b2+ ions, one with a mass increase of +16 Da (mono oxidized, indicated as Met ox) and another with the mass increase of +32 Da (di-oxidized, indicated as Met (ox)$_2$). The second insert shows two fragments with m/z 1123.57 and 1139.25, which may represent the y9+ ions for the unmodified and oxidized form of Tyr3; the ratio of these peaks suggests that the peptide containing oxidized Met1 and DOPA in position 3 is far less abundant than the unmodified peptide (a fraction of less than 10%).

However, this value does not necessarily reflect a lower rate of DOPA formation compared to Met sulfone; rather, it may indicate a higher reactivity of DOPA, resulting in DOCH and further cross-linked products. A spectrum of the doubly charged peptide ion with m/z 678.34 in Figure S3 shows such an example where Tyr is oxidized to DOCH based on a mass increase of 14 Da, which corresponds to the incorporation of one oxygen atom and loss of two hydrogen atoms, albeit at very low abundance of both y9+ and b3+-H_2O sequence-indicating ions.

We were also able to detect a characteristic loss of methane sulfenic acid (CH_3SOH, -64 Da, compared with the mono oxidized sequence) from the N-terminus oxidized Met (Figure S4), which was suggested earlier as diagnostic for identifying peptides containing oxidized Met [26, 33].

Met36 is located in a solvent exposed domain of IFNβ-1a [7, 31]. Figure S5a and S5b represent the MS/MS spectra matching the sequence M$_{36}$NFDIPEEIK$_{45}$, in which either Met36 or Phe38 is oxidized. These two products are not resolved by our HPLC method and the respective spectra overlay, as evidenced by the presence of two potential fragment ions for y8+ at m/z 990.48 and 1006.44 (Figure S5b) for oxidized Met36 and oxidized Phe38, respectively. As additional evidence for oxidized Phe38, the ion at m/z 228.18 can be considered, which most likely represents the b2+-H_2O ion (indicated as F ox) from the parent peptide with oxidized Phe38 (Figure S5c). Though the fraction of the peptide with oxidized Phe38 is rather small (ca. 3-5% based on the relative abundance of fragment ion y8+ in Figure S5b, indicated as F ox), it

is noteworthy that in the control spectra (non-oxidized IFNβ-1a) both fragments at 1006.44 and at 228.18 were not observed (Figure S6a-S6c). Altogether, these data suggest that untreated IFNβ-1a already contains some oxidized Met36 residues, whereas oxidized Phe38 is only present in oxidized IFNβ-1a.

Met62 is a buried residue [7, 31]. It was resistant to oxidation by hydrogen peroxide [31], however, in conditions of MCO, we detected MS/MS spectra for the sequence $M_{62}LQNIFAIFR_{71}$ containing the oxidized Met62 (Figure S7). Again, the spectrum for the loss of methane sulfenic acid in position 62, indicative of Met62 oxidation, was observed (Figure S8).

Met117 is a solvent exposed residue [7, 31] and is most susceptible to oxidation (under mild oxidative conditions via hydrogen peroxide), as compared to Met1, Met36 and Met62 [31]. Figure S9 represents a spectrum for the sequence $L_{116}MSSLHLK_{123}$, where besides Met117, His121 is oxidized to 2-oxo-His. The distinct $b2^+$ and $y3^+$ ions indicate oxidation of Met117 and His121, respectively.

Phenylalanine oxidation

The fragment containing oxidized Phe38 ($M_{36}NF_{38}DIPEEIK_{45}$) has been described above in the section Methionine oxidation.

Phe50 is adjacent to several Gln residues, which may undergo deamidation, during digestion or MCO. Figure S10 presents a typical MS/MS spectrum indicating the oxidation of Phe50, in addition to deamidation of one of the Gln residues. Formation of pyroglutamic acid, which should be attributed only to the cyclization of N-terminal Gln in the peptide (-18 Da) during the LC-MS analysis, was also detected.

Histidine and tyrosine oxidation

His oxidation to 2-oxo-His is well documented and has been measured in proteins such as insulin [34-36]. Stadtman et al. showed that His oxidation also can yield asparagine as a potential product [37].

The sequence $N_{86}LLANVYHQINHLK_{99}$ obtained after digestion with a combination of trypsin and endoproteinase Glu-C, contains two His residues in positions 93 and 97 and one Tyr residue in position 92. Several observed

combinations of His93 or His97 mono oxidation (+16 Da on His), oxidation of both His residues (+32 Da), and formation of Asn in positions 93 and 97 are shown in Figures S11, S12, S13, S14(a-b), S15(a-b), where the respective modifications are proven by $b7^+$, $b8^+$, and $b12^+$ ions, as well as $y3^+$, $y7^+$, $y8^+$, and $y12^{++}$ ions. In Figure S15 are shown only the ions which differ from Figure S14.

His121 and Tyr3 oxidation was already demonstrated for the fragments L_{116}MSSLHLK$_{123}$ and M_{1}SYNLLGFLQR$_{11}$, respectively, as discussed above.

Sequence I_{129}LHYLK$_{134}$ contains two potential sites for oxidation: His131 and Tyr132. The ions highlighted in Figure S16a fit to oxidation of either His131 or Tyr132, whereas in Figure S16b, based on multiple fragments for the $y4^+$ ion, oxidation of both amino acid residues is suggested. The ions $y4^+$-H_2O and $b4^+$ in Figure S16b, at m/z 574.26 and m/z 559.29, respectively, support the simultaneous oxidation of His and Tyr. Also, the region of the spectrum between 200 and 400 m/z contains two peaks identified as $b3^+$ in which His (Figure S16b, H ox, m/z 380.28) or Tyr (Figure S16c, Y ox, m/z 364.11) are oxidized. Furthermore, in Figure S16c is depicted the ion $y3^{++}$ supporting Tyr oxidation. Altogether, it seems that three different peptides containing either oxidized His131, oxidized Tyr132, or both amino acids oxidized, were detected simultaneously.

In the sequence E_{137}YSHCAWTIVR$_{147}$, containing a missed cleavage site and 2 additional oxygen atoms, there are 3 potential oxidation sites: Tyr138, His140, and Trp143. In the spectrum shown in Figure S17 the $y5^+$-H_2O ion at m/z 656.96 rules out Trp oxidation, $y8^{++}$ at m/z 501.36, in the insert, supports His oxidation, and $b3^+$ and $b4^{++}$ are in accordance with Tyr oxidation to DOCH. Further evidence for His140 oxidation is shown in Figure S18, where Cys is derivatized with iodoacetamide, and N-terminal Glu (E) is cleaved-off by Glu-C.
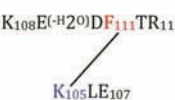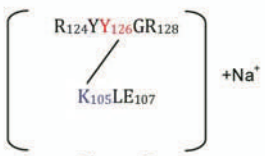
Tryptophan oxidation

Trp22 oxidation was detected in both the oxidized and the control samples, suggesting a spontaneous oxidation of this residue during protein storage or handling. Spectra presented in Figures S19 and S20 show the formation of the major oxidative products, hydroxyl Trp (probably hydroxylated at position 5), and N-formyl kynurenine [38-39], respectively, as evidenced through the

presence of the sequence-indicative ions y3[+]-y7[+] and some b[+] ions, particularly b3[+]. In addition to Trp oxidation, Asn25 deamidation was detected in peptide L$_{20}$LWQLNGR$_{27}$ from both oxidized and control IFNβ-1a, which could be expected given the highly deamidation prone N$_{25}$G sequence [40].

Cross-links

This section focuses on MS/MS identification of covalent cross-links formed through 1,4- and/or 1,6-type addition to the electrophilic product of Tyr oxidation, i.e. DOCH, resulting from MCO of IFNβ-1a. As we have only MS/MS but no NMR data of our reaction products, we have to assume that both reactions are possible. Table III lists all the cross-linked peptides detected by MS/MS and Figures 6a, 7a, 8a and 9a represent the corresponding MS/MS spectra along with the peptide cross-links. The structures displayed in Figures 6b, 7b, 8b and 9b representatively show only the products of 1,4-addition. Data will be presented starting from the first chemically modified electrophilic amino acid measured, i.e. DOCH in the first observed position (Tyr3) of IFNβ-1a. Data reported were obtained from IFNβ-1a that was oxidized, reduced, alkylated and digested.

Table III. Cross-links measured by LC-ESI-MS/MS in peptides derived from oxidized IFNβ-1a after reduction, alkylation and enzymatic digestion (in blue nucleophilic amino acids, in red amino acids oxidized to DOCH).

Cross-link*	Experimental m/z	Charge	Theoretical mass	Δ m
M_1SYNLLGFLQR$_{11}$ | Y_{30}C$^{(IAM)}$LK$^{(IAM)}$DR$_{35}$ Figure 6	756.02	3	2265.11	-0.05
M_1SYNLLGFLQR$_{11}$ D$_{54}$AALTIY_{60}E$_{61}$ Figure 7	756.03	3	2265.11	-0.02
K$_{108}$E$^{(-H2O)}$DF_{111}TR$_{113}$ K_{105}LE$_{107}$ Figure 8	598.3	2	1194.60	+0.00
[R$_{124}$YY_{126}GR$_{128}$ K_{105}LE$_{107}$] +Na$^+$ Figure 9	569.79	2	1137.56	+0.02

* (IAM): alkylation with iodoacetamide; (-H_2O): loss of 1 water molecule.

Met1 – Tyr30

The free N-terminus of Met1 is a good nucleophile for 1,4- or 1,6-type addition under our applied conditions (pH 7.2), where the N-terminal amino group is in part deprotonated. Figure 6a shows the MS/MS spectrum corresponding with the cross-link between Met1 and Tyr30 in the respective tryptic peptides, as illustrated by the scheme in Figure 6b. The series of y-ions in the peptide A (where the prefix A indicates the ions that belong to the peptide M_1SYNLLGFLQR$_{11}$) of the cross-link (Ay2$^+$ to Ay9$^+$) together with Ab1$^+$ in the lower insert (zoomed region m/z 1046 -1060) demonstrate that the

peptide A is linked to peptide B through the Met1 amino group, whereas Bb1+-H$_2$O and Bb1+-NH$_3$ ions (zoomed m/z region 1498-1514) indicate the involvement of Tyr30 of the peptide B in the cross-link formation. Note that in the sequence Y$_{30}$CLKDR$_{35}$ both Cys and Lysine (Lys) residues are in alkylated form (displayed by the mass of the parent peptide and fragment ion Bb5++). Furthermore, we noticed that both Met1 and Tyr3 in the cross-linked peptide are not oxidized, suggesting that the cross-link may protect these residues from oxidation.
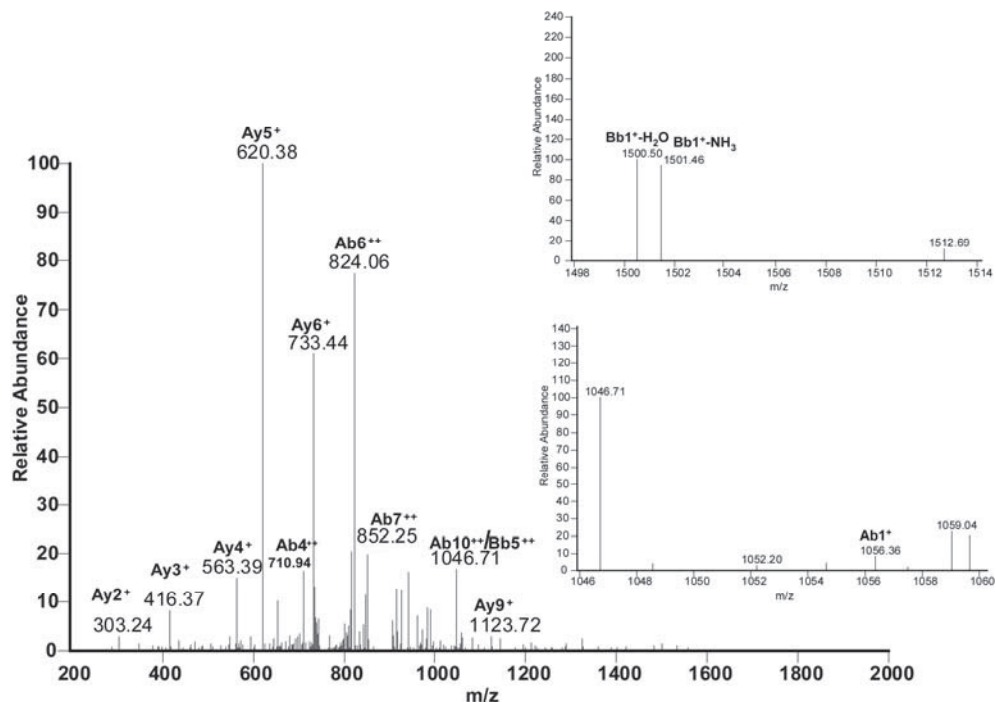
Figure 6a. MS/MS spectrum for the sequence M$_1$SYNLLGFLQR$_{11}$ cross-linked through Met1 to the sequence Y$_{30}$C(IAM)LK(IAM)DR$_{35}$.
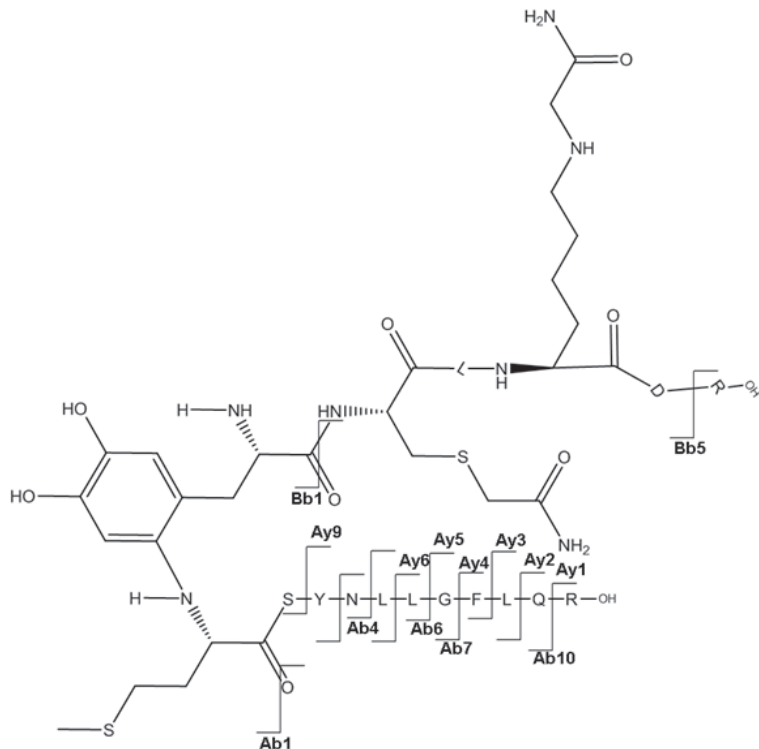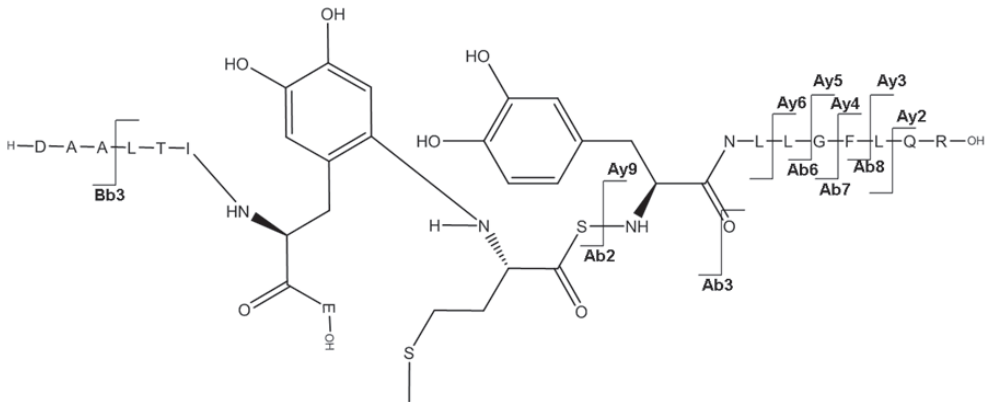
Figure 6b. Suggested chemical structure of the cross-linked sequence (theoretical *mass* 2265.11, experimental *m/z* 756.02, [M+H]$^{3+}$, Δm from parent peptide -0.05).

Met1 – Tyr60

Figure 7a, reports the MS/MS spectrum for the cross-link involving Met1 and Tyr60. The structure provided in Figure 7b, where Met1 is covalently bound to Tyr60, was drawn for the position 6 of the aromatic ring of DOPA (or DOCH), which is sterically less hindered than the positions 1 and 2. In the peptide $M_1SYNLLGFLQR_{11}$, an additional mass shift of +16 should be attributed to Tyr3 oxidation to DOPA, as suggested by the presence of ion Ab3^{++}, ruling out the oxidation of another sensitive residue, Phe8. The ion Ab2$^+$ supports the structure shown in Figure 7b. In addition, fragments matching ions Ay9$^+$ and Bb3$^+$ disprove the involvement of sequences $Y_3NLLGFLQR_{11}$ and $D_{54}AA_{56}$ in the cross-link formation.
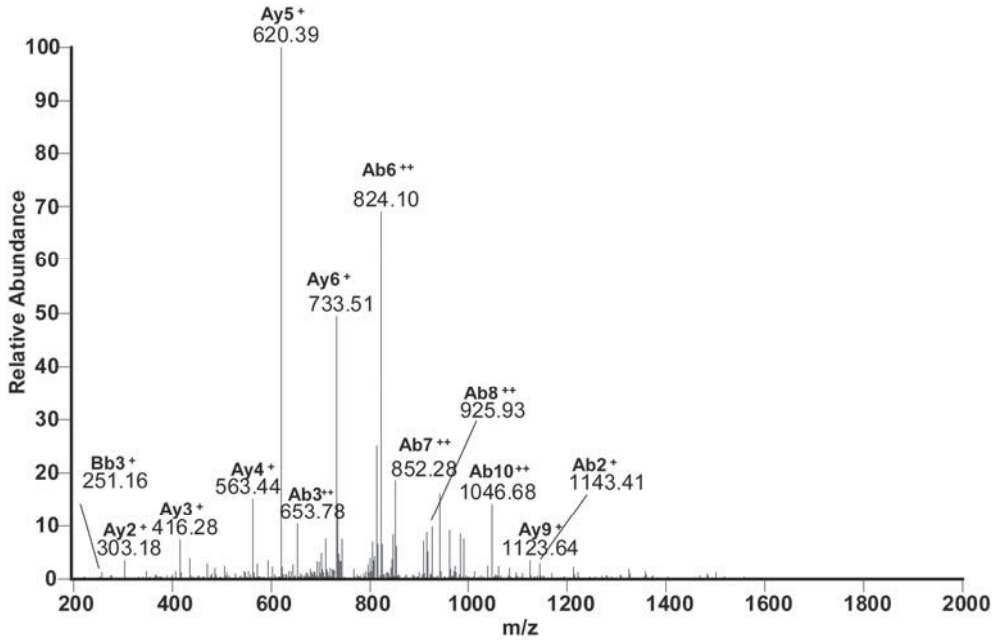
Figure 7a. MS/MS spectrum of sequence $M_1SYNLLGFLQR_{11}$ cross-linked through Met1 to the sequence $D_{54}AALTIY_{60}E_{61}$.

Figure 7b. Suggested chemical structure of the cross-linked sequence (theoretical *mass* 2265.11, experimental *m/z* 756.03, $[M+H]^{3+}$, Δm from parent peptide -0.02).

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Lys105 – Phe111

Figure 8a displays the MS/MS spectrum consistent with a cross-link between Lys105 and Phe111. It must be noticed that the mass for this cross-link is 18 Da lower than the mass that would be expected. This is due to loss of water on Glu109 in the sequence $K_{108}EDFTR_{113}$, which can arise from the cyclization with its N-terminal amino group or with the more nucleophilic amino group of Lys108, which would lead to the formation of a 1,4 diazocine-like structure as shown by Koriatopoulou et al [41]. The presence of an intense fragment ion $Bb1^{++}$ in particular shows that the sequence $K_{108}EDFTR_{113}$ is covalently attached to Lys105, as illustrated in Figure 8b. Furthermore, the fragment ion $By2^+$ proves that neither Leu106 nor Glu107 of the sequence $K_{105}LE_{107}$ are involved in the cross-linked structure.
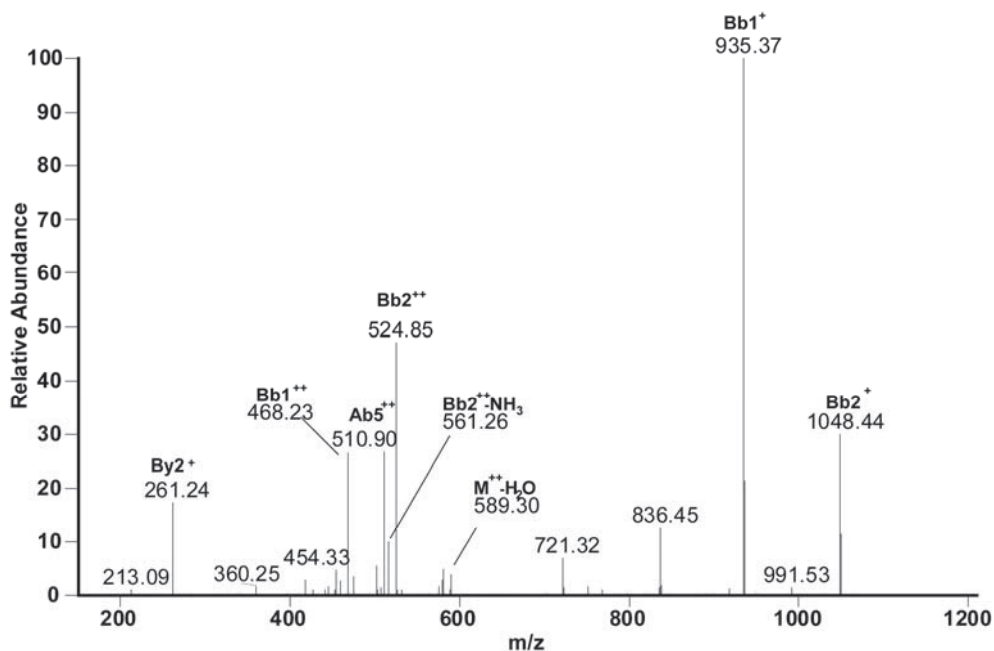
Figure 8a. MS/MS spectrum of sequence $K_{105}LE_{107}$ cross-linked through Lys105 to the sequence $K_{108}E^{(-H2O)}DF_{111}TR_{113}$.
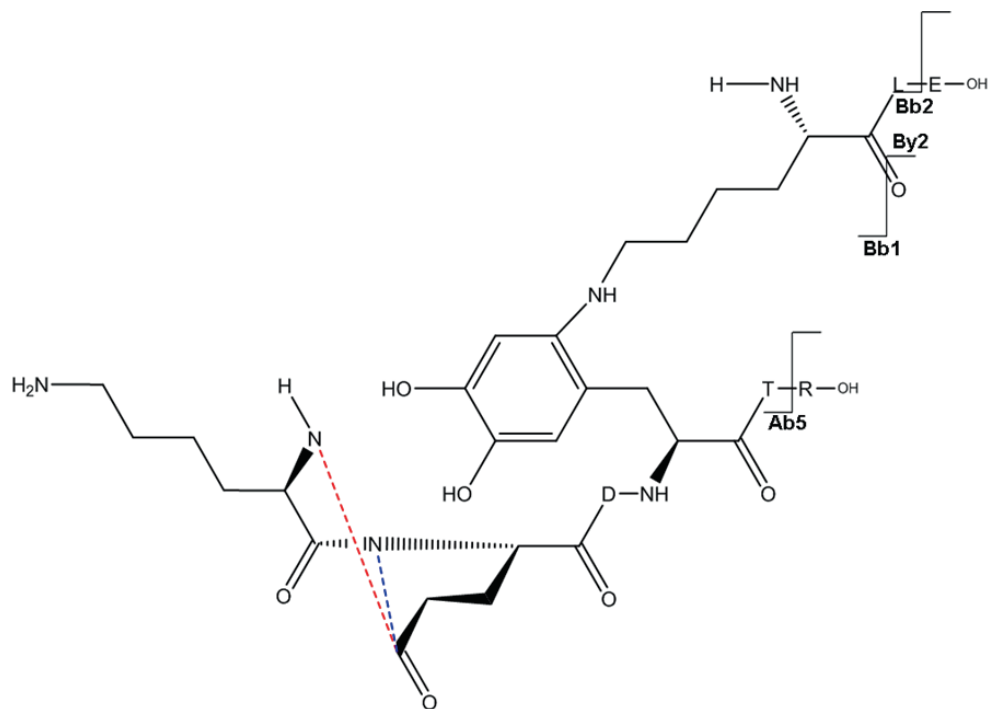
Figure 8b. Suggested chemical structure of the cross-linked sequence (theoretical *mass* 1194.60, experimental *m/z* 598.30, [M+H]$^{2+}$, Δm from parent peptide +0.00).

Lys105 – Tyr126

The cross-linked sequence $R_{124}YYGR_{128}$ with $K_{105}LE_{107}$ through Lys105 and Tyr126 is presented in Figure 9. Here there are two adjacent Tyr residues (in $R_{124}YYGR_{128}$) which can be involved in the cross-link. The Ay3^{++} and Ay4^{++} related ions however suggest that Tyr126 (and not Tyr125) serves as electron acceptor in the cross-link. [Bb1+ Na]$^{+}$ and [Bb2 + Na]$^{+}$ fragments prove the involvement of Lys105 in the peptide cross-linking. The sodiation of the respective fragment ions (except for By2^{+}) is due to complex formation between the C-terminal carboxyl of Arg128 and residual sodium ions present in samples, likely due to incomplete removal of sodium phosphate buffer during protein dialysis after MCO.
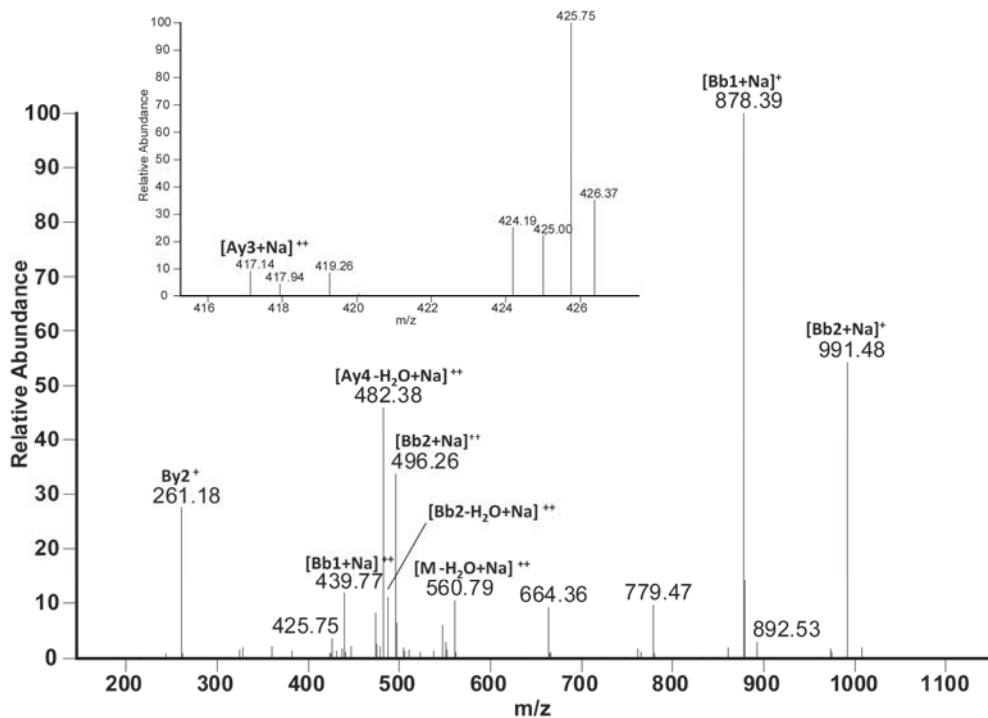
Figure 9a. MS/MS spectrum of sodiated sequence $K_{105}LE_{107}$ linked through Lys105 to the sequence $R_{124}YY_{126}GR_{128}$ (the inset show the MS/MS spectrum zoomed in the m/z range 415-428.
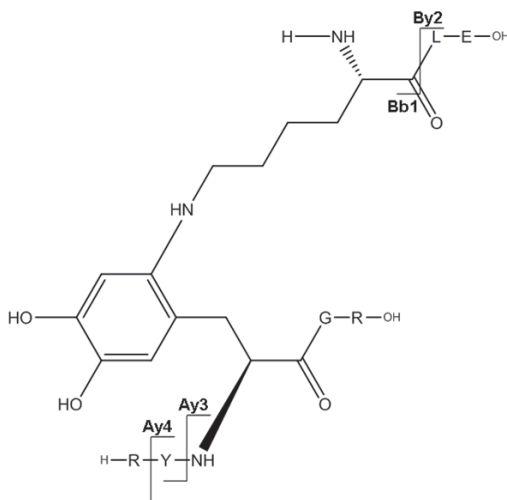

Figure 9b. Suggested chemical structure of the cross-linked sequence (theoretical *mass* 1137.56, experimental *m/z* 569.79, [M+H]$^{2+}$, Δm from native parent peptide +0.02).

Discussion

Oxidative chemical modification of amino acid residues may alter the secondary and tertiary protein structure, favoring interaction between protein surfaces and subsequently leading to non-covalent aggregation. Indeed, MCO (using the oxidative system presented in this paper) of proteins, such as growth hormone [42], IFNα-2b [14], IFNβ-1a [8], IgG1 [16], IgG2 [43], insulin and PEGylated insulin [19], relaxin [44], recombinant SHa (29–231) prion protein [45], and superoxide dismutase [46], have been shown to lead to extensive aggregation. Moreover, protein aggregates generated by MCO have been shown to be particularly immunogenic.

For instance, Hermeling et al. [14-15], using a transgenic immune tolerant mouse model, discovered that aggregated IFNα-2b, oxidized via metal catalysis was more immunogenic than IFNα-2b aggregates that were produced otherwise.

More recently, van Beers and colleagues hypothesized that a particular combination of oxidation and covalent aggregation could be responsible for the immune response against IFNβ-1a exposed to MCO, though no particular mechanisms for chemical cross-linking were demonstrated [8]. Similarly, MCO of an IgG1 was found to produce immunogenic aggregates [16].

Here, performing an extensive mass spectrometric characterization of IFNβ-1a oxidized under conditions identical to those in the above examples (i.e., Cu^{2+}/ascorbate catalysis), we describe chemical modifications of multiple residues that can affect the structure of IFNβ-1a. Results of the current study, in particular, reveal that the oxidative modification of Phe and Tyr results in an electron acceptor structure, namely DOCH, which may be involved in IFNβ-1a cross-linking through a 1,4- or 1,6-type addition mechanism of primary amines to DOCH. The results reported in this work are in agreement with the data we recently published using insulin as a model protein [17]. However, since Met and Trp are not present in insulin, we did not know whether these are involved in covalent cross-linking of other proteins such as IFNβ-1a. Although we detected oxidation of 4 Met residues and 1 Trp residue in oxidized IFNβ-1a, we found no evidence of Met or Trp being involved in covalent cross-links. These data are in contrast with the common opinion that Trp oxidation could lead to new carbonyl groups and subsequent cross-linking via Schiff base formation [47]. However, the imine structure generated through

Schiff base formation is reversible unless it is reduced with sodium borohydride or cyanoborohydride to form a secondary amine group [48], which may explain why Trp oxidation did not seem to be involved in aggregation of IFNβ-1a.

Our data on ABS derivatization of the control samples (Figure 1B) support the hypothesis that DOCH is an intermediate in the covalent cross-links of IFNβ-1a aggregates induced by MCO. SEC analysis under reducing conditions did not point to disulfide-mediated covalent cross-links. Furthermore, fluorescence spectroscopy data and MS/MS analysis helped to exclude other potential mechanisms of aggregation, such as dityrosine formation. This suggests that the reaction between two tyrosyl radicals is not favorable under our experimental conditions [49].

In addition to the putative cross-links via a 1,4- or 1,6-type addition mechanism, extensive oxidative modifications of numerous amino acid residues were induced during MCO: Met in positions 1, 36, 62 and 117 were found to be oxidized to sulfoxides. Met1 was also oxidized to a higher oxidation state to form Met sulfone. His oxidation to 2-oxo-His, as observed before in insulin and other proteins exposed to similar oxidative conditions [50], targeted IFNβ-1a residues in positions 93, 97, 121, 131 and 140. Moreover, oxidative modification to Asn was detected for His93 and His97.

Unfortunately, we failed to detect reliable spectra for ABS-derivatized peptides derived from oxidized IFNβ-1a (unpublished data). This could be attributed to either relatively low abundance of such modifications or further chemical reactions, which may occur during the analysis, thus interfering with the detection based on a mass increase of 179, 196 and 366 Da [27].

Table II demonstrates that not all of the potential targets for MCO were found oxidized. Most of the unmodified amino acids, such as Phe70, Trp79 and Tyr125, are in fact residues which are responsible for stabilizing the hydrophobic core of the molecule through a broad network of hydrogen bonds [7]. This could protect them from MCO and chemical cross-linking. A similar explanation could be given for the relatively low number of detected cross-linked peptides, which is far below the theoretically possible number of cross-links.

Besides the chemical modifications discussed above, based on far-UV CD measurements we noticed that MCO leads to changes in IFNβ-1a's secondary

structure resulting in the conversion of alpha helices to disordered structures [29, 51]. Interestingly, this was also seen upon MCO of IFNα-2b [14], but not of IgG1, which has only minor alpha helical content and for which its beta-sheet content seemed to be largely maintained after MCO [16]. Nevertheless, MCO of IgG1 did result in immunogenic aggregates [16], comparable to the observations with IFNα-2b and IFNβ1-a. This suggests that the change in secondary structure in oxidized IFNα-2b and IFNβ-1a is not a major factor contributing to the immunogenicity of these products. Rather, the combination of covalent aggregation and chemical modifications, which are probably very similar across the different proteins, likely contribute to the immunogenicity of proteins exposed to MCO. The structural changes observed in the present study, i.e. oxidation, conformational changes and aggregation, are comparable to the ones previously measured in IFNα-2b, although at that time a detailed MS/MS analysis was not performed and only Met oxidation was investigated [14]. However, recently DOPA formation in IFNα-2b was measured by our group (unpublished data), and, considering that both interferons share similar structural features, we suggest that the same type of chemical modifications and cross-links could be expected in IFNα-2b.

Conclusion

In this work we mapped the modifications of the primary structure in oxidized and immunogenic aggregates of IFNβ-1a, focusing on the amino acids potentially involved in covalent cross-linking. Several oxidative modifications were identified, especially in oxidation prone solvent-exposed amino acids. The primary role of DOPA and DOCH, i.e. Tyr and Phe oxidation products, as electron acceptors for 1,4- or 1,6-type addition, has been confirmed. These results are in agreement with the mechanistic studies previously performed on insulin, and potentially might be extended to other proteins exposed to similar oxidative conditions.

Acknowledgements

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Chapter 7

Immune mechanisms underlying immunogenicity of aggregated recombinant human interferon alpha-2a in immune tolerant mice

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Abstract

Purpose. To investigate the immune mechanism responsible for the immunogenicity of aggregated recombinant human interferon alpha-2a (rhIFNα) and to study if the presence of aggregated rhIFNβ increases rhIFNα's immunogenicity.

Methods. Transgenic mice immune tolerant for human interferon alpha were treated with native or aggregated rhIFNα. After a washout, the mice were rechallenged with aggregated or native rhIFNα to study immunological memory. Mice depleted from CD4+ T-cells were used to test for CD4+ T-cell involvement in immunogenicity. Furthermore, the mice were treated with a formulation containing aggregated rhIFNβ and native rhIFNα to test whether aggregated rhIFNβ acts as an adjuvant for rhIFNα.

Results. Native rhIFNα did not elicit antibodies, demonstrating the immune tolerant status of the transgenic mice. Aggregated rhIFNα was immunogenic in immune tolerant mice but did not induce immunological memory. Blocking CD4+ T-cells abolished the antibody response against aggregated rhIFNα. Aggregated rhIFNβ did not increase immunogenicity of native rhIFNα.

Conclusions. An atypical immune response with T-cell dependent and T-cell independent characteristics appears to be involved in the formation of antibodies elicited by aggregated rhIFNα, in line with previous results obtained with rhIFNβ. Moreover, highly aggregated rhIFNβ does not act as adjuvant for native rhIFNα.

Introduction

The number of recombinant human protein drugs entering the market is expanding greatly. Due to their intrinsic low toxicity and high versatility they are excellent drugs to treat various diseases [1]. Nonetheless, protein drugs do have a major disadvantage, namely immunogenicity [2], i.e., during treatment some patients will form anti-drug antibodies (ADAs). Those ADAs can interfere with kinetics, compromise efficacy, lead to infusion reactions and even cause life-threatening side-effects [3-5]. So, immunogenicity poses a serious health risk and increases costs of therapy.

In order to lower immunogenicity, either by developing products with minimal immunogenicity, by adjusting treatment regimen, or by identifying patients at risk, more insight into the underlying immune mechanisms is needed. Immune tolerant mouse models have proven to be very suitable to assess which product-related characteristics, such as aggregates, lead to ADA formation and to study the immune processes responsible for this [6-9]. We have previously shown that for highly aggregated recombinant human interferon beta-1b (rhIFNβ, Betaferon®) the immune response leading to ADA formation shares characteristics of both a T-cell independent response, i.e., involvement of marginal zone B-cells and no apparent immunological memory formation, and characteristics of a T-cell dependent immune response, i.e., involvement of CD4+ T-cells [10].

The primary aim of this study was to determine if such an atypical immune response would also be responsible for the ADA formation against another highly aggregated and immunogenic protein, metal catalyzed oxidized recombinant human interferon alpha-2a (rhIFNα). We therefore performed a set of experiments in an immune tolerant mouse model for human interferon alpha-2a, in which we studied the presence of an immunological memory response upon rechallenge with either native or aggregated rhIFNα and the involvement of CD4+ T-cells in the formation of ADAs. In addition, we studied whether immunogenic, highly aggregated rhIFNβ, when mixed with native rhIFNα, would induce or enhance an ADA response against native rhIFNα.

Materials and Methods

Materials

RhIFNα was provided as liquid formulation (1.5 mg/mL in 25 mM ammonium acetate, 120 mM NaCl, acetic acid, pH 5) by Hoffmann-La Roche (Basel, Switzerland). Betaferon® (Bayer Schering Pharma AG, Berlin, Germany) was reconstituted with 0.54% NaCl, according to the instructions in the package insert.

Tris, glycine, SDS, L-ascorbic acid, copper (II) chloride, disodium hydrogen phosphate, hydrogen peroxide and EDTA were purchased from Sigma–Aldrich, Schnelldorf, Germany. Slide-A-Lyzer dialysis cassettes, molecular weight cut-off 10 kDa, were purchased from Thermo Fisher Scientific, Breda, the Netherlands. All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use. Lithium heparin gel tubes were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, the Netherlands).

Preparation of native and aggregated rhIFNα formulations

RhIFNα, as received from the provider, was dialyzed against 10 mM sodium phosphate buffer, pH 7.4 (PB). Aggregation of rhIFNα was induced by metal catalyzed oxidation (hereafter refer to as aggregated rhIFNα) [11], performed after dilution of rhIFNα to 0.3 mg/mL, and by subsequent incubation with 40 µM $CuCl_2$ (previously prepared in 400 µM stock solution in PB) for 10 minutes. Subsequently, ascorbate (previously prepared in 400 mM stock solution in PB) was added to a final concentration of 4 mM. After three hours the reaction was quenched by adding 100 mM EDTA (previously dissolved in PB) to a final concentration of 1 mM, as previously reported [12-13]. The aggregated samples were dialyzed using 10 kDa cut-off dialysis cassettes against PB for 24 hours. Both formulations were subsequently characterized for protein concentration and structure (see below).

Mixture of rhIFNα and Betaferon®

The mixture of native rhIFNα with Betaferon® was obtained by mixing 0.66 mL of rhIFNα (0.15 mg/mL in PB) with 0.33 mL of Betaferon® 0.15 mg/mL, yielding final concentrations of 0.1 mg/mL of rhIFNα and 0.05 mg/mL rhIFNβ, pH 7.0. The formulation was subsequently analyzed by SEC (size exclusion chromatography) and SDS-PAGE (sodium dodecyl sulfate epolyacrylamide) to verify the non-aggregated state of rhIFNα in the mixture.

Analytical characterization of the formulations

UV/VIS absorption spectroscopy

UV/VIS absorption measurements were performed with an Agilent 8453 UV/VIS spectrophotometer (Waldbronn, Germany), which included a Peltier element for temperature control and a magnetic stirrer. The Peltier element was stirred by an Agilent 89090A controller. Quartz cells with a path length of 1 cm were used for all measurements. Scans were taken from 200-900 nm with 1 nm intervals. The protein concentration was determined by measuring the absorbance at 278.5 nm, using an extinction coefficient (0.1 %; 1 cm; 278.5 nm) of 1.06 [14].

Far-UV circular dichroism (far-UV CD) spectroscopy

Far-UV CD spectra were recorded from 190 to 250 nm using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-mm path length quartz cuvette at 20 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 6 repeated scans, background corrected with the corresponding buffer spectrum. The CD signals were converted to molar differential extinction coefficient, $\Delta\varepsilon$.

Intrinsic steady state fluorescence

Intrinsic fluorescence was measured in 96-well plates using the plate reader unit of the FS920 fluorescence spectrometer (Edinburgh Instruments, Livingston , UK). All the formulations were diluted to a protein concentration of 0.1 mg/ml to avoid inner filter effects. The concentration after dilution was

confirmed with a BCA assay. Tryptophan residues were selectively excited at 295 nm. Emission spectra were recorded from 305 to 500 nm using a step size of 2 nm, gain of 115, Z-position of 20 mm, number of flashes 100 with a frequency of 400 Hz. Triplicates for each sample were analyzed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gradient gels (4-20%, tris-HCl), were run under reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) and non-reducing (sample buffer without β-mercaptoethanol) conditions at room temperature. The electrophoresis buffer was 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine, and 0.1% (w/v) SDS. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, the Netherlands). Samples analyzed under reducing conditions were boiled for 2 min before application to the gel to favor the reduction of disulfide bonds. A low-range molecular weight standard (Biorad) was included on the gel for determination of molecular weight.

Western blotting

Protein bands in SDS-PAGE gels were blotted onto a polyvinylidene difluoride immuno blotting membrane with a mini trans-blot electrophoretic transfer cell (Biorad, Veenendaal, the Netherlands). Blots were blocked for 2 hours at room temperature with 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) with constant orbital shaking. After washing with 0.1% (v/v) Tween 20 in PBS and with water, the blots were incubated with polyclonal rabbit anti-hIFNα (PBL Biomedical Laboratories, Piscataway, NJ, US) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water and further incubated with peroxidase goat anti-rabbit IgG (Sigma-Aldrich) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Afterwards blots were again washed with 0.1% (v/v) Tween 20 in PBS and with water, and then incubated in a solution of 4-chloro-1-naphtol (Sigma- Aldrich) in methanol (20% (v/v)), water, and H_2O_2 (0.015%

(v/v)). After color development the blots were stored overnight to increase the intensity of the bands.

Size-exclusion chromatography (SEC)

Samples (100 μg protein/mL) were analyzed by SEC with a TSK-GEL 3000 column (TOSOH BIOSCIENCE GmbH, Stuttgart, Germany), using a mobile phase of 50 mM sodium phosphate and 200 mM sodium chloride, pH 7.2, filtered through a 0.2-μm filter prior to use, at a flow rate of 0.50 mL/min by a Waters 2695 controller equipped with an autosampler and a Waters 2996 photodiode array detector (Waters, Milford, MA, USA). The column was calibrated by analyzing protein molecular weight standards obtained from Sigma-Aldrich (i.e., thyroglobulin, BSA, ovalbumin, α-chymotrypsin, and myoglobin).

Mouse studies

Breeding

Heterozygous FVB/N transgenic (TG) mice immune tolerant for hIFNα and their non-transgenic (NTG) littermates were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). The genotype of the offspring was determined by PCR showing the presence or absence of the hIFNα gene in chromosomal DNA isolated from ear tissue. Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available *ad libitum,* and all animal experiments were performed according to Institutional Ethical Committee Regulations of Utrecht University, the Netherlands.

Immunogenicity and immunological memory response

TG and NTG mice were injected intraperitoneally (i.p.) with 10 μg of native rhIFNα or aggregated rhIFNα (n= 24 per group) for 5 days per week during 3 consecutive weeks (days 0-4, days 7-11 and days 14-18). After an injection-free period of 6 weeks, half of the TG and NTG mice (n=12) were rechallenged with 10 μg native rhIFNα and the other half (n=12) were rechallenged with 10 μg aggregated rhIFNα on 2 consecutive days (days 63 and 64). Blood was collected via cheek puncture before the start of the experiment, on different

days during the 3 treatment weeks (days 7, 11, 14 and 18), during washout (days 21, 28 and 42) and before rechallenge (days 56, 58 and 60). At each blood sampling time point, blood was collected before injection to prevent interference of ADA-drug complexes in antibody determination, and was taken from 7 out of 24 mice per group per time point to prevent oversampling. At 13 days after rechallenge (day 77) all mice were sacrificed and blood was isolated. Plasma was obtained from blood after centrifugation in lithium heparin tubes (3000 g, 10 min) and stored at -20 °C until further analysis.

Involvement of CD4+ T-cells in the formation of ADAs

TG and NTG mice (n=18 per group) were treated with 10 µg aggregated rhIFNα or 5 µg ovalbumin adsorbed to aluminum hydroxide gel (Sigma-Aldrich BV, Zwijndrecht, the Netherlands) in 100 µl PBS (ova) for 5 days per week during 3 consecutive weeks (days 0-4, days 7-11 and days 14-18). Ova is a T-cell dependent antigen and served as control for a CD4+ T-cell immune response. As control for a T-cell independent immune response, TG and NTG mice were treated with Pneumovax® (n=18 per group), according to Scheikl and colleagues [15]. Primary i.p. immunization was performed with 1 µg of Pneumovax® in 100 µl PBS on day 0, followed by a second i.p. injection with another 1 µg of Pneumovax® on day 11.

To study involvement of the CD4+ T-cells, half of the mice (n=9 per group) received 3 i.p. injections of rat anti-CD4 antibody GK 1.5 (150 µg in 100 µl PBS) (Bioceros, Utrecht, the Netherlands) before the start of treatment. Depletion was maintained by administration of 150 µg GK 1.5 every 3 to 4 days during the 3 treatment weeks. Blood was collected via cheek puncture before the start of the experiment and during treatment (days 9 and 16) from 3 or 6 mice out of 9. On day 23 all mice were sacrificed and blood was isolated. During the experiment, 2 TG and 2 NTG mice died due to fighting. Numbers of remaining mice can be found in Figure 5.

To confirm depletion of CD4+ T cells, additional groups of mice (n=22) were treated with GK 1.5 or PBS following the same procedure as described before. During every treatment week, some of these mice were sacrificed and single-cell suspensions of spleens were tested by flow cytometry for depletion by a non-competing anti-CD4 monoclonal RM4-4 antibody (BD Bioscience, the Netherlands). Measurements were taken using a FACSCanto II® (BD

Bioscience) and analysis was performed with the FACSDiva software v6.1.1 (BD Bioscience). Depletion efficiency was on average >95% throughout the experiment (data not shown).

Immunogenicity of rhIFNα when co-administered with Betaferon®

TG and NTG mice were injected i.p. with 10 µg of (i) native rhIFNα, (ii) aggregated rhIFNα, or (iii) the mixture of native rhIFNα and Betaferon® (n=6 per group) for 3 weeks, 5 days per week (days 0-4, days 7-11 and days 14-18). Blood was drawn via cheek puncture from 3 out of 6 mice per time point to prevent oversampling, before the start of the experiment and on days 9 and 14. On day 21, all mice were sacrificed and blood was isolated. Plasma was obtained after centrifugation in lithium heparin tubes (3000 g, 10 min) and stored at -20°C until further analysis.

Determination of antibody titers by enzyme-linked immunosorbent assay (ELISA)

Plasma was analyzed for anti-rhIFNα antibodies, anti-rhIFNβ antibodies (Betaferon®) and anti-ova antibodies by ELISA, as described in detail by Hermeling et al [7, 16]. In the case of Pneumovax®, anti-pneumococcal polysaccharide antibodies were detected by the following method. Mouse plasma was preadsorbed to pneumococcal cell wall polysaccharide (CWPS) antigens to capture non-specific antibodies against CWPS, a known impurity of the Pneumovax® vaccine [15]. In brief, mouse plasma was diluted 1:100 in PBS, mixed with 2 µg of CWPS and the mixture was incubated for 30 min at room temperature. Adsorbed samples (100 µl) were added to 0.05 µg/well Pneumovax® coated microtiter plates, which were then incubated for two hours followed by incubation with primary antibody and secondary antibody. After each step plates were washed 3 times with washing buffer (0.05 % (w/v) Tween 20 in PBS). After adding the secondary antibody a color reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine (Roche, Almere, the Netherlands) and stopped after 10 minute incubation time by adding 0.18 M sulfuric acid. Optical density values for all ELISAs were measured at 450 nm wavelength with a microplate reader (Novopath; Biorad, the Netherlands) and data analysis was performed with GraphPad Prism 4.03 software (San Diego,

CA, USA). Samples were defined positive if their mean absorbance values were at least three times higher than the 95th percentile value of negative control plasma. OD450-log dilution plots were fitted to a sigmoidal dose-response curve and the reciprocal of the dilution corresponding to 50% of the maximal signal (EC50 value) was defined as the titer. Negative samples were assigned a titer of 0, and were taken along in data-analyses.

Statistics

For the immunological memory experiment, differences in anti-rhIFNα antibody titers between initial treatments were assessed using a nonparametric Kruskal-Wallis test. The same test was used to assess difference in ADAs before and after rechallenge. First an overall effect of time (before and after rechallenge) was assessed. If this overall effect was significant, further tests were performed to specify which groups differed. For the CD4^{+} T-cell depletion study and co-administration of native rhIFNα and Betaferon® experiment also a nonparametric Kruskal-Wallis test was used to assess statistical difference in antibody titers between groups. For all comparisons an overall effect of treatment (all time points combined) was determined; if statistically different, further statistical testing was performed to determine differences in titers per time point. A p value ≤0.05 was considered significant.

Results and Discussion

Physicochemical characterization of native rhIFNα, aggregated rhIFNα and the rhIFNα and Betaferon® mixture

Two batches of native rhIFNα and aggregated rhIFNα were prepared. Reported analytical data represent the average ± lower/upper value for the two batches and are summarized in Table I. Our results are in fair agreement with those reported for rhIFN alpha-2b (which contains Arg in position 23 instead of Lys, as in rhIFN alpha-2a used in the present study) by Hermeling et al. [16] and are briefly discussed below.

The mixture of native rhIFNα with Betaferon® was prepared in duplicate and analyzed by SEC and SDS-PAGE to verify the monomeric nature of rhIFNα.

Characteristics of native and aggregated rhIFNα

The chromatographic behavior of the aggregated protein indicated a substantial reduction in the monomer content, next to a large fraction of higher molecular weight species, when compared to native rhIFNα, which contained 99% of monomeric protein besides a small amount of dimer according to SEC (Table I). SDS-PAGE showed an apparent molecular weight of about 17 kDa under reducing and non-reducing conditions for native rhIFNα, whereas aggregated rhIFNα was characterized by the presence of higher molecular weight species, which appeared to be mainly mediated by non-reducible covalent bonds (Figure 1, panel a and b). Western blotting indicated that that the aggregated protein still contained native epitopes (Figure 1, panel c).

Table I. Summary of SEC results and spectroscopic features of rhIFNα and its aggregated form.

Sample	SEC			UV		Far-UV CD			Fluorescence maximum	
	Monomer[1] (soluble fraction)	Oligomers[2] (soluble fraction)	Insoluble fraction[3]	OD ratio 280/260 nm	OD at 350 nm	208 nm (Δε)	222 nm (Δε)	Δε ratio 208/222 nm	λ nm	Intensity (a.u.)
Native rhIFNα	99.0 ± 0.6	1.1 ± 0.6	0	1.85 ± 0.03	0.006 ± 0.002	-5.34 ± 0.97	-5.03 ± 0.96	-1.06 ± 0.01	326 ± 1	19.7 ± 0.7
Aggregated rhIFNα	55.3 ± 8.5	44.7 ± 8.5	0	1.10 ± 0.15	0.22 ± 0.07	-3.12 ± 1.28	-2.89 ± 1.16	-1.08 ± 0.01	330 ± 1	7.9 ± 1.3

[1] Percentages were calculated based on SEC peak areas relative to the total peak area of native rhIFNα: $AUC_{peak}/AUC_{native,\ total}$ x 100.

[2] For the aggregated protein due to the broad overlapping peaks, an exact estimation of the molecular weight of the multimers was not achievable.

[3] The insoluble fraction was defined as the fraction that was not recovered by SEC; percentages were calculated from the total peak area in SEC and the total peak area of native rhIFNα: $(AUC_{native,\ total}\text{-}AUC_{sample,\ total})/AUC_{native,\ total}$ x 100.
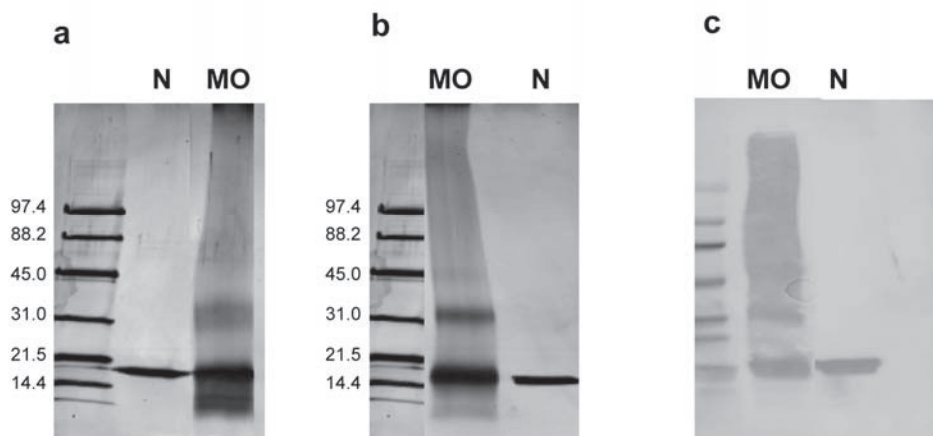
Figure 1. SDS-PAGE of native (N) and aggregated by metal catalyzed oxidation (MO) rhIFNα-2a under (a) reducing conditions and (b) non-reducing conditions, and (c) Western Blotting obtained from a gel run under non-reducing conditions. Numbers on the left indicate molecular weight (kDa) of the markers.

UV spectra of aggregated rhIFNα showed a decrease in the A280/A260 ratio, besides an increase in the optical density at 350 nm, providing further evidence of the presence of aggregates in the stressed formulation via metal catalysis [17]. Intrinsic steady state fluorescence suggested a more hydrophilic environment of the tryptophan residues in the stressed sample, where a lower intensity and a red-shifted spectrum (emission maximum 330 ± 1 nm), was recorded in comparison to the native protein (emission maximum 326 ± 1 nm). Far-UV CD measurements pointed to a decrease in α-helical content for aggregated rhIFNα but no major changes towards other secondary structures, since no statistical difference between the 208/222 nm intensity ratio of native and stressed rhIFNα was measured (Table I).

Characteristics of the rhIFNα and Betaferon® mixture

The association state of rhIFNα in the formulation containing rhIFNα and Betaferon® was investigated by SEC. The chromatograms show that rhIFNα was maintained in its monomeric state (Figure 2). Furthermore, no changes in the soluble fraction of Betaferon® were observed before and after the addition of rhIFNα (Figure 2), highlighting that mixing of rhIFNα with rhIFNβ did not lead to major changes in the aggregation status of either of the two proteins.

Using SDS-PAGE, it was confirmed that rhIFNα recovered from the rhIFNα and Betaferon® mixture, was indeed in its monomeric non-aggregated state, as demonstrated by the detection of a single band with the same molecular weight of native rhIFNα (data not shown). Similarly, Betaferon® alone and in combination with rhIFNα showed similar bands. Based on Western blotting, native rhIFNα and rhIFNα recovered from the combined rhIFNα and Betaferon® solution (not shown) contained intact epitopes.
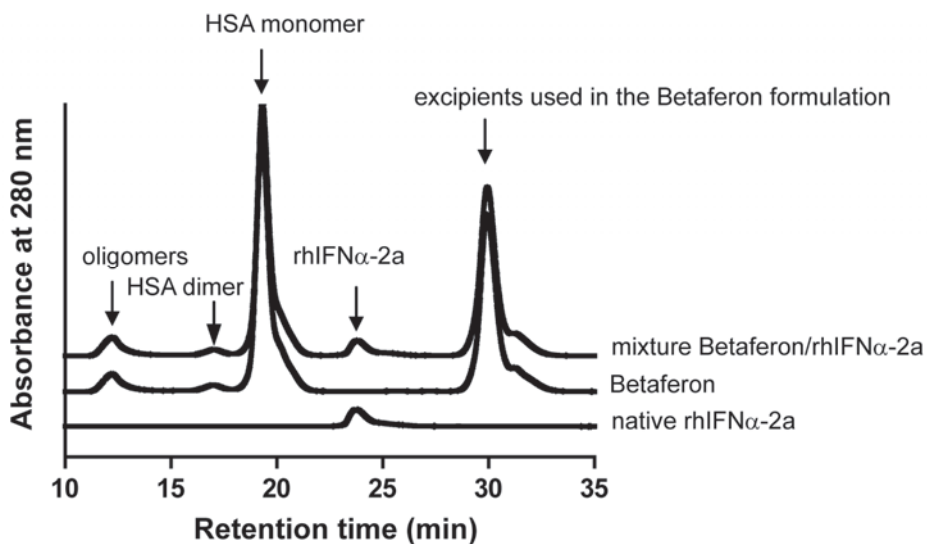

Figure 2. SEC of native rhIFNα, Betaferon and the mixture Betaferon/rhIFNα. The identity of the peaks from left to right is: oligomers, HSA dimer, HSA monomer, rhIFNα-2a, excipients used in the Betaferon formulation. Note that no rhIFNβ peak could be identified, probably because the protein is highly aggregated [18].

Mouse studies

Immunogenicity of native and aggregated rhIFNα

TG and NTG mice were treated with either native rhIFNα or aggregated rhIFNα for 3 weeks followed by a treatment-free period of 6 weeks (washout). Native rhIFNα caused an ADA response in all NTG mice (Figure 3a), while it was very poorly immunogenic in the TG mice (2 out of 24 mice had detectable antibody levels, each at only 2 time points (t= 18 and t=42 days), illustrating the immune tolerant status of these mice towards rhIFNα. Since the two TG mice with detectable antibody levels displayed similar antibody responses as the NTG mice at only two time points, the antibody titers were considered statistical outliers and these two mice were excluded from further analyses.
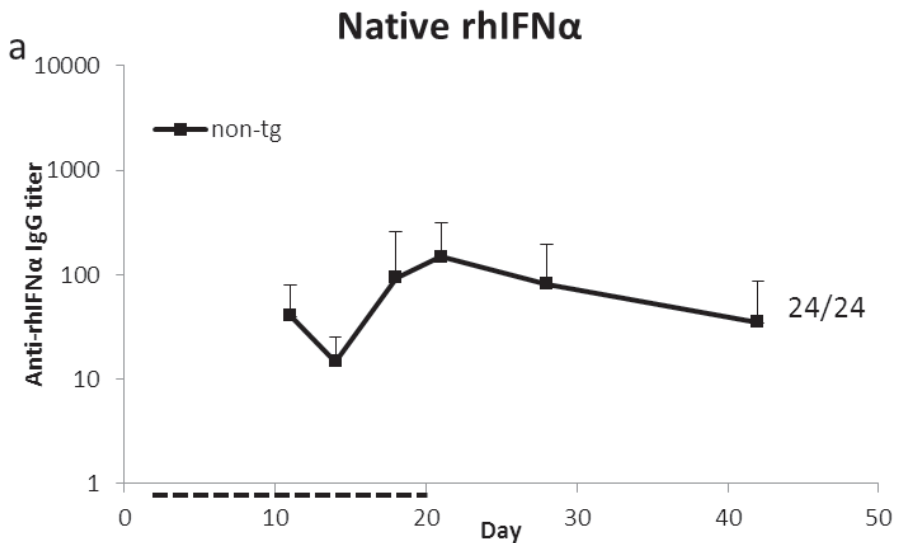
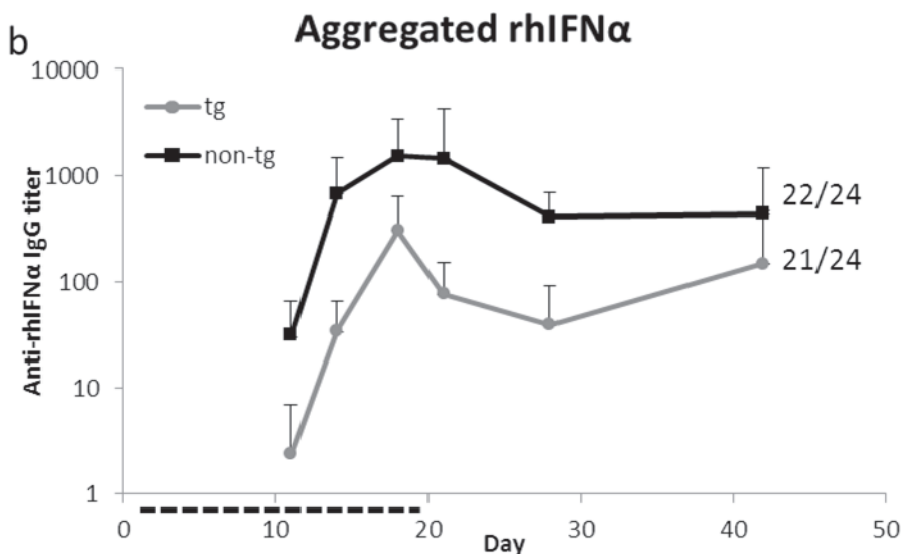**Figure 3a.** Anti-rhIFNα IgG titers of NTG (non-tg, black lines) mice treated with native rhIFNα during the initial three treatment weeks (dashed line below x-axis) and washout period. Two out of 24 TG mice treated with native rhIFNα showed detectable antibody titers on days 18 and 42, these mice were considered outliers and are not presented. The number of antibody positive mice out of the total number of mice per group is given. Data represent mean + standard deviation per time point (n= 24).

In contrast, aggregated rhIFNα caused an antibody response in both the NTG (22 out of 24 responders) and TG mice (21 out of 24 responders) (Figure 3b). For the NTG mice, treatment with aggregated rhIFNα gave higher titers than treatment with native rhIFNα (p=0.002, Figure 3).

These results show that native rhIFNα is poorly immunogenic in the TG mice and that by aggregation immunogenicity is strongly enhanced. This corresponds to our previous findings with recombinant human interferon alpha-2b [16, 19], and to our recent results with human monoclonal IgG [8], showing that metal catalyzed oxidation of therapeutic proteins results in aggregation and enhanced immunogenicity.

Figure 3b. Anti-rhIFNα IgG titers of TG (tg, grey lines) and NTG (non-tg, black lines) mice treated with aggregated rhIFNα during the initial three treatment weeks (dashed line below x-axis) and washout period. The number of antibody positive mice out of the total number of mice per group is given. Data represent mean + standard deviation per time point (n= 24).

Immunological memory response

NTG mice displayed low antibody titers before rechallenge independent of the initial treatment, while TG mice had detectable titers before rechallenge only when initially treated with aggregated rhIFNα (Figure 4). NTG mice initially treated with native rhIFNα showed increased antibody levels after rechallenge with native and aggregated rhIFNα (overall effect, p=0.038, Figure 4, panel a). This was most apparent in NTG mice initially treated and rechallenged with native rhIFNα, (p<0.001). NTG mice initially treated with aggregated rhIFNα had higher antibody titers after rechallenge with both rhIFNα products (overall effect p<0.001, Figure 4, panel b); this effect was most pronounced in mice rechallenged with aggregated rhIFNα (p=0.004). In TG mice initially treated with aggregated rhIFNα, no statistically significant increase in antibody titers was present upon rechallenge with native or aggregated rhIFNα (overall effect, p=0.077, Figure 4, panel b).
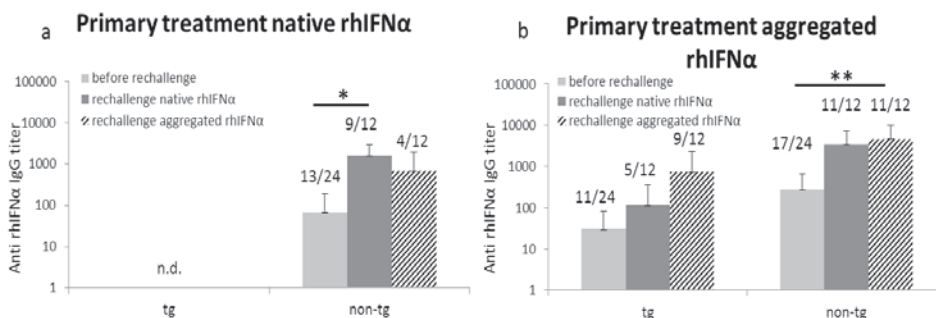

Figure 4. Anti-rhIFNα IgG titers of TG (tg) and NTG (non-tg) mice receiving primary treatment with native rhIFNα (a) and aggregated rhIFNα (b). Bars show titers before rechallenge (light grey) and after rechallenge with native rhIFNα (dark grey) or with aggregated rhIFNα (striped). Data represent mean + standard deviation. Above each bar the number of antibody positive mice out of the total number of mice per group is given. * indicates p<0.05. ** indicates p<0.01. n.d.=not detectable.

These results suggest that for NTG mice a memory response is present when either native or aggregated rhIFNα is given as primary treatment, and when either of the two products is given during rechallenge. Interestingly, antibody titers after rechallenge appear to be higher when the same product is given during initial treatment and rechallenge. This indicates that there is some

level of specificity in the immunological memory response and that there are distinct features in native or aggregated rhIFNα responsible for this specificity. This could be due to differences in exposed epitopes between aggregated and native rhIFNα and/or to the formation of new epitopes in aggregated rhIFNα as a result of the oxidation; it was recently shown for insulin that the same oxidation mechanism used to create rhIFNα aggregates leads to numerous chemical changes and cross-links in insulin [13].

For the TG mice there was no statistically significant immunological memory response observed, although there is a trend towards increased antibody levels after rechallenge (Figure 4, panel b). This apparent lack of immunological memory is similar to our previous observations with rhIFNβ in immune tolerant mice for hIFNβ [10]. However, studies assessing the presence of memory T- and B-cells should be performed to provide more definite proof on the presence or absence of immunological memory formation.

Involvement of CD4+ T-cells in the formation of ADAs

To study whether CD4+ T-cells are involved in the antibody response against aggregated rhIFNα, TG and NTG mice depleted from their CD4+ T-cells were treated with aggregated rhIFNα (Figure 5, panel a). Control groups of TG and NTG mice were treated with ova (a T-cell dependent antigen, Figure 5, panel b) or Pneumovax® (a T-cell independent antigen, Figure 5, panel c). CD4+ T-cell depletion in both TG and NTG mice treated with aggregated rhIFNα resulted in an almost abolished antibody response (overall effect, p=0.009 for TG and p=0.003 for NTG) with most apparent effects at the end of testing. Only 1 out of 8 NTG mice showed detectable antibody levels after depletion. As expected, CD4+ T-cell depletion also diminished antibody titers against the T-cell dependent antigen ova in both TG and NTG mice (overall effect p<0.001 for both), while the antibody response against the T-cell independent antigen Pneumovax® remained unaffected (overall effect p=0.408 for TG and p=0.741 for NTG).

These results indicate that the antibody response against aggregated rhIFNα is dependent on CD4+ T-cells. Together with the previous observation of an apparent lack of immunological memory, it appears that the immune mechanisms underlying immunogenicity of aggregated rhIFNα resemble the mechanisms underlying immunogenicity of Betaferon® [10]; both appear to lack

immunological memory and both involve CD4+ T-cells. However, it has to be noted that for the antibody response against Betaferon®, early involvement of marginal zone B-cell in particular led to the hypothesis of a T-cell independent trigger of immunogenicity, and of ancillary T-cell involvement in actual antibody production [10].
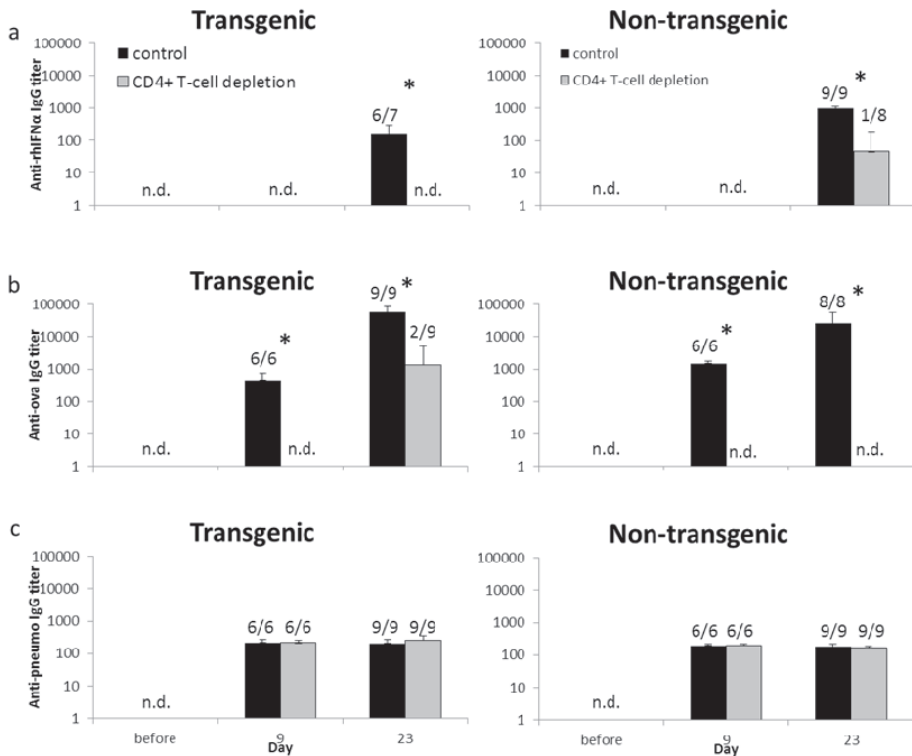

Figure 5. Total anti-rhIFNβ IgG titers (a), total anti-ova IgG titers (b) and total anti-pneumo IgG titers (c) of TG and NTG mice treated with aggregated rhIFNα, ovalbumin or Pneumovax®, respectively. Data represent mean + standard deviation per time point. Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Black bars represent control mice with CD4+ T-cells, grey bars indicate mice depleted from CD4+ T-cells. * shows statistical difference in antibody titers between control mice and mice without CD4+ T-cells (p<0.05). n.d.= not detectable.

Nonetheless, this is the first study showing that the immune mechanisms underlying immunogenicity of two highly aggregated proteins might be comparable. Moreover, this and the previous study [10] indicate the importance of clinical studies looking into the immune mechanisms in humans involved in immunogenicity of therapeutic proteins. Sparse patient data on re-induction of treatment with interferon beta and monoclonal antibodies indicate that immunological memory might also be absent in patients [20], but this needs to be confirmed in larger patient cohorts.

Immunogenicity of rhIFNα when co-administered with Betaferon®

TG and NTG mice were treated with either native rhIFNα, aggregated rhIFNα or the combined rhIFNα and Betaferon® solution. Figure 6 shows the corresponding anti-rhIFNα IgG titers of the TG and NTG mice. For the TG mice, only aggregated rhIFNα was capable of inducing ADAs (overall effect p<0.0001, Figure 6a), which was most apparent on day 21. Native rhIFNα and the combined rhIFNα and Betaferon® solution did not cause detectable ADA formation in these mice.
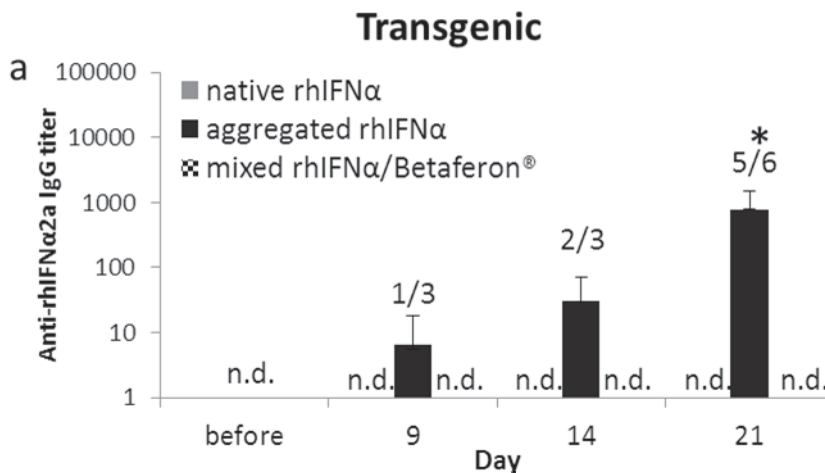

Figure 6a. Anti-rhIFNα IgG titers of TG mice treated with either native rhIFNα (grey bars), aggregated rhIFNα (black bars) or the mixture of rhIFNα/Betaferon® (black and white pattern). Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Data represent mean + standard deviation per time point. * indicates p<0.05 between the three treatment groups. n.d = not detectable.

For the NTG mice (Figure 6b) ADAs were formed for all 3 treatments, however no differences in ADA titers between treatments were found (p=0.122). This confirms some of our previous studies [19] in which NTG mice, in contrast to TG mice, could not discriminate between poorly and highly immunogenic formulations.
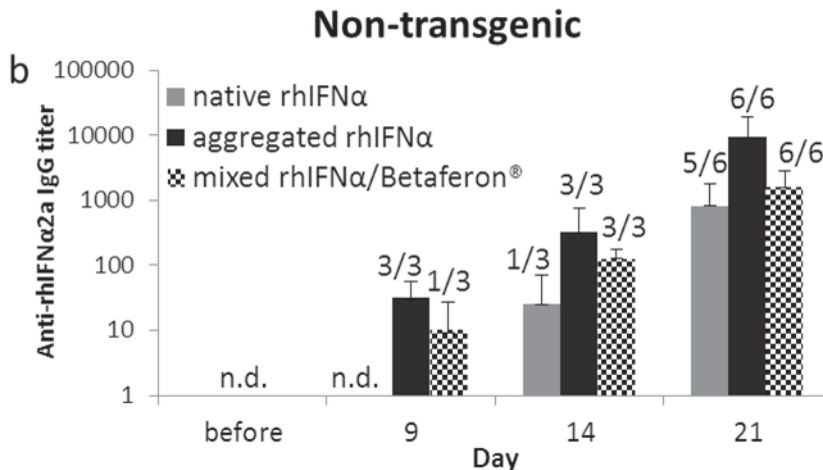

Figure 6b. Anti-rhIFNα IgG titers of NTG mice treated with either native rhIFNα (grey bars), aggregated rhIFNα (black bars) or the mixture of rhIFNα/Betaferon® (black and white pattern). Above each bar the number of antibody positive mice out of the total number of sampled mice per group is given. Data represent mean + standard deviation per time point. n.d = not detectable.

Both TG and NTG mice treated with combined rhIFNα and Betaferon® solution formed antibodies against rhIFNβ (data not shown), confirming that immune tolerance was specific for rhIFNα.

These findings show that Betaferon® does not trigger immunogenicity of rhIFNα, although it causes the formation of anti- rhIFNβ antibodies. It can therefore be concluded that activation of the immune pathways involved in immunogenicity of Betaferon® does not lower the threshold for immunogenicity of native rhIFNα. Betaferon® is therefore not functioning as adjuvant. It would be interesting to study if the lack of stimulatory effect by Betaferon® is also applicable to other therapeutic proteins, and if well-known

adjuvants are actually capable of enhancing immunogenicity of native rhIFNα and other non-immunogenic proteins in the immune tolerant mouse model.

In the current experiment we co-administered the two protein species in one formulation, however they were not chemically linked, as was confirmed by SEC (Figure 2). Several studies have shown that depending on the immunostimulatory molecules, linkage might be important in exerting their effect [21-22]. It might therefore be worth studying immunogenicity of rhIFNα when covalently bound to immunogenic rhIFNβ aggregates.

Conclusion

The immune mechanisms underlying immunogenicity of aggregated rhIFNα resemble the immune mechanisms underlying immunogenicity of Betaferon®; an apparent lack of immunological memory and involvement of CD4+ T-cells. Immunogenic and highly aggregated Betaferon® does not act as adjuvant for non-immunogenic rhIFNα.

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Chapter 8

Development of a transgenic mouse model to study the immunogenicity of recombinant human insulin

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Abstract

Mouse models are increasingly used to assess the immunogenicity of recombinant human (rh) therapeutic proteins and to investigate the immunological processes leading to anti-drug antibodies (ADAs). In 1994 a transgenic (TG) mouse model for studying the immunogenicity of insulin variants was described (Ottesen et al., Diabetologia 37:1178-1185). However, this model requires the use of complete Freund's adjuvant (CFA), which changes the formulation and thus limits its applicability for studying the effect of formulation-related factors on immunogenicity. The aim of this work was to develop an adjuvant-free TG mouse model for evaluating the immunogenicity of rh insulin (insulin) formulations. Intraperitoneal administration of insulin (20 μg/dose, 12 doses over a period of 4 weeks) did not break the immune tolerance of the TG mice, whereas it did elicit antibodies in non transgenic (NTG) mice. This tolerance in TG mice could be circumvented by insulin covalently bound to 50 nm polystyrene nanoparticles as well as by oxidized and aggregated insulin and to a lesser extent by commercially available insulin products.

Introduction

With the development of recombinant DNA technology it has become possible to produce well-defined recombinant human (rh) therapeutic proteins [1-2]. Despite the fact that these proteins are structurally very similar to their endogenous counterparts, almost all rh proteins for therapeutic use are immunogenic [3-4].

Many factors influence the immunogenicity of a protein drug, which can be categorized into patient-dependent (e.g., type of disease, genetic background), treatment-dependent (e.g., dose, dosing schedule, route of administration, co-medication) and product-dependent factors [5-6]. An increasing number of publications support aggregation of therapeutic proteins as one of the major product-related factors influencing immunogenicity [7-8].

For rh interferon alpha (IFNα) and rh interferon beta (IFNβ), transgenic immune tolerant mouse models have been shown to be a valuable tool to study the product-related factors contributing to the immunogenicity and the underlying mechanisms [9-11]. For human insulin, the first transgenic (TG) mouse model was described in 1994 by Ottensen et al. [12] to study the impact of the modifications of the insulin sequence on the formation of new epitopes.

However, while they used complete Freund's adjuvant (CFA) to trigger the immune system, we prefer CFA free models to study formulation-related factors because the addition of CFA alters the formulation, CFA may affect protein's structure (unpublished observation from our lab) and likely affects the biodistribution of the protein.

The primary aim of this work was to create a CFA free TG mouse model to study the immunogenicity of rh insulin (insulin) formulations. Next, to study the applicability of the mouse model, we compared the immunogenicity of oxidized and aggregated insulin, oxidized non-aggregated insulin, and three different commercially available formulations of insulin variants (i.e. Levemir®, Insulatard®, Actrapid®).

Materials and Methods

Materials

Insulin containing 0.4% (w/w) zinc ions was provided by Merck (Oss, the Netherlands). Three commercially available insulin formulations, i.e.

Insulatard® (long acting insulin suspension obtained with zinc and protamine), Levemir® (long acting insulin modified on Lys B 29 with a fatty acid) and Actrapid® (neutral unmodified insulin solution) were a gift from the Leiden University Medical Center. Copper(II) chloride, ascorbic acid, ethylenediamine tetraacetic acid (EDTA), ammonium bicarbonate (ABI), sodium citrate, citric acid, arginine, disodium hydrogen phosphate, n-hydroxysulfosuccinimide sodium salt (NHS-sulfo), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDAC), 2-N-morpholino-ethanesulfonic acid (MES), were bought from Sigma–Aldrich (Schnelldorf, Germany). Glacial acetic acid and acetonitrile were purchased from Boom (Meppel, the Netherlands). 3.5 kDa Slide-A-Lyzer dialysis cassettes were purchased from Thermo Fisher Scientific (Etten-Leur, the Netherlands). Polystyrene nanoparticles (NP), (diameter 50 nm) that contain surface carboxyl groups, were purchased from Polysciences GmbH (Eppelheim, Germany). All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use.

Preparation of unmodified insulin solutions

Insulin solutions of 1 mg/mL were prepared by dissolving insulin in 0.1 M HCl. Subsequently, 50 mM sodium phosphate buffer (PB), pH 7.4, was added up to 1 mL and the pH adjusted to 7.4. The concentration was calculated by UV spectroscopy, using a molecular weight of 5.8 kDa and an extinction coefficient of 6200 M^{-1} cm^{-1} at 276 nm [13]. Insulatard®, Levemir® and Actrapid® were diluted to 0.2 mg/mL with PB, prior to injection.

Preparation of oxidized aggregated and oxidized non-aggregated insulin

Oxidized and aggregated insulin (PB-MCO) was prepared as previously reported [14]. To obtain oxidized non-aggregated insulin, metal catalyzed oxidation (MCO, via the oxidative system Cu^{2+}/ascorbate) was performed in 50 mM sodium citrate buffer, pH 3.0 (CB) for 3 hours at room temperature. To this end, insulin solution of 1 mg/mL was prepared by dissolving insulin directly in CB. MCO was performed by addition to 1 mL of 1 mg/mL insulin, 100 µL of 0.4 mM $CuCl_2$ in CB to a final concentration of 40 µM. The reaction

was performed in 2-mL Eppendorf tubes covered with aluminum foil to protect the reaction mixture from light. After 10 min of incubation of insulin with Cu^{2+}, to allow copper to bind to insulin, the oxidation reaction was started by the addition of 11 μL of 400 mM ascorbic acid in CB to a final concentration of 4 mM. The reaction was quenched after 3 h of incubation at room temperature by adding 11.2 μL of a 100 mM EDTA in CB, pH 3 to a final concentration of 1mM. The oxidized sample was extensively dialyzed at 4 °C against CB. Next, a second dialysis against 250 mM ammonium bicarbonate (ABI) buffer, pH 8.0, for 24 h, was performed. These procedures, as previously reported [14], prevented insulin aggregation. Further we will refer to this oxidized non-aggregated insulin as ABI-MCO.

Preparation of insulin covalently bound to 50 nm polystyrene nanoparticles

Insulin was covalently bound onto 50-nm polystyrene nanoparticles (NP) following the procedure described by Kalkanidis et al [15]. Briefly, to prepare 9.00 mL of 1 mg/mL covalently bound insulin, 3.6 mL of NP (2.5 % w/v, aqueous suspension) were suspended in 2.25 mL of 0.2 M MES. Then 1.125 mL of 28.80 mg/mL EDAC in water, were added dropwise over a period of 10 minutes. After that, 1.125 mL of 360 mM NHS-sulfo in water, were added and the reaction mixture was left to equilibrate for 2 hours at room temperature on a rotating plate (final pH was 6.0). After 2 hours, the pH was brought to 6.9-7.0 with 1 M NaOH and 900 μL of 10 mg/mL insulin in PB pH 7.0 were dissolved in the mixture (final insulin concentration was 1 mg/mL, pH 7.0). Coupling was carried out overnight at room temperature before 24 hours dialysis against 50 mM PB, pH 7.4 (further we will refer to this suspension as NP-ins). In order to verify whether insulin was covalently bound to the NP or only adsorbed, an insulin control (hereafter called insulin adsorbed on NP (ADS-ins)) was prepared following a similar procedure but without NHS-sulfo and without EDAC. Because of the nature of the reagents (NHS-sulfo and EDAC), carboxylic groups in insulin can be potentially activated during coupling with NP. Hence, a second control (hereafter called cross-linked insulin (CR-ins)) was prepared in the same way as described above for the preparation of NP-ins, but without the addition of NP.

SDS-PAGE

Acrylamide gradient gels (10-20% tris-tricine, Bio-Rad, Veenendaal, the Netherlands) were run under reducing and non-reducing condition as described before [16]. The cathode electrophoresis buffer was 0.1 M tris(hydroxymethyl) aminomethane, 0.1 M tricine, and 3 mM SDS, pH 8.3. The anode electrophoresis buffer was 0.1 M Tris pH 8.9. Gel electrophoresis was performed with a Biorad Protean III system (Biorad). Samples were boiled for 2 min before application to the gel. A polypeptide marker solution (Biorad) was included for determination of the molecular weight.

Centrifugation

Centrifugation in presence of SDS was performed prior to size exclusion chromatography (SEC) analysis for NP-ins and ADS-ins (not CR-ins, which was centrifuged as described below), to investigate the amount of insulin adsorbed and covalently bound, calculated by comparison with native insulin. 2 mL of NP-ins and ADS-ins (final insulin concentration 1 mg/mL), were loaded into Ultra–Clear centrifuge tubes (1/2 x 2 in., 13 x 51 mm, Beckman Coulter, Inc, Brea, CA), in presence of 20 mg SDS (i.e., 1% w/v SDS). Native insulin, 2 mL of 1 mg/mL, was loaded in another tube without SDS. Next, the tubes were centrifuged with a BECKMAN Titanium Centrifuge Type 70 Ti (Ultra Fixed Angle Rotor) for 30 minutes at 50000 rpm, (250000 g). (Beckman Coulter, Fullerton, CA). Supernatant was diluted two-fold in 50 mM PB, pH 7.4, prior to SEC analysis. CR-ins was centrifuged for 10 minutes, 4 °C at 16162 g with a bench centrifuge (Sigma 1-15 bench centrifuge, Shropshire, UK), without SDS, to remove insoluble aggregates that might block the SEC column.

Size exclusion chromatography (SEC)

SEC experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA), consisting of a 1200 series HPLC pump, degasser, autosampler G1329A, and a variable wavelength detector G1316A. The SEC eluent was composed of a mixture of 1 g/L arginine in water:acetonitrile:glacial acetic acid 65:20:15 (v/v/v), as described in the United States and European Pharmacopoeias [17-18], and chromatograms were acquired using UV absorption at 276 nm.

Dynamic light scattering (DLS)

DLS measurements were performed with a Malvern Zetasizer Nano ZS (Malvern, Herrenberg, Germany) equipped with a 633-nm He-Ne laser and operating at an angle of 173°. The software used to collect and analyze the data was the Dispersion Technology Software version 6.01 from Malvern. Each sample, containing 5 μl of NP, NP-ins or ADS-ins, diluted with 495 μl water, was measured in single-use polystyrene half-micro cuvettes (Fisher Emergo, Landsmeer, The Netherlands). Water as a dispersant (viscosity 0.8872 cP and RI 1.330) was used to measure different formulations of nanoparticles, mentioned above. To measure plain insulin, 500 μl of 10 mg/mL human insulin in 50 mM PB, pH 7.4, and PB as a solvent (viscosity 1.0200 cP and RI 1.335) was used. The measurements were made at a position of 4.65 mm from the cuvette wall with an automatic attenuator and at a controlled temperature of 25°C. For each sample, 15 runs of 10 s were performed, with three repetitions. The intensity size distribution, the Z-average diameter (Z-ave) and the polydispersity index (PdI) were obtained from the autocorrelation function using the "general purpose mode". The default filter factor of 50% and the default lower threshold of 0.05 and upper threshold of 0.01 were used.

Circular dichroism spectroscopy (CD)

Far-UV CD measurements were performed as previously described [16].

Mouse studies

Breeding

Balb/c mice, transgenic for the human insulin gene, were obtained from Dr. J. Kapp. TG animals were bred with non-transgenic (NTG) Balb/c mice to obtain heterozygous TG offspring and NTG littermates, used in the *in vivo* studies presented here. Animals were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). NTG Balb/c mice were purchased (since breeding did not result in sufficient NTG littermates) at the Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available *ad libitum*. The presence or absence of the human insulin gene was determined by real

time PCR (rtPCR) in chromosomal DNA isolated from ear tissue. All animal experiments described were approved by the Animal Ethical Committee of the Utrecht University.

Functional immune system: response to foreign protein

To test whether the transgene interfered with the general capacity to produce antibodies we immunized both TG (n=4) and NTG (n=4) animals with 5 μg of human serum albumin (HSA, i.p.) on five consecutive days for three weeks, according to Hermeling et al. Blood was taken before the start of the immunization and on days 9 and 18 by cheek puncture. On day 28 mice blood was collected via heart puncture under isoflurane anesthesia followed by cervical dislocation. The obtained plasma was stored at -80°C until further analysis.

Validation of the insulin mouse model: Immunogenicity of insulin and NP-ins

TG and NTG mice were treated for 4 weeks with 3 i.p. injections per week (Monday/Wednesday/Friday) of 20 μg of unmodified insulin (19 mice per group). The same schedule and number of animals was used to study if insulin bound to nanospheres (NP-ins) would be able to induce antibody formation Blood was drawn from all mice per group by cheek puncture before the injection schedule started and additionally on day 14. On day 28, blood was collected via heart puncture under isoflurane anesthesia followed by cervical dislocation for all mice. Plasma was stored at -80°C until further analysis.

Application of the insulin mouse model: Immunogenicity of insulin formulations

98 animals, 49 TG and 49 NTG, were used to compare the potential immunogenicity of PB-MCO, ABI-MCO, Levemir®, Insulatard® and Actrapid®, with insulin and NP-ins. To this end, 7 mice per group were used and the same injection schedule was used as described above. Blood was drawn before the injection schedule started and on day 28 via heart puncture under isoflurane anesthesia followed by cervical dislocation for all mice. Storage was performed at -80 °C until further analysis.

Binding antibody assay

Plasma was analyzed for binding antibodies against insulin or HSA by direct ELISA, as described in detail by Hermeling et al [10]. Microtiter plates were coated with 100 μl of 5 μg/ml native insulin or 5 μg/ml HSA, depending on the assay, overnight at 4°C. Samples were considered positive when the OD at 450 nm of the 20 or 10 fold diluted plasma was higher than the cut-off value, calculated according to Mire-Sluis et al [19]. Positive and negative control samples were included in each ELISA plate. The plots of OD 450 versus dilution were fitted to a sigmoidal curve and the reciprocal of the dilution at the midpoint was considered the titer of the plasma. Sigmoidal curves were calculated using a fourth order polynomial degree regression curve, elaborated with GraphPad version 4.03. Each plate contained negative plasma from NTG animals (i.e., before injection of any formulation) and positive plasma from NP-ins treated NTG and TG animals. To confirm the specificity of the antibodies for insulin, and their cross-reactivity, selected antibody-positive plasma samples of TG and NTG mice (2 samples per group) were spiked for 1 hr with increasing amounts of insulin (100 μg max), bovine serum albumin (BSA, 100μg max), bovine insulin, bovine insulin's chain A or Chain B, before adding the samples to the plate and performing the ELISA as described before. Samples from NTG mice treated with NP-ins were diluted 200-fold while samples from TG mice treated with NP-ins and from NTG and TG treated with insulin, were 20-fold diluted.

Statistical analysis

Antibody titers were first checked for normal distribution. After determining they were not normally distributed a non-parametric Mann-Whitney U test was used to compare antibody titers between groups. Significant differences between groups in the number of responders were determined with a Fisher's test. A calculated probability (p) value equal than or below 0.05 was considered to be statistically significant.

Results and Discussion

Physicochemical characterization of NP-INS, PB-MCO and ABI-MCO

Conjugation of human insulin to carboxylated polystyrene nanoparticles: evaluation of covalent binding, conjugation efficiency and characterization of particle size

Chemical covalent linkage between insulin and polystyrene nanoparticles (NP) was achieved following a previously described procedure [15]. To investigate the nature of the coupling, the amount of insulin covalently coupled versus the adsorbed one, and to exclude the presence of protein aggregates, we used SDS-PAGE, SEC and DLS analysis. Results suggest that mainly particles coated with covalently bound human insulin, with a Z-average of 55.21 ± 0.11 were present . No substantial increase in particle size was observed when insulin was mixed with non-activated particles (50.37 ± 0.21) indicating little or noncovalent binding of insulin to the NP.

Furthermore, the presence of free insulin in the mixture of ADS-ins likely explains the slightly lower average diameter. The low PdI measured with DLS (<0.1 for all NP formulations studied) points to the absence of NP aggregates.

The absence of aggregates in NP-ins and the successful conjugation of insulin to NP was further confirmed with SDS-PAGE (Figure 1, panel A, B and C). In particular panel A indicates the covalent nature of the bond between insulin and NP, since free insulin was barely detected. Panel B shows that the broad band at the top of Figure 1, panel A is solely due to NP and provides evidence that the coupling procedure with NHS-sulfo and EDAC does not induce aggregates formation as for these reagents used with insulin but without NP (Figure 1, panel C).
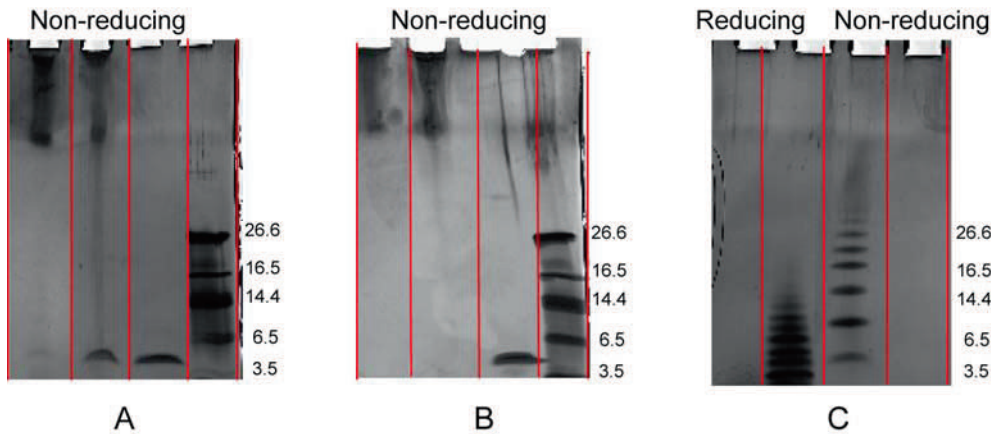
Figure 1. SDS-PAGE results: A, from left to right: nanoparticles-insulin (NP-ins), insulin adsorbed on nanoparticles (ADS-ins), rh insulin (insulin), polypeptide standard (M.W. indicated in kDa); B, from left to right: NP-ins, polystyrene nanoparticles (NP), insulin, polypeptide standard; C, from left to right: buffer, reducing SDS-PAGE of cross-linked insulin (CR-ins), non-reducing SDS PAGE of CR-ins, buffer.

Aggregate content and structural changes in PB-MCO and ABI-MCO

PB-MCO has been extensively characterized before [14, 16]. SEC and CD analysis were used to confirm our previous results: PB-MCO insulin contained 23.1 ± 3.6% aggregates and its secondary structure was substantially perturbed, as demonstrated by the reduction of the intensity of the entire spectrum and an increased 208/223 nm ratio, relative to insulin (PB-MCO 1.30 ± 0.03; insulin 1.19 ± 0.01), consistent with our previous data. ABI-MCO showed an amount of aggregates comparable to that of insulin, based on SEC analysis (ABI-MCO 0.7 ± 0.2%; insulin 0.4 ± 0.2%). CD analysis instead suggested that the content of alpha helix in ABI-MCO had decreased, parallel to an increase in random coil structure [20], based on the higher 208/223 ratio, which was 1.40 ± 0.02. This is likely due to oxidation of amino acid residues on insulin chain A and B which are normally involved in forming an alpha-helix structure, namely Tyr A14 and A19 and, His B10 and Tyr B16 [21].

Furthermore, the introduction of new oxygen atoms on His, Phe and Tyr residues, may alter the native hydrogen bond network of insulin, inducing insulin to assume a non-native like secondary structure [22].

Validation of the insulin mouse model and immunogenicity of insulin formulations

Immunization of transgenic and non-transgenic mice with human serum albumin

Mice were injected with 5 µg HSA daily for five consecutive days for three consecutive weeks. Anti-HSA IgG titers at days 11 and 18 were determined. TG animals showed an antibody response against HSA which was comparable to that in NTG animals, showing that the transgene did not interfere with the general capacity of the TG animals to produce antibodies. This is in line with previous TG mouse models for IFNα-2b [9, 23], IFNβ-1a [10-11] and insulin [12], which were also shown to be similarly responsive to the administration of a foreign protein as their NTG counterpart.

Immunogenicity of insulin and NP-ins

NTG and TG mice were injected with 20 µg of native insulin for 4 weeks (3 i.p. inj./week). Figure 2 shows the total IgG titers at day 28.
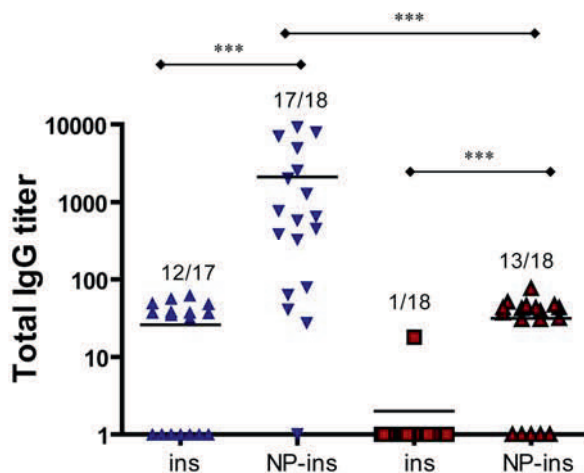

Figure 2. Total anti-insulin IgG titers after 28 days in NTG (blue) and TG (red) animals treated with rh insulin (ins) and nanoparticles-ins (NP-ins). Non-responders were assigned an arbitrary titer of 1 and they have been included in the calculation of the average titers depicted in this figure. Values above the bars represent the number of positive out of total mice. *** indicates p < 0.001. Five animals died during the studies for unknown reasons.

Of the TG mice injected with insulin only 1 out of 18 develop antibodies against human insulin. In contrast, nearly 50% of the NTG mice treated with insulin developed measurable antibody titers after two weeks of treatment (data not shown) which increased to about 70% after 4 weeks of treatment.

This shows that the TG mice are tolerant for human insulin, while NTG mice are not. To validate that TG mice (and the NTG controls) can form anti-human insulin antibodies they were treated with NP-ins. This formulation contains a particulate form of insulin which is likely to expose repetitive epitopes.

Several studies have demonstrated that presentation of such structures to the immune system, can result in antibody formation [7, 24].

Indeed, both NTG and TG animals do develop antibody against human insulin by the administration of NP-ins. For the TG mice 50% and 72% showed antibodies after 14 (data not shown) and 28 days, respectively. 94.4 % of NTG mice were antibody positive after 14 (data not shown) and 28 days. Based on Fisher's test, the number of responders (both TG and NTG) was higher for NP-ins treated mice compared to mice treated with insulin (p=0.022).

In addition to the differences in number of responders, we also found differences in antibody titers. When combining all treatment data, NTG mice displayed significantly higher antibody titers than TG mice. In more detail, NTG mice treated with NP-ins had higher antibody titers compared to (i) NTG mice treated with insulin (p<0.001, at days 14 and 28) and to (ii) NP-ins treatment in TG mice (p<0.001) (Figure 2). Similarly, TG mice treated with insulin show significantly lower antibody titers as with NP-ins (p<0.001). The immunogenicity of NP-ins, where insulin is covalently bound on the surface of the particles is likely due to the particulate character [25] and to the exposure of repetitive native-like epitopes [7, 24].

As observed before in other mouse models [10, 26], the same formulation induced a higher antibody titer in NTG mice than in TG mice, likely due to the absence of the human insulin gene in NTG mice. Furthermore, human and murine insulin feature high homology (i.e., > 92%, because of 4 different amino acid residues). Hence, there might be cross reactivity between insulin (either mouse or human, which are in theory both produced in TG animals) and ADAs. This could compromise detection of anti-insulin antibodies by ELISA due to binding between the circulating insulin and anti-human insulin antibodies, as observed by Thomas et al [27].

In addition, murine and human insulin contain an identical A-chain loop, which is a well known antigenic determinant on insulin [28-29]. Furthermore, due to the high sequence homology between human and murine insulin and a comparable rigid structure of the A-chain loop, human insulin might not be as immunogenic for NTG animals as we see for other foreign proteins with lower degree of homology. Perhaps this is why not all NTG mice immunized with human insulin were seropositive and the antibody titers in the seropositive ones were low. Concluding, differences in titers among TG mice may arise from differences in their transgene number (for human insulin).

Specificity of the antibodies for human insulin

Spiking plasma with increasing amounts of insulin and BSA confirmed the specificity of the antibodies measured: whereas spiking with insulin significantly diminished the OD at 450 nm for all positive mice ($p<0.05$), BSA did not inhibit the OD 450 signal ($p>0.05$), confirming the specificity of the antibodies for native insulin (Figure 3).
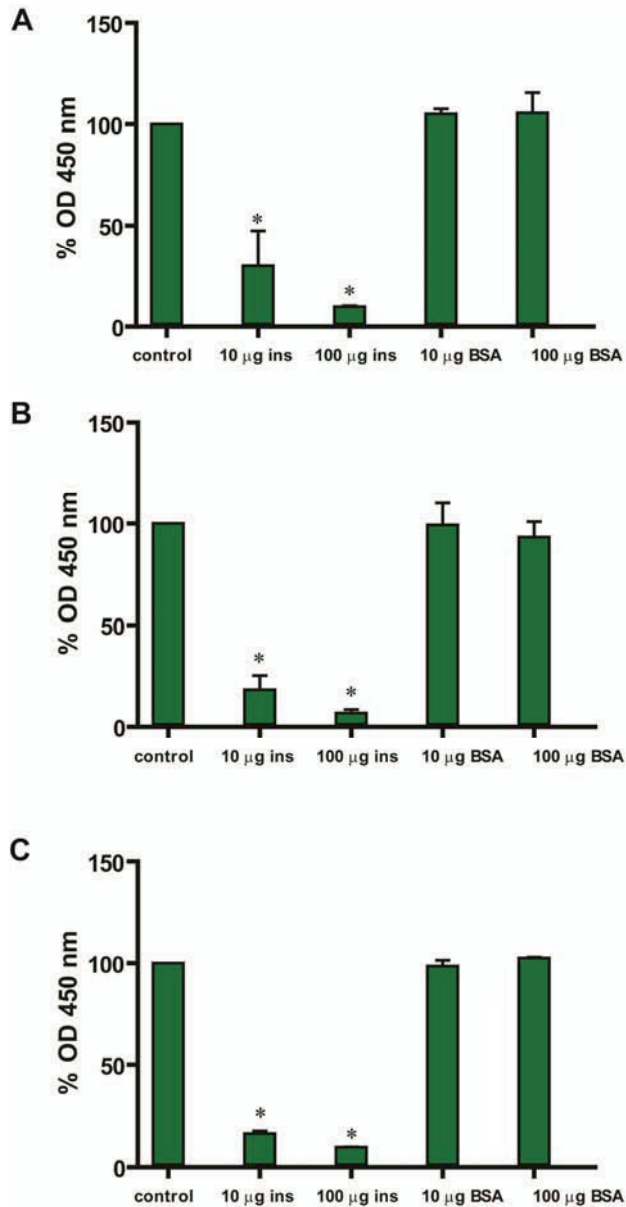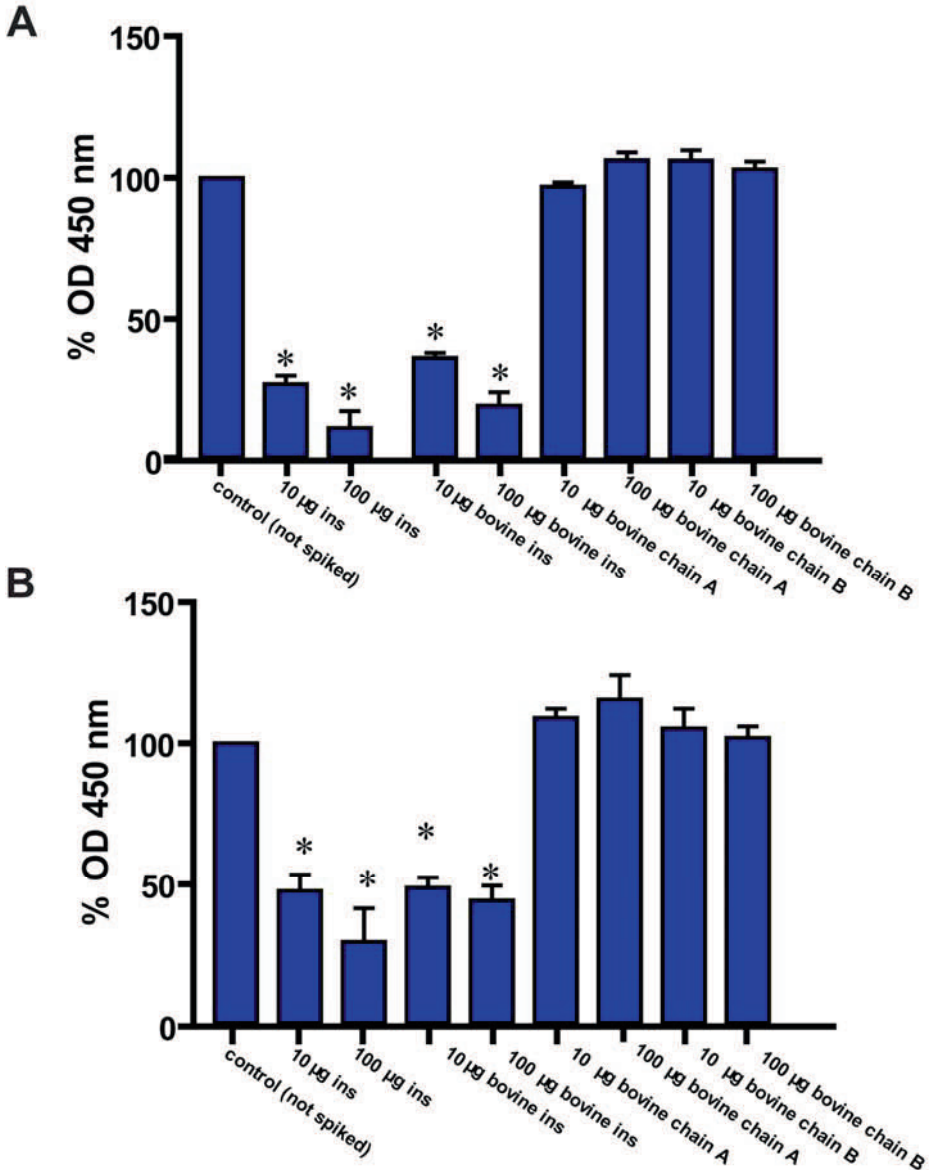
Figure 3. Spiking of plasma of NTG mice treated with nanoparticles-ins (NP-ins) (panel A), TG mice treated with NP-ins (panel B), NTG mice treated with rh insulin (ins) (panel C). Plasma was incubated with 10 or 100 µg ins or bovine serum albumin (BSA), as indicated at the x-axis. In the control sample no protein was added. Two antibody positive mice were used for each group. Data were normalized against the control and represented as mean + upper value. * indicates that the OD % was significantly lower than the control ($p<0.05$).

Cross-reactivity of the antibodies

In order to check whether the antibodies raised against human insulin cross-react with bovine insulin or its separate chains A and B, positive plasma samples were spiked with increasing amounts of human insulin, bovine insulin, bovine insulin's chain A and bovine insulin's chain B (Figure 4).
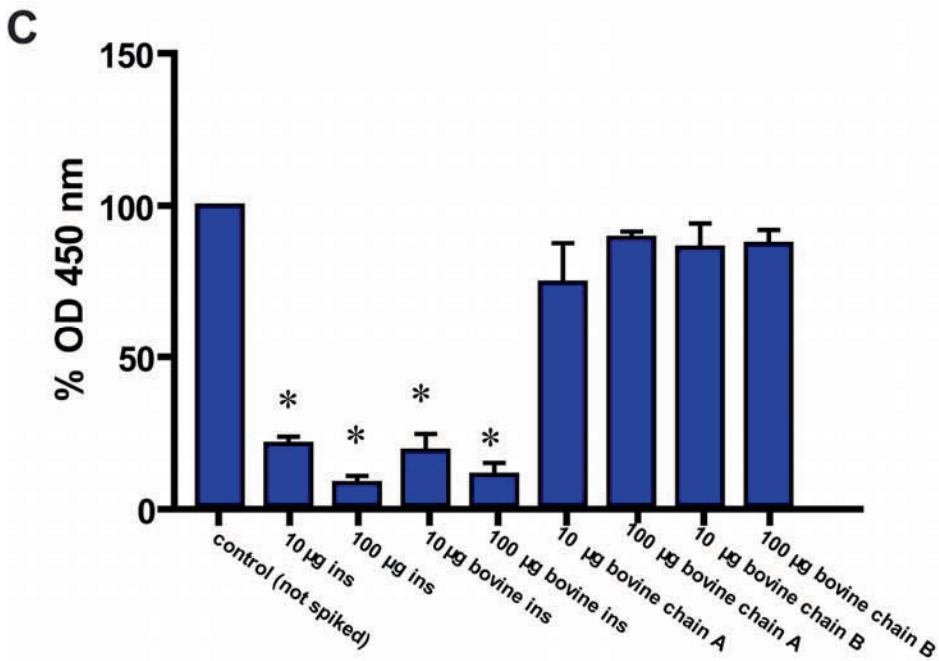
Figure 4. Spiking of plasma of NTG mice treated with nanoparticles-ins (NP-ins) (panel A), TG mice treated with NP-ins (panel B), NTG mice treated with rh insulin (ins) (panel C). Plasma was incubated, separately with ins, bovine insulin, bovine insulin's chain A and bovine insulin's chain B (i.e. 10 and 100 µg). In the control sample no protein was added (not spiked). Two antibody positive mice were used for each group. Data were normalized against the control and represented as mean + upper value. * indicates that the OD % was significantly lower than the control (p<0.05).

While bovine insulin, which shares with human insulin ~ 94% of the primary structure (i.e. 48 over 51 amino acids are identical) (Figure 5) as well as higher order structural features (i.e. secondary and tertiary structure), was recognized by the anti-human insulin antibodies, separated insulin's chains from bovine source were not. These findings suggest that the antibodies cross-react with bovine insulin and recognize conformational epitopes rather than linear epitopes.

A-CHAIN

	1	11	21
Human	GIVEQCCTSI	CSLYQLENYC	N
Bovine	GIVEQCCASV	CSLYQLENYC	N
Mouse/Rat	GIVDQCCTSI	CSLYQLENYC	N
I+II			

B-CHAIN

|	1	11	21	30
Human	FVNQHLCGSH	LVEALYLVCG	ERGFFYTPKT	
Bovine	FVNQHLCGSH	LVEALYLVCG	ERGFFYTPKA	
Mouse/Rat I	FVKQHLCGPH	LVEALYLVCG	ERGFFYTPKS	
Mouse/Rat II	FVKQHLCGSH	LVEALYLVCG	ERGFFYTPMS	

Figure 5. The amino acid sequences of human, bovine, mouse and rat insulin. Differences with human insulin are highlighted in red.

Immunogenicity of insulin formulations

In panel A and B of Figure 6 are shown the total anti insulin IgG titer in NTG and TG animals respectively. As expected, TG mice did not develop antibodies against native insulin, while 50% of the mice treated with NP-ins were antibody positive, in agreement with what was observed during our validation experiment. Similarly, half of the TG animals treated with PB-MCO were antibody positive and had comparable titers to those treated with NP-ins. This is in agreement with earlier results where IFNα-2a (unpublished results from our group), IFNα-2b [23], IFNβ-1a [26] and IgG1 [30], oxidized and aggregated with the oxidative system Cu^{2+}/ascorbate, induced ADAs. Although ABI-MCO formulations lacked aggregates, 33% of the animals were found to be positive.

This suggests that the chemical modifications induced during oxidation and/or the perturbation of the secondary structure contributed to the slightly enhanced immunogenicity of this formulation. Levemir® and Insulatard® were poorly immunogenic in TG animals (1 mouse over 7 was positive for both formulations), while Actrapid® did not elicit any immune response.

Significantly lower titers (p<0.05) were detected in TG animals treated with NP-ins, PB-MCO and Insulatard® compared to NTG (Figure 6, panel B) mice treated with these formulations. In ABI-MCO and Levemir® treated animals, the titers of TG and NTG mice were comparable.
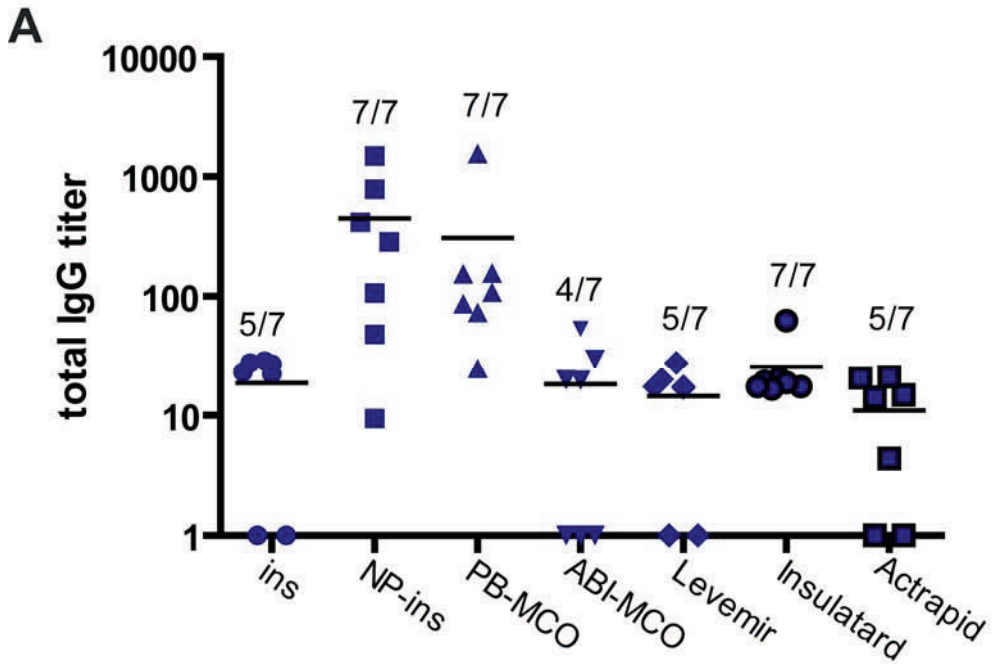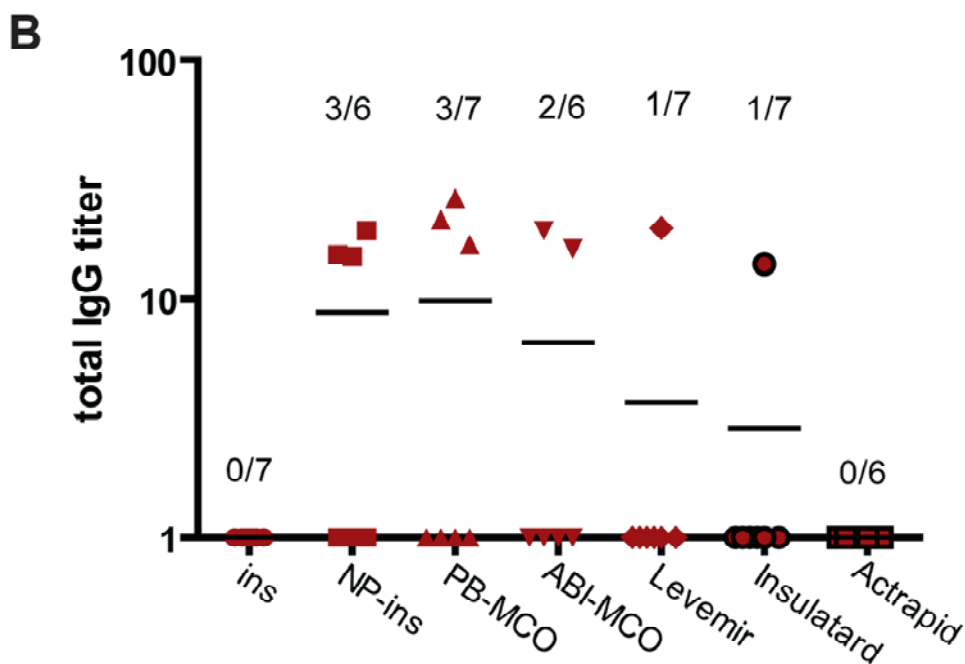
Figure 6. Total anti-insulin IgG titer after 28 days in NTG animals (panel A). Non-responders were assigned an arbitrary titer of 1 and they have been included in the calculation of the titers depicted in this figure. Values above the bars represent the number of positive out of total mice. Samples: rh insulin (ins), nanoparticles-insulin (NP-ins), oxidized and aggregated insulin (PB-MCO), oxidized non aggregated insulin (ABI-MCO), long acting insulin modified on Lys B 29 with fatty acid (Levemir®), long acting insulin obtained as a zinc suspension (Insulatard®), fast acting insulin (Actrapid®). Total anti-insulin IgG titer after 28 days in TG animals (panel B). Animal treatment and graph layout are the same of panel A. Please note that since TG mice treated with ins and Actrapid® had no detectable antibody and no responders (variance of the group=0), statistical testing involving this two groups was not allowed. Titers among groups with detectable antibody and responders were comparable. Three animals died during the studies for unknown reasons.

Conclusion

Here we reported the validation and application of a TG mouse model, immune tolerant for human insulin, for the study of product-related factors influencing the immunogenicity of insulin. In these studies it was confirmed that the combination of covalent aggregation and chemical modifications, induced via the oxidative system Cu^{2+}/ascorbate, likely contributes to the immunogenicity of therapeutic proteins. This mouse model represents a

promising tool to study in more detail the immune mechanisms of antibody formation against therapeutic proteins, using insulin as a model protein.

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Chapter 9

Summary and perspectives

Summary

Recombinant human protein therapeutics have unique structural and biological features which make them useful to treat several diseases [1].

However, a drawback of this class of drugs is that they are immunogenic, i.e. a proportion of patients develop anti-drug antibodies (ADAs) during treatment [2]. Among the many factors involved in protein immunogenicity, protein aggregation has been identified as an important risk factor [3]. Aggregates that are generated through oxidative stress have been found to be particularly immunogenic [4-7]. The aim of this thesis was to investigate the mechanisms behind protein aggregation as a result of metal catalyzed oxidation (MCO) [8], and to study the relationship between oxidation, aggregation and immunogenicity of therapeutic proteins.

Chapter 2 is a literature review of the structural and pharmaceutical consequences of oxidation observed in protein and peptide therapeutics, focusing on antibodies, calcitonin, granulocyte colony-stimulating factor, growth hormone, interferon alpha and beta, insulin, oxytocin and parathyroid hormone.

Chapter 3 deals with the aggregation behavior under stress conditions of PEGylated insulin compared with its unpegylated counterpart. Recombinant human insulin was conjugated on lysine B29 with 5-kDa PEG and subjected to heating at 75 °C, metal-catalyzed oxidation, and glutaraldehyde cross-linking. Under each of the applied stress conditions, insulin and PEGylated insulin showed comparable degradation profiles. All the stressed samples were shown to contain submicron aggregates in the size range between 50 and 500 nm. Several stressed samples also contained micron sized particles. Mainly covalent aggregates composed of protein molecules with different degrees of modification in the secondary and tertiary structure were measured, depending on the stress method applied. PEGylation, however, was shown not to affect the sensitivity of insulin towards aggregation [9].

Therapeutic proteins such as recombinant human interferon alfa, recombinant human interferon beta and monoclonal antibodies form highly immunogenic

aggregates induced via copper catalyzed oxidation. In **Chapter 4** the chemical mechanism responsible for insulin aggregation induced by MCO with Cu^{2+}/ascorbate, is described. Oxidized insulin was shown to contain DOPA (3,4-dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid), originated from Phe and Tyr residues. DOCH, being an electron acceptor for 1,4- or 1,6-type addition (i.e. Michael addition), was found responsible for new cross-links resulting in covalent aggregation of insulin during MCO. Specifically, 1,4- or 1,6-type addition products were detected between DOCH at positions B16, B26, A14, and A19, and free amino groups of the N-terminal amino acids Phe B1 and Gly A1, and side chains of Lys B29, His B5 and His B10. Fragments originating from peptide bond hydrolysis were also measured [10].

Chapter 5 describes studies on the anti-oxidant properties of several excipients during Cu^{2+}/ascorbate catalyzed oxidation of human insulin. Among the excipients studied, 100 µM triethylenetetramine (TETA) was the only one to inhibit almost completely oxidation-induced insulin aggregation, fragmentation and structural changes. TETA also prevented aggregation and fragmentation of a monoclonal IgG1 under identical oxidative conditions, indicating its general applicability as anti-oxidant for copper-sensitive proteins. In conclusion, TETA is a promising candidate excipient for formulations of oxidation-sensitive proteins.

Oxidation via Cu^{2+}/ascorbate of recombinant human interferon beta-1a (IFNβ) leads to highly immunogenic aggregates. In **Chapter 6** a study is presented on the identification of the oxidation sites and covalent cross-links in IFNβ exposed to MCO. Oxidation products of Met, His, Phe, Trp and Tyr residues were identified throughout the primary sequence. Similar to insulin (**Chapter 4**), covalent cross-links via 1,4- or 1,6-type addition between primary amines and DOCH were detected, while there was no evidence of disulfide bridge, Schiff base, or dityrosine formation. The chemical cross-links identified are most likely responsible for the formation of immunogenic covalent aggregates of IFNβ induced by oxidation.

In **Chapter 7** the immune mechanism responsible for the immunogenicity of aggregated recombinant human interferon alpha-2a (IFNα-2a) and the influence of aggregated IFNβ on IFNα's immunogenicity is studied. Transgenic mice immune tolerant for IFNα were treated with native or aggregated IFNα. After a washout period, the mice were rechallenged with aggregated or native IFNα to test for CD4+ T-cell involvement in immunogenicity. Furthermore, the mice were treated with a formulation containing aggregated IFNβ and native IFNα to test whether aggregated IFNβ acts as an adjuvant for IFNα. After a washout period and a rechallenge with either aggregated or native IFNα an apparent lack of immunological memory was observed. When we blocked CD4+ T-cell function in immune tolerant mice, aggregated rhIFNα failed to induce ADAs. Finally, immune tolerant mice treated with a mixture of aggregated IFNβ and native IFNα did not develop an antibody response against IFNα. These results are in line with previous results obtained with IFNβ [11].

Chapter 8 reports the validation of an adjuvant-free mouse model, which is intended as a tool to evaluate the immunogenicity of insulin formulations. Administration of human insulin (20 μg/dose, 12 doses over a period of 4 weeks) resulted in an immune response in non-transgenic animals, whereas the transgenic mice proved to be immune tolerant. This tolerance in transgenic mice could be circumvented when they were treated with insulin that was covalently bound to 50 nm polystyrene nanoparticles. This transgenic mouse model for human insulin was applied for comparing the immunogenicity of insulin formulations and may be of use to further investigate the mechanism behind the immunogenicity of therapeutic proteins, using insulin as a model protein.

Perspectives

The scientific work presented in this thesis aimed to contribute to the development of safer, less immunogenic biopharmaceuticals, by describing the mechanisms behind MCO-induced aggregation of therapeutic proteins and its relationship with immunogenicity. Further research is however needed to better prevent degradation of therapeutic proteins and better understand the factors contributing to their immunogenicity. This section provides some

perspectives for future research, focusing on: strategies to prevent protein degradation and analytical tools for protein characterization.

Strategies to prevent protein degradation

During the last two decades, industry became increasingly aware of the risk of degraded products, particularly aggregates, in protein formulations, justifying the search for solutions to prevent or reduce aggregation [3].

In general, two approaches can be employed to prevent protein degradation. The first is to improve protein stability by modifying the protein molecule, either by genetic engineering [12] or by chemical modification [13] (i.e. bio-conjugation like PEGylation). The second option is to improve stability by proper formulation [14]. An example of the first approach is the production of a human interleukin-1 where Asn36 is mutated into Ser36 [15]. The three-dimensional structure of this genetically engineered protein was shown to be identical to that of the native protein with dichroic studies and proton NMR. The mutant was resistant against base-catalyzed and temperature-induced deamidation [15].

Chemical modification, like conjugation of proteins with polymers (i.e. PEG), in some cases can be a solution as demonstrated for instance by the higher stability of PEGylated IFN proteins relative to their unmodified counterparts [16]. PEGylation has been used to increase the half life, resistance to enzymatic degradation and to prevent formation of fibrillar aggregates [17-19]. However, as shown in chapter 3, PEGylation did not prevent degradation of insulin in a liquid formulation. In our studies however we used a 5 kDa PEG, therefore we cannot exclude that other forms of PEGylation may improve the stability.

Excipients are widely employed to protect proteins from aggregation and oxidation [14]. In chapter 5, several anti-oxidant molecules were screened for their potential protective effect towards MCO-induced aggregation and fragmentation of insulin and a monoclonal antibody. Among the molecules screened triethylenetetramine [20] (TETA) turned out to be the most promising one in preventing MCO. However, it is currently unknown if TETA can prevent oxidation of amino acid residues not involved in aggregation, such as methionine and tryptophan, which are not present in insulin. Future studies, with the aid of tandem mass spectrometry, should verify the protective effect of TETA against oxidation of such amino acid residues.

Surfactant excipients, like polysorbate 80, are widely employed in protein formulation and useful for preventing aggregation [21]. One of the major drawbacks of polysorbates is that they contain alkyl chains that could auto-oxidize, generating protein-damaging peroxides. Given that peroxide formation is enhanced in aerobic environment and further promoted by light [21], a first strategy to reduce the amount of these reactive molecules in polysorbates would be preventing any contact with air/oxygen during storage.

Next, the addition of anti-oxidant excipients can be beneficial for inhibiting or minimizing generation of radicals. In this case chelating agents like TETA should not be used since these anti-oxidants are not able to scavenge radicals. Instead, molecules like methionine, vitamin E or glutathione could be considered, as shown by Ha et al [22]. Concluding, to prevent degradation a thorough understanding of the mechanisms involved is required. In chapter 4, for instance, by using high sensitive tandem mass spectrometry analysis and selective fluorogenic tagging [23-24], we showed that insulin aggregation under our oxidative system was due to tyrosine oxidation products that can react with amino groups in the insulin molecule. Based on these findings we further proposed two strategies to inhibit or reduce aggregation (chapter 4).

Similarly, mechanisms involved in several other pathways of degradation should be analyzed with the final goal of preventing any type of protein degradation.

Analytical tools for protein characterization

When a protein degrades, generally a heterogeneous mixture of degradation products with different physico-chemical features are formed, such as aggregates with different size, shape, protein conformation and chemical modifications, as well as structurally modified monomers and fragments [9]. Potentially any of these species can contribute to unwanted side reactions.

The availability of robust and reliable analytical tools capable of characterizing all of these species is required in research as well as for quality control (QC) purposes. For aggregate characterization, most of the routine analytical techniques approved for QC analysis, focus on the size range >1 μm and <100 nm, which obviously leave a big gap in the subvisible size range [25]. Techniques like nanoparticle tracking analysis [26] (NTA, used in several of the studies presented in this thesis) and flow imaging, allow visualization and

quantification of particulate aggregates and provide size information. These methods are increasingly used a characterization tools to fill the size gap previously mentioned [27]. Furthermore, an accurate mapping of chemical modifications of protein drugs can be achieved with high sensitive MS/MS analysis. Nonetheless, some chemical modifications like Trp oxidation can be artifact generated during sample preparation [28]. To prevent misinterpretation of the results, selective chemical derivatization (like the fluorogenic derivatization shown in chapter 4 that can be performed on oxidized Trp as well) before MS analysis, can be considered.

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Appendix 1

Nederlandse samenvatting (Dutch summary)

Samenvatting

Recombinante humane therapeutische eiwitten hebben unieke structurele en biologische eigenschappen waardoor ze bruikbaar zijn voor de behandeling van verschillende aandoeningen. Een nadeel van deze klasse geneesmiddelen is dat ze immunogeen zijn, wat wil zeggen dat sommige patiënten anti-geneesmiddel antilichamen (ADAs) tijdens de behandeling ontwikkelen. Van de vele factoren die betrokken zijn bij eiwit-immunogeniciteit is aggregatie van eiwitten een belangrijke risicofactor.

Aggregaten die door oxidatieve stress worden gevormd zijn bijzonder immunogeen gebleken. Het doel van dit proefschrift was om de mechanismen achter eiwitaggregatie ten gevolge van gekatalyseerde oxidatie te onderzoeken (MCO), en daarnaast om de relatie tussen oxidatie, aggregatie en immunogeniciteit van therapeutische eiwitten te bestuderen.

Hoofdstuk 2 beschrijft een literatuuronderzoek van de structurele en biologische gevolgen van oxidatie waargenomen in therapeutische eiwitten en peptiden, met name gericht op antilichamen, calcitonine, granulocyt kolonie-stimulerende factor, groeihormoon, interferon alfa en bèta, insuline, oxytocine en bijschildklierhormoon.

Hoofdstuk 3 vergelijkt het aggregatiegedrag van gepegyleerde en niet-gepegyleerde insuline onder stresscondities. Recombinant humaan insuline werd aan lysine B29 geconjugeerd met 5-kDa PEG en onderworpen aan verhitting bij 75 °C, metaal gekatalyseerde oxidatie en glutaaraldehyde cross-linking. Onder elke stressconditie toonden insuline en gepegyleerd insuline vergelijkbare degradatieprofielen. Alle gestreste monsters bleken aggregaten te bevatten in de ordegrootte tussen 50 en 500 nm. Ook microndeeltjes werden in diverse gestreste monsters gevonden. Afhankelijk van de toegepaste stress werden voornamelijk covalente aggregaten samengesteld uit eiwitmoleculen met verschillende mate van modificatie in de secundaire en tertiaire structuur gemeten. Pegylatie bleek echter de gevoeligheid van insuline voor aggregatie niet te beïnvloeden.

Therapeutische eiwitten zoals recombinant humaan interferon alfa, recombinant humaan interferon bèta en monoklonale antilichamen vormen

zeer immunogene aggregaten geïnduceerd onder invloed van koper gekatalyseerde oxidatie. In **Hoofdstuk 4** wordt het chemische mechanisme beschreven van insulineaggregatie door MCO, geïnduceerd door middel van Cu^{2+}/ascorbinezuur. Geoxideerde insuline bleek DOPA (3,4-dihydroxyfenylalanine) en DOCH (of propionzuur) te bevatten, afkomstig van Phe en Tyr residuen. DOCH, dat een elektronenacceptor is voor 1,4 - of 1,6-type toevoeging (d.w.z. Michael-additie), bleek verantwoordelijk te zijn voor nieuwe cross-links die resulteerden in covalente aggregatie van insuline tijdens MCO. Specifiek werden 1,4 - of 1,6-type additie producten gedetecteerd tussen DOCH op posities B16, B26, A14 en A19, en de vrije aminogroepen van de N-terminale aminozuren Phe B1 en Gly A1 en de zijketens van Lys B29, His B5 en His B10. Fragmenten afkomstig van gehydrolyseerde peptidebindingen werden ook gemeten.

Hoofdstuk 5 beschrijft het onderzoek naar de anti-oxiderende eigenschappen van verschillende hulpstoffen tijdens Cu^{2+}/ascorbaat gekatalyseerde oxidatie van humaan insuline. Van de onderzochte hulpstoffen bleek 100 μM triethyleentetramine (TETA) de enige stof die vrijwel volledig door oxidatie-geïnduceerde insulineaggregatie, -fragmentatie en structurele veranderingen van insuline verminderde. TETA voorkwam ook aggregatie en fragmentatie van een monoklonaal IgG1 onder identieke oxidatieve omstandigheden, wat de algemene toepasbaarheid van TETA aangeeft als anti-oxidant voor koper-gevoelige eiwitten. Concluderend is TETA een veelbelovende kandidaat als hulpstof in formuleringen van oxidatie-gevoelige eiwitten.

Oxidatie via Cu^{2+}/ascorbaat van recombinant humaan interferon bèta-1a (IFNβ) leidt tot zeer immunogene aggregaten. In **Hoofdstuk 6** wordt een studie gepresenteerd naar de identificatie van de oxidatieplaatsen en covalente cross-links in IFNβ, blootgesteld aan MCO. Oxidatieproducten van Met, His, Phe, Trp en Tyr restgroepen werden geïdentificeerd in de primaire structuur van het eiwit. Vergelijkbaar met insuline (**Hoofdstuk 4**) werden covalente crosslinks via 1,4 - of 1,6-type addities tussen primaire amines en DOCH gevonden, terwijl er geen bewijs was voor de vorming van een disulfide brug, Schiff base, of dityrosine. De geïdentificeerde chemische cross-links,

veroorzaakt door oxidatie, zijn hoogstwaarschijnlijk verantwoordelijk voor de vorming van immunogene covalente aggregaten van IFNβ.

In **Hoofdstuk 7** is het immunologische mechanisme bestudeed dat verantwoordelijk is voor de immunogeniciteit van geaggregeerd recombinant humaan interferon alfa-2a (INFα-2a) en de invloed van geaggregeerd INFβ op IFNα's immunogeniciteit. Transgene muizen die immunotolerant zijn voor IFNα werden behandeld met natief (in oorspronkelijke staat verkerend) of geaggregeerd IFNα. Na een uitwasperiode werden de muizen opnieuw blootgesteld aan natief of geaggregeerd IFNα om de betrokkenheid van CD4+ T-cellen in de immunogeniciteit te testen. Voorts werden de muizen behandeld met een formulering die geaggregeerd IFNβ en oorspronkelijk IFNα bevatte om te testen of INFβ werkt als een adjuvans voor IFNα. Na een uitwasperiode en een hernieuwde blootstelling met geaggregeerd of natief IFNα werd een schijnbaar gebrek aan immunologisch geheugen waargenomen. Wanneer we de CD4+ T-cell functie blokkeerden in immunotolerante muizen werden geen ADAs geïnduceerd door geaggregeerd rhIFNα. Tenslotte ontwikkelden immunotolerante muizen, behandeld met een mengsel van geaggregeerd IFNβ en natief IFNα, geen antilichaamreactie tegen IFNα. Deze resultaten komen overeen met eerdere resultaten verkregen met IFNβ.

Hoofdstuk 8 rapporteert de validatie van een adjuvans-vrij muismodel, dat is bedoeld als hulpmiddel om de immunogeniciteit van insulineformuleringen te evalueren. Toediening van humaan insuline (20 µg/dosis, 12 doseringen over een periode van 4 weken) resulteerde in een immuunrespons in niet-transgene dieren, daar waar de transgene muizen immuuntolerant bleken te zijn. Deze tolerantie in transgene muizen kon worden omzeild indien ze werden behandeld met insuline dat covalent is gebonden aan polystyrene nanodeeltjes met een diameter van 50 nm. Dit transgene muismodel voor humane insuline werd toegepast om de immunogeniciteit van insulineformuleringen te vergelijken en kan van nut zijn voor verder onderzoek naar het mechanisme achter de immunogeniciteit van therapeutische eiwitten, met insuline als modeleiwit.

Perspectieven

Het wetenschappelijk werk dat in dit proefschrift is gepresenteerd heeft als doel een bijdrage te leveren aan de ontwikkeling van veiligere, minder immunogene biofarmaca, door het beschrijven van de mechanismen achter MCO-geïnduceerde aggregatie van therapeutische eiwitten en de relatie met immunogeniciteit. Verder onderzoek is echter nodig om de degradatie van therapeutische eiwitten beter te verhinderen en een beter inzicht te krijgen in de factoren die bijdragen aan hun immunogeniciteit.

Dit gedeelte biedt een aantal perspectieven voor toekomstig onderzoek, gericht op strategieën om eiwitafbraak te verhinderen en analytische methoden ontwikkelen voor eiwitkarakterisering.

Strategieën om eiwitaggregatie te verhinderen

In de laatste twee decennia is de farmaceutische industrie zich meer bewust geworden van de risico's van afbraakproducten in eiwitformuleringen, voornamelijk aggregaten, wat de zoektocht naar oplossingen voor het voorkomen en verminderen van aggregatie rechtvaardigt. Doorgaans kunnen twee strategieën ingezet worden om eiwitdegradatie te voorkomen. De eerste strategie richt zich op het verbeteren van de eiwitstabiliteit door het eiwitmolecuul te modificeren, door genetische of chemische modificatie (bijvoorbeeld door bio-conjungatie zoals pegylatie). De tweede optie is het formuleren van een eiwitmolecuul. Een voorbeeld van de eerste strategie is de productie van een humaan interleukin-1 eiwit waar Asn36 gemuteerd is tot Ser36. De driedimensionale structuur van dit genetisch gemanipuleerde eiwit bleek identiek te zijn aan die van het oorspronkelijke eiwit, zoals aangetoond met circulair dichroïsme en proton NMR. De eiwitmutant was resistent tegen base-gekatalyseerde en hitte-geïnduceerde deamidatie.

Chemische modificatie, zoals conjugatie van eiwitten met polymeren (bijvoorbeeld PEG), kan in sommige gevallen een oplossing zijn, zoals dat bijvoorbeeld is aangetoond door de relatief hogere stabiliteit van gepegyleerde IFN eiwitten ten opzichte van hun ongemodificeerde varianten. Pegylatie is ingezet om de halfwaardetijd te verhogen om resistentie tegen enzymatische degradatie te vergroten en om de vorming van fibrillaire aggregaten tegen te gaan. Maar zoals is aangetoond in hoofdstuk 3 werd de

degradatie van insuline in een vloeibare formulering niet verhinderd door pegylatie. In onze studies hebben wij echter een 5-kDa PEG gebruikt, waardoor wij niet kunnen uitsluiten dat andere vormen van pegylatie de stabiliteit ten goede kunnen komen. Hulpstoffen worden veelal ingezet om eiwitten te beschermen tegen aggregatie en oxidatie. In hoofdstuk 5 zijn verschillende anti-oxiderende moleculen getest op hun potentieel beschermende effect tegen MCO-geïnduceerde aggregatie en fragmentatie van insuline en een monoclonaal antilichaam. Eén van de geteste moleculen, triethyleentetra-amine (TETA), bleek het meest veelbelovend te zijn om MCO te verhinderen. Het is op dit moment echter niet bekend of TETA oxidatie kan verhinderen van aminozuurresiduen die niet betrokken zijn bij aggregatie, zoals methionine en tryptofaan, welke niet aanwezig zijn in insuline.

Toekomstige studies zouden het beschermende effect van TETA moeten verifiëren tegen oxidatie van zulke aminozuurresiduen met hulp van tandem-massaspectrometrie. Oppervlakte-actieve hulpstoffen zoals polysorbaat 80 worden veel ingezet in eiwitformuleringen en zijn tevens nuttig om eiwit aggregatie te verhinderen. Eén van de grote nadelen van polysorbaten is dat zij alkyl ketens bezitten die kunnen auto-oxideren, waardoor eiwit beschadigende peroxides ontstaan. Aangezien peroxidevorming sterker is in een aërobe omgeving en verder versterkt wordt door licht, zou een eerste strategie om de hoeveelheid van deze reactieve moleculen in polysorbaten terug te dringen zijn om elk contact met lucht/zuurstof tijdens opslag te vermijden. Vervolgens kan de toevoeging van anti-oxiderende hulpstoffen behulpzaam zijn om de vorming van radicalen te verminderen of te minimaliseren. In dit geval zouden chelatoren zoals TETA niet gebruikt moeten worden, omdat deze anti-oxidantia niet in staat zijn om radicalen weg te vangen. Daarentegen kunnen moleculen zoals methionine, vitamine E of glutathion overwogen worden.

Concluderend, om degradatie te verhinderen is een sterk inzicht nodig in de achterliggende mechanismen. In hoofdstuk 4 bijvoorbeeld, door het gebruik van hoog gevoelige tandem-massaspectrometrische analyse en selectieve fluorogene labels, toonden wij aan dat insulineaggregatie in ons oxidatieve systeem plaatsvond door oxidatieproducten van tyrosine die kunnen reageren met aminogroepen in het insulinemolecuul. Gebaseerd op deze bevindingen stelden wij twee strategieën voor om aggregatie te verhinderen of te

reduceren (hoofstuk 4). De mechanismen die betrokken zijn bij verscheidene andere degradatieprocessen zouden op gelijke manier geanalyseerd moeten worden om elke andere manier van eiwitdegradatie tegen te gaan.

Analytische hulpmiddelen voor eiwitkarakterisatie

Wanneer een eiwit degradeert, vormt zich over het algemeen een heterogene mix van degradatieproducten met verschillende fysisch-chemische eigenschappen, zoals aggregaten met verschillen in grootte, vorm, eiwitconformatie en chemische modificaties, alsmede qua structuur gemodificeerde monomeren en fragmenten. Elk van deze soorten kan mogelijk bijdragen aan ongewilde nevenreacties.

Voor een adequate karakterisatie van elk van deze soorten degradatieproducten moet men de beschikking hebben over betrouwbare, robuuste analysemethoden. De meeste van de routineuze analytische technieken voor de karakterisatie van aggregaten bij kwaliteitscontrole, richten zich op een deeltjesgrootte van >1 µm en <100 nm, welke duidelijk een groot gat laten in het subvisuele deeltjesgroottebereik. Technieken zoals nanoparticle tracking analyse (NTA, welke gebruikt is in verscheidene studies gepresenteerd in dit proefschrift) en flow imaging microscopie maken het mogelijk om deeltjesaggregatie te visualiseren en kwantificeren, en geven informatie over de deeltjesgrootte. Deze methoden worden in toenemende mate gebruikt als karakterisatiehulpmiddelen om het eerder genoemde gat in het deeltjesgroottebereik te dichten.

Daarnaast kan een nauwkeurig beeld worden gevormd van chemische modificaties van eiwitgeneesmiddelen met hoog gevoelige MS/MS-analyse. Sommige gedetecteerde chemische modificaties zoals Trp-oxidatie kunnen echter artefacten zijn die onstaan tijdens de monstervoorbereiding. Selectieve chemische derivatisering voorafgaand aan de MS-analyse kan overwogen worden om misinterpretatie van de resultaten te voorkomen.Bijvoorbeeld, een vergelijkbare fluorogene derivatisatie zoals in hoofdstuk 4 wordt getoond kan op geoxideerde Trp-residuen toegepast worden.

Appendix 2

Abbreviations

Abbreviations

ABI	ammonium bicarbonate
ABS	4-(aminomethyl) benzenesulfonic acid
ADAs	anti-drug antibodies
Ag	antigen
ANOVA	analysis of variance
Asn	asparagine
AUC	area under the curve
BABs	binding antibodies
BAM	benzylamine
CB	sodium citrate buffer
CD	circular dichroism
CDRs	complementarity determining regions
CFA	complete Freund's adjuvant
CHO	Chinese hamster ovary
CSB	sodium citrate sucrose buffer
CT	calcitonin
Cu^{2+}	bivalent copper ion
Cys	cysteine
DHB	dihydroxybenzoic acid
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DO	dissolved oxygen
DOCH	2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid
DOPA	3,4-dihydroxyphenylalanine
DTT	DL-dithiothreitol
E. coli	Escherichia coli
EDAC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization mass spectrometry
ESI-ToF	electrospray ionization time of flight
Fab	fragment antigen-binding
Fc	fragment crystallisable
FcRn	neonatal Fc receptor
FDA	Food and Drug Administration

FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
G-CSF	granulocyte colony stimulating factor
GH	growth hormone
GM-CSF	granulocyte macrophage colony stimulating factor
GnHCl	guanidine hydrochloride
GPC	gel permeation chromatography
GSH	reduced glutathione
His	histidine
HMWO	high-molecular-weight oligomers
HSA	human serum albumin
IAM	iodoacetamide
IFNα-2a	interferon alpha-2a
IFNα-2b	interferon alpha-2b
IFNβ-1a	interferon beta-1a
IgG	immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LOPC	light obscuration particle counting
Lys	lysine
mAbs	monoclonal antibodies
MCO	metal catalyzed oxidation
MeO-PEG-NHS	Alpha-metoxy omega-carboxylic acid succinimidyl ester poly(ethylene glycol)
MES	2-N-morpholino-ethanesulfonic acid
Met	methionine
Nab	neutralizing antibody
NHS-sulfo	N-hydroxysulfosuccinimide sodium salt
NP	polystyrene nanoparticles
NTA	nanoparticle tracking analysis
NTG	non transgenic
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PB	sodium phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	polydispersity index
PEG	polyethylene glicol

Phe	phenylalanine
PTH	parathyroid hormone
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
RP-HPLC	reversed-phase high pressure liquid chromatography
rtPCR	real time PCR
SA	sinapinic acid
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
SEM	standard error of the mean
Td	T-cell dependent
TETA	triethylenetetramine
TFA	trifluoroacetic acid
TG	transgenic
Ti	T-cell independent
TMB	3,3′,5,5′-tetramethylbenzidine
Trp	tryptophan
Tyr	tyrosine
USP	United States Pharmacopeia
UV	ultraviolet
v/v	volume/volume
w/w	weight/weight
Z-ave	Z-average diameter

Appendix 3

List of publications

List of publications

2013

R. Torosantucci, V. S. Sharov, M. van Beers, V. Brinks, C. Schöneich, W. Jiskoot. Identification of oxidation sites and covalent cross-links in metal catalyzed oxidized interferon beta-1a: potential implications for protein aggregation and immunogenicity. *Molecular Pharmaceutics* **2013**, *10*, 2311-2322.

R. Torosantucci, D. Weinbuch, R. Klem, W. Jiskoot. Triethylenetetramine prevents insulin aggregation and fragmentation during copper catalyzed oxidation. *European Journal of Pharmaceutics and Biopharmaceutics* **2013**, *84,* 464-471.

R. Torosantucci, V. Brinks, G. Kijanka, L. Andhyk Halim, M. Sauerborn, H. Schellekens and W. Jiskoot. Development of a transgenic mouse model to study the immunogenicity of recombinant human insulin. *Submitted for publication.*

R. Torosantucci, C. Schöneich, W. Jiskoot. Oxidation of therapeutic proteins and peptides: structural and biological consequences. *Submitted for publication.*

M. Sauerborn[#], **R. Torosantucci**[#], L. Boon, W. Jiskoot, H. Schellekens, V. Brinks. Immune mechanisms underlying immunogenicity of aggregated recombinant human interferon alpha 2a in immune tolerant mice. *Submitted for publication.*

# authors contributed equally

2012

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

2011

R. Torosantucci, B. Kükrer, A. Mero, M. Van Winsen, R. Tantipolphan, W. Jiskoot. Plain and mono-pegylated recombinant human insulin exhibit similar stress-induced aggregation profiles. *Journal of Pharmaceutical Sciences* **2011**, *100*, 2574-2585.

2010

R. Tantipolphan, S. Romeijn, J. Engelsman, **R. Torosantucci**, T. Rasmussen, W. Jiskoot. Elution behavior of insulin on high-performance size exclusion chromatography at neutral pH. *Journal of Pharmaceutics and Biomedical Analysis*, **2010**, *52*, 195-202.

2009

F. De Simone, J. Andres, **R. Torosantucci**, J. Waser. Catalytic formal homo-Nazarov cyclization. *Organic Letters* **2009**, *11*, 1023-1026.

2008

M. Faccini, M. Balakrishnan, M. B. J. Diemeer, **R. Torosantucci**, A. Driessen, D. N. Reinhoudt, W. Verboom. Photostable nonlinear optical polycarbonates. *Journal of Materials Chemistry* **2008**, *18*, 5293-5300.

M. Faccini, M. Balakrishnan, **R. Torosantucci**, A. Driessen, D. N. Reinhoudt, W. Verboom. Facile attachment of nonlinear optical chromophores to polycarbonates. *Macromolecules* **2008**, *41*, 8320-8323.

Appendix 4

Curriculum Vitae

Curriculum Vitae

Riccardo Torosantucci was born in Rome, Italy, 22nd September 1980. In July 2008 he received his master degree in Chemistry and Pharmaceutical Technologies from the University of Rome "La Sapienza". He completed the first internship of his master studies working for six months in supramolecular chemistry, in particular on the synthesis of calixarenes at the Department of Biologically Active Substances of University of Rome "La Sapienza" under the supervision of Prof. Dr. Bruno Botta. Next, in July 2007, Riccardo was awarded the "Master's thesis abroad travel grant" and he joined for six months the group of Supramolecular Chemistry and Technology (SMCT) directed by Prof. Dr. David Nicholas Reinhoudt at Twente University, The Netherlands. During this period he worked on the synthesis of new polymers for second-order nonlinear optics. After his master thesis defence in July 2008, from October until December 2008, Riccardo worked at the Laboratory of Catalysis and Organic Synthesis (LCSO) of the École Polytechnique Fédérale de Lausanne (EPFL), Switzerland, under the supervision of Prof. Dr. Jérôme Waser, focusing mainly on the synthesis of heterocyclic compounds. In March 2009 Riccardo started his PhD at the Division of Drug Delivery Technology at Leiden University, The Netherlands, under the supervision of Prof. Dr. Wim Jiskoot, Prof. Dr. Huub Schellekens and Dr. Vera Brinks, on a project entitled "Unwanted immunogenicity of therapeutic proteins". In May 2011, as a part of his PhD project, he spent 5 months at the Department of Pharmaceutical Chemistry of Kansas University, Lawrence, USA, in the group directed by Prof. Dr. Christian Schöneich, where he worked on mass spectrometric characterization of therapeutic protein aggregates induced via metal catalyzed oxidation. Since May 2013 Riccardo is working as a project manager at Coriolis-Pharma in Munich, Germany.

Grants & Awards

2012

- First prize for oral presentation at the ninth biennial meeting of the Globalization of Pharmaceutics Education Network, Monash University, Melbourne, Australia.

- GlaxoSmithKline Housing Grant

- Merck & Co. Travel Grant

- Nederlandse Stichting voor Farmacologische Wetenschappen (NSFW) travel grant *2012*

2011

- CASSS Separation Science Society travel grant

- Leids Universiteits Fonds travel grant (Leiden University, NL)

- Nederlandse Stichting voor Farmacologische Wetenschappen (NSFW) travel grant *2011*

2007

- "Master's thesis abroad travel grant", University of Rome "La Sapienza"

2006

- Scholarship at the Department of Pharmaceutical Chemistry, University of Rome "La Sapienza"

Appendix 5

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Thanks to Dr. John den Engelsman for providing me with the insulin used in these studies and for inviting me to present our results to his scientific group at Organon NV/Merck.