

Metal Ion Leachates and the Physico-Chemical Stability of Biotherapeutic Drug Products

Sandeep Kumar¹, Shuxia Zhou^{1, #} and Satish K. Singh^{1, *}

¹Pfizer Inc., Biotherapeutics Pharmaceutical Sciences, 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA

Current address: Johnson & Johnson, Inc., 145 King of Prussia Road, Radnor, PA 19087, USA

Abstract: Metal ions that leach into biotherapeutic drug product solution during manufacturing and storage, result in contamination that can cause physico-chemical degradation of the active molecule. In this review, we describe various mechanisms by which metal ion leachates can interact with therapeutic proteins and antibodies. Site-specific modifications due to metal catalyzed oxidation (MCO) of the therapeutic proteins cause them to become destabilized and potentially increasingly aggregation prone. We have examined the molecular sequences and structures for three case studies, human relaxin (hRLx), human growth hormone (hGH) and an IgG2 mAb to rationalize the experimental findings related to their MCO. The analysis indicates that metal-binding sites lie in close spatial proximities to predicted aggregation prone regions in these molecules. From the perspective of pharmaceutical development of biotherapeutic drugs, this link between molecular origins of MCO and subsequent aggregation is undesirable. This article further suggests molecular design strategies involving disruption of APRs that may also help mitigate the impact of metal ion leachates on biotherapeutic drug products as well as improving their solubility.

Keywords: Metal ion, aggregation, oxidation, protein, biotherapeutics.

INTRODUCTION

Metal leachables are one of many classes of potential contaminants that may be introduced into a biopharmaceutical product as a result of direct and/or indirect contact with materials used in manufacturing, packaging, storage, and delivery of the therapeutic drugs [1]. Appropriate steps have to be taken to minimize such contamination since they have the potential to alter the physico-chemical properties and reduce stability of the product, and in worst-case scenarios, also impact safety and efficacy of the drug. The US requirements are governed by 21 CFR parts 210 and 211. Current Good Manufacturing Practice in Manufacturing, Processing, Packing or Holding of Drugs: General and Current Good Manufacturing Practice for Finished Pharmaceuticals, Subpart D-211.65, which states "Equipment shall be constructed so that surfaces that contact components, in-process materials, drug products shall not be reactive, additive, or adsorptive so as to alter the safety, identity, strength, quality, or purity of the drug product beyond the official or other established requirements.

Contact materials for biopharmaceuticals fall into three major categories: glass, plastics/elastomers, and stainless steel [1]. The major organic leachables arise from the plastics/elastomers, while the main inorganic leachables are metal ions and trace elements [1, 2] such as iron, copper, zinc, nickel, manganese, aluminum, magnesium, calcium, etc. [1]. The primary sources of these leachables are glass containers and manufacturing equipment. Metal complexation agents such as buffer species (citrate, lactate), antioxidant (ascorbic acid) and chelating agents (EDTA, DTPA) can accelerate the leaching process of ions from metal contact surfaces during the storage or manufacture of biotherapeutics [3].

Contamination of biotherapeutic products with metal ions can have a significant impact on the stability and viability of these products. Trace levels of metal ions can result in oxidation [4-7], aggregation [6, 7] or the formation of insoluble particulates [8] in protein products. Site-specific metal binding can also induce secondary and tertiary structure changes resulting in the formation of

protein aggregates [9, 10]. Trace amounts of transition metal ions have also been found to induce protein fragmentation [4].

Markovic [11] has reported on examples from regulatory experience where the quality of protein drug products was negatively impacted by metal leachables. N-terminal degradation was induced by divalent metal cations from rubber stoppers in a liquid formulation of a therapeutic protein. Barium was found to leach from glass vials and react with sulphate ions present in the formulation of a biotherapeutic resulting in precipitates apparent as visible particles - the latter situation would not be limited to a biotherapeutic though [11]. Salts of tungsten oxide have been reported to be extracted from pre-filled syringes (PFS) into product solution resulting in precipitation of a monoclonal antibody [8]. Seidl *et al.* [12] recently reported that tungsten from PFS caused protein denaturation and aggregation, potentially leading to increased immunogenicity in an investigational clinical trial of an EPO biosimilar product (discussed later). Iron ions introduced into product solution through contact with stainless steel components in filling and storage equipment have been implicated in increased oxidation and aggregation [13, 14]. Nickel, copper and zinc ions leaching from a metal hose-barb fitting were found to cause precipitation of a conjugate vaccine [15]. The system comprised of a carrier protein conjugated with multiple copies of antigen peptide. The individual components (carrier protein or peptide) by themselves were not susceptible to precipitation by the ions. It was hypothesized that specific binding of ions to the His residues present in the antigen peptide leads to the formation of inter-conjugate metal complexes. These complexes, in turn, lead to formation of multi-mers that exceed the solubility limit for the conjugate vaccine [15].

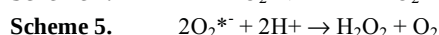
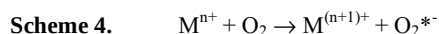
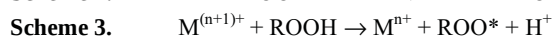
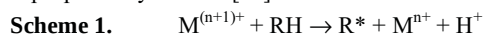
Oxidation and aggregation are two key pathways by which metal ions can impact a biotherapeutic drug. Often, these phenomena occur simultaneously. The mechanisms of metal ion interaction with proteins have been studied extensively, although primarily in the context of their *in vivo* function. The objective of this review is to examine interaction of therapeutic proteins with metal ions that underpins the biotherapeutic drug product degradation. We describe selected examples from the literature where contribution of metal ions towards oxidation of the therapeutic proteins has been studied experimentally. Oxidation reaction schemes are discussed, along

*Address correspondence to this author at the Pfizer Inc., AA4, 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA; Tel: +1-636-247-9979; E-mail: satish.singh@pfizer.com.

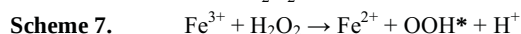
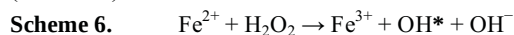
with various aspects of metal-binding to proteins as well as impact of metal-binding and oxidation on structure, flexibility and stability. Metal catalyzed oxidation (MCO) often destabilizes therapeutic protein structures, increasing their aggregation propensity. Thus, amino acid sequences and macromolecular structures were examined in three case studies to understand the structural rationale behind the experimental observations. Furthermore, this review suggests potential strategies to improve the robustness of biotherapeutic drug products with respect to metal ion leachates. While most of the examples discussed in this review are therapeutic proteins, impact of metal ions on a few additional proteins, especially amyloidogenic ones, is also discussed.

METAL CATALYZED OXIDATION OF PROTEINS

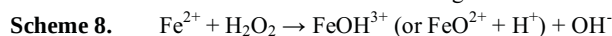
Metal catalyzed oxidation (MCO) refers to a set of complicated reaction schemes involving various reactive oxygen species (ROS) generated by successive metal-catalyzed one-electron reduction of molecular oxygen (O_2^* , H_2O_2 , $^*OH$, ROO^* , $^1O_2$) [16] (see Scheme III in Hovorka and Schoneich [17]). Common transition metal contaminants [e.g. Fe(II) and Cu(I)] can result in the formation of free radicals via redox reactions with a variety of inorganic or organic substrates (Scheme 1), hydroperoxides (Schemes 2, 3), or molecular oxygen (Schemes 4, 5) [18–20]. Electron donors (pro-oxidants) may be needed to regenerate M^{n+} from $M^{(n+1)+}$ and drive these reactions at measurable rates. Ascorbic acid, thiols (e.g. dithiothreitol), or phenols nominally considered as antioxidants, can thus react as reducing agents and promote the formation of ROS. The Ascorbate/Cu(II) or Ascorbate/Fe(III) have been used as model oxidizing systems. However, Ascorbate is not a common ingredient in biopharmaceutical formulations and therefore the H_2O_2 /Fe(II) system is proposed by Ji *et al.* [21].



Fenton chemistry is actually used to destroy organic compounds through the hydroxyl free radical which is a non-selective oxidant. Fe(II) is oxidized by hydrogen peroxide to Fe(III), a hydroxyl radical and a hydroxyl anion (Scheme 6). Fe(III) is then reduced back to Fe(II), a by disproportionation with hydrogen peroxide (Scheme 7).



The hydroxyl radical (OH^*), a freely diffusible species, is therefore not selective in its impact. The ferryl ion (represented as FeO^{2+}) is the metal-bound equivalent of the hydroxyl radical and is also formed during the reduction of peroxide by Fe(II) (Scheme 8). The ferryl ion does not diffuse freely but preferentially reacts with the immediate ligand sphere of the metal complex and therefore results in more site-specific oxidation than the hydroxyl radical [16]. Thus, in a protein, the Fe(II) [or Cu(I)] binds to the metal-binding site and the protein: metal complex reacts with H_2O_2 (Scheme 8) to generate locally an activated oxygen species that reacts with the side chains of the metal-binding site residues.



The nature and location of the oxidizing species as well as the accessibility of the amino acid residues determine their actual impact on the protein integrity. In the case of site-specific oxidation (e.g. with the ferryl ion), the geometry of the metal-binding site, the size of the metal ion, and the relative reactivity of the specific amino acids in the metal-binding vicinity determine the outcome. Site-specific oxidation mechanisms are, therefore, generally resistant to inhibition by free radical scavengers since the radical does not have to diffuse far for causing the oxidation [22]. However,

perturbation of the metal-binding site (e.g. by cosolvents) can inhibit the binding and, thereby, the oxidation [17].

Cysteine (Cys), methionine (Met), tryptophan (Trp), histidine (His), and tyrosine (Tyr) are the most susceptible residues to oxidation in that order. Of these, Cys, Met and His are considered most susceptible to MCO, whereas Trp and His are also significantly impacted by light.

The thiol group is highly reactive and free Cys is thus not commonly found in biotherapeutics protein products [21]. Oxidation of Cys leads to formation of inter- or intra-molecular disulfide bonds and the reaction is strongly catalyzed by transition metal ions. In the absence of a nearby thiol group, monomolecular products such as sulfenic ($RSOH$), sulfinic (RSO_2H) and sulfonic (RSO_3H) acids can form, especially at elevated temperatures [18]. The reaction is favored at higher pH when the thiol group is deprotonated with an optimum around pH 6 ($-SH$ group in a free Cys has pKa value of 9.1 [23]). The local environment determines the pKa of the Cys group in the folded structure, thus impacting the oxidation susceptibility of the residue. Oxidation of Met to methionine sulfoxide [$RS(O)CH_3$] is commonly observed in therapeutic proteins. Under extreme conditions, methionine sulfone [$RS(OO)CH_3$] may be detected.

Histidine residues are susceptible to MCO due to their metal-binding property, forming 2-oxo-histidine (2-oxoimidazoline) [24]. Other products seen are Asparagines and Aspartates, although detailed mechanistic information for the latter products is not well understood. Similar to Cys, the oxidation susceptibility of His is dependent on the pH (in relation to its pKa in the structure), with the deprotonated form being more labile; deprotonation of imidazole increases its metal binding capacity. Tryptophan oxidation by ROS can yield a host of species such as 5-hydroxytryptophan, 7-hydroxytryptophan, ox-indole alanine, kynurenine and N-formylkynurenine. MCO of Tyrosine yields di-tyrosine which can result in intra- / inter- molecular cross-linking of protein molecules. Photo-oxidation also yields 3, 4-dihydroxyphenylalanine and di-tyrosine. The high pKa of tyrosine residues implies that it is significantly reactive only when it is ionized under very alkaline conditions.

PROTEIN STRUCTURAL ASPECTS OF METAL BINDING AND METAL CATALYZED OXIDATION

Metal-binding sites in proteins represent a diverse range of geometries based on the coordination number of the metal involved. Functional groups such as α -NH₂, side-chain carboxyl, O-(peptide), N-(peptide), N-(imidazole), side-chain O-(hydroxyl), S-(sulfhydryl), S-(thioether) and bridging S-(disulfide), create the binding sites [25]. However, metals also have specific ligand preferences. Calcium and magnesium ions prefer side-chain oxygen atoms of Asp and Glu residues. D-block metals such as zinc and iron mainly bind to nitrogen- and sulfur- containing residues such as His and Cys [26]. Analysis of known metal-binding sites shows that Asp, Glu and His followed by Cys are the most common residues present at these sites. The analysis also shows that for metals with high coordination numbers (e.g. Fe, Co, Ni, Cr, Ti) amino acid residues may alone not be able to satisfy valency requirements; water molecules also participate. Residues such as Asp and Glu are bidentate and can thus provide two ligand atoms for coordination [26], probably explaining their frequent appearance in binding sites. Divalent metals thus prefer Asp, Glu, His and Cys, binding through the side-chain atoms of these residues.

MCO is also selective for site and geometry in terms of the residues that are impacted. For example, MCO (Ascorbate/ O_2 /Trace Fe) of glutamine synthetase resulted in modification of a specific His (out of 16) only, even though the particular His is found in a part of the sequence rich in oxidation-sensitive residues (Met-His₂₅₆-Cys-His-Met). In this case, the enzyme also loses its catalytic activity as a consequence of the oxidation [9, 27, 28]. In general,

enzymes that are highly sensitive to modification by MCO systems are also dependent on metal ions for their catalytic activity. They perform contain a metal ion binding site. For example, preferential oxidation of Met4 in the B chain of human relaxin (hRLx) over that of Met25 in the same chain was observed in an MCO [Ascorbic acid/Cu(II) or Fe(III)/O₂] system. This trend is opposite to the observed trend for oxidation by H₂O₂ or light [19, 29]. A complete structural model of hRLX derived from a crystal structure of hRLx, available from the Protein Data Bank (PDB, www.rcsb.org, [30], PDB entry: 6RLX), was used to rationalize the above issue by measuring the solvent exposure of the two Met residues. Percent solvent accessible surface area (ASA) of the Met residues in hRLX structure was calculated using protein properties module in MOE2012.10 software from Chemical Computing Group (www.chemcomp.com). Met 4 is less solvent exposed (ASA = 17%) than Met 25 (ASA = 32%), but it lies in close proximity to metal-binding residues in the relaxin structure. Besides the solvent exposure, protein conformational flexibility is another important consideration in non-site specific protein oxidation as well as in site specific MCO [31]. Because MCO requires well-defined geometrical orientations for oxidation prone residue(s) and its (their) metal binding neighbor(s), the regions with lower conformational flexibility (greater rigidity) may be at greater risk of MCO. Debye-Waller factors, also known as B-factors, obtained from protein crystallization experiments denote mean displacements of the atoms about their average positions. In particular, B-factors for C^α atoms have been used as a measure of protein flexibility and stability [32-35]. C^α atom B-factor for Met 4 is 27.25 Å² which is lower than that for Met 25 (41.02 Å²). His 12 in chain A of the hRLx is also modified by MCO and its C^α atom B-factor is 20.20 Å². MCO of His12 in the chain A results in structural modifications and precipitation of the protein (discussed later). Lam *et al.* (2011) [36] reported of an unusual Trp oxidation in a Fab due to its presence in close proximity to three His residues. Replacement of the His31 closest to Trp50 with a non-metal binding residue eliminated the oxidation induced by the combination of peroxides from surfactant and trace metal contaminants.

In studies with a series of model peptides containing Met and His, presence of His increased the oxidation rate of Met with maximum enhancement observed for the sequence His-X-X-Met [37, 38]. Similarly, Met oxidation by an MCO [Fe(II)/H₂O₂] system in a model peptide resulted in a 2:1 preference for oxidation of the unrestricted Met compared to a conformationally restricted Met-analog (Nor). In contrast, incubation of peptide with H₂O₂ alone yielded a 1:1 ratio of oxidation of the two Met residues. The flexible C-terminal Met has better access to the peptide-bound iron compared to the N-terminal Nor [16].

There is also a degree of specificity within various MCO systems. For example, Cu²⁺ but not Fe²⁺, Ni²⁺, Co²⁺, Cr³⁺, or Mn²⁺ can catalyze the oxidation of B-chain Histidines in Insulin or Zn²⁺-Insulin [39]. Similarly, the [H₂O₂/Cu(II)] system results in binding-site specific oxidation of albumin as opposed to more random oxidation by the [H₂O₂/Fe(II)/EDTA] system [40]. The stronger binding of copper to albumin results in site-specific attack near the copper-binding site. Weaker binding of [Fe(II)/EDTA] results in non-specific attack from radicals generated by free complex [Fe(II)/EDTA] in solution. The [Ascorbate/Fe(II)/EDTA] system led to preferential oxidation of Trp residues in bovine serum albumin (BSA) compared to His residues with [Ascorbate/Cu(II)] system [41]. Strong and specific interaction of Cu(II) with His residues has been shown [42]. In the case of Trp, the oxidative modification is due to its intrinsic reactivity to free radicals than to any specific chelation or binding behavior. Oxidation of free amino acids by the Fenton reagents [Fe(II)/H₂O₂] was found to be completely dependent on the presence of bicarbonate ion and also enhanced by sub-stoichiometric amounts of iron chelators [43].

PREVENTION OF METAL CATALYZED OXIDATION

Stabilization/protection of protein therapeutics against MCO using formulation approaches generally relies on addition of anti-oxidants or chelators. The choice of an optimum antioxidant depends on the oxidation mechanism involved. MCO-based degradation cannot, in general, be protected by addition of antioxidants (oxygen scavengers / reducing agents) such as ascorbic acid, propyl gallate or bisulfites. Hydroxyl radical scavengers such as formate, ethanol, mannitol may also not be effective. These can function as pro-oxidants and are used as model oxidizing systems. Thiols (e.g. cysteine, glutathione, thioglycerol) which function as chain terminators for free-radical reactions are expected to be effective [44]. Free methionine (a thioether) added to solution can function as a sacrificial antioxidant and protects against Fe(II) induced free-radicals also by chain-termination [13]. Free tryptophan was also found to be effective against MCO [Fe(II)/EDTA] of PTH [21].

Chelating agents would also be expected to be effective as stabilizers against MCO systems. Commonly used chelators are (salts of) EDTA and DTPA, although citric acid, phosphoric acid (weak), tartaric acid also possess chelating properties. Dicarboxylic amino acids (glutamic and aspartic acids) have also been shown to function as metal ion chelators [45], a property that is reflected in these residues being often found in metal-binding sites [26]. However, EDTA is not always effective in the complete conversion of metal ions, particularly iron, into redox inert forms and may even accelerate the process of oxidation. EDTA is effective in preventing oxidation when copper is the catalytic species by completely sequestering the ion. The EDTA-Fe complex, however, has an open structure allowing hydroxyl radicals to escape [46] since it cannot cover all five valences of iron. Stadtman [47] also proposes that in cases where the protein possesses a tight metal binding site, sequestration of the metal in solution by EDTA would be protective. However, it is likely that this would not be effective in the case of the [Fe(II)/EDTA] system as discussed above. In cases where EDTA is actually required for oxidation, it is proposed that these proteins lack a tight metal binding site but can bind the EDTA-metal complex. EDTA would thus carry the metal to the binding site and promote site-specific oxidation at the EDTA binding site [47]. Poly-aminocarboxylate metal chelators (NTA, EDDA, EDTA, EGTA, DTPA) were found to impair site-specific oxidation of Met in model peptides, but allowed non-site specific oxidation through ROS species different from the species formed at peptide metal binding sites [48]. Specific iron chelators such as desferrioxamine can, however, completely shut down the iron-catalytic effect. EDTA and DTPA have however also been shown to be effective in protecting an IgG2 mAb against iron induced oxidation when present in stoichiometric amounts sufficient to bind all iron [3]. Lam *et al.* [36] were also able to eliminate the oxidation of Trp50 in the Fab (discussed above) by adding EDTA. In summary, the use of EDTA as an antioxidant for MCO should be assessed carefully. It is likely that when addressing the trace amounts of metals present as contaminants from leaching, EDTA would be found to be effective.

Stabilization against oxidation by protein engineering approaches is possible by disrupting metal-binding sites if activity and structural / chemical stability can be preserved, while not enhancing immunogenic potential. Another possibility is to mutate away oxidation-susceptible residues under the constraints of same activity and stability. An example of the latter situation was analyzed by Kim *et al.* [49], who examined the four Met residues (Met 26, Met 32, Met 65 and Met98) in staphylococcal nuclease A. Oxidation (by H₂O₂) destabilized the molecule significantly, when measured as free energy difference (ΔΔG) between oxidized and un-oxidized forms (ΔΔG = 5.4 – 1.5 = 3.9 kcal/mole) by chemical (guanidine hydrochloride) denaturation experiments (Table 2 in Kim *et al.* [49]). Substitutions at Met26 or Met32 did not result in significant destabilization of the protein as compared to the wild-type (Table 1 in Kim *et al.* [49]). The mutants Met26Ile or Met32Ile were destabi-

lized to similar extent as the wild-type upon oxidation (Table 2 in Kim *et al.* [49]). A structural model of staphylococcal nuclease A was obtained from the PDB entry 1EY0 and optimized via implicit solvent energy minimizations using MOE2012.10 software from Chemical Computing Group (www.chemcomp.com). The resulting structure indicates that Met26 and Met32 are solvent exposed with their solvent accessible surface areas (ASA) being 25%. In contrast, Met65 and Met98 are solvent inaccessible (ASA for Met65 = 7% and ASA for Met98 = 13%). Consistent with the structural location of Met98, oxidation of this residue requires significant protein destabilization. Experimental data from Kim *et al.* [49] shows that Met98Leu mutation destabilizes the protein by 2.5 kcal/mole ($\Delta\Delta G = \Delta G_{H_2O}(WT) - \Delta G_{H_2O}(Met98Leu) = 5.4 - 2.9 = 2.5$ kcal/mole) as compared to the wild-type. However the $\Delta\Delta G$ difference between oxidized and un-oxidized forms of the Met98Leu mutant is lower at 2.0 kcal/mole in contrast with the 3.9 kcal/mole reduction for the wild-type protein. This implies that use of Met98Leu against oxidation would require further substitutions at other sites to enhance structural stability to counter the destabilization by the mutation itself. Met65Leu on the other hand destabilizes the protein by only 0.8 kcal/mole as compared to the wild-type, and the $\Delta\Delta G$ difference between oxidized and un-oxidized forms of Met65Leu mutant is 2.0 kcal/mole (as compared to 3.9 kcal/mole for the wild-type protein), implying an overall benefit of this mutation. Other substitutions such as Met65Ile/Ala offered no advantages over oxidative destabilization. Besides these observations, there may have been a correlation between solvent exposure of Met residues and their oxidation levels. Unfortunately, the data on oxidation levels of individual Met residues in Staph Nuclease A was not reported [49]. Nonetheless, this example illustrates the complex interplay of protein structure, flexibility and stability involved in engineering out the undesirable effects of oxidation. Therefore, such approaches should be used only when a significant benefit is expected. It is also pertinent to mention that the experimental data on the free energy changes in protein stability upon mutations such as those described in the above example can be obtained from ProTherm database [50].

IMPACT OF METAL CATALYZED OXIDATION ON PROTEIN STRUCTURAL INTEGRITY

MCO leads to changes in local chemical structure of the amino acid residues, which in turn can impact overall physico-chemical stability and activity of the protein. Oxidized residues become more polar and hydrophilic. For example, oxidation of Met to Met-sulfoxide replaces a flexible hydrophobic side chain with a larger, hydrophilic and less flexible one [51]. Oxidation of His alters the characteristics of a basic amino acid. Chemical modification of residues will also impact the packing arrangement in the vicinity. Cys oxidation changes the charge. The oxidation mediated changes in size, polarity, charge etc. of amino acid residues also alters the balance of forces responsible for maintaining the native structure of a protein. The changes in protein native structure can lead to the loss of potency/activity as well as solubility, leading to formation of aggregates or precipitates. In hRLx, MCO [Ascorbate/Cu(II)/O₂] impacts His 12 in the chain A, resulting in structural changes that lead to physical instability, aggregation and precipitation at higher pH. The pH-dependent solubility behavior of the MCO products of hRLx also suggests a difference in His degradation profile with pH [6, 52]. A complete structural model of hRLx indicates that His12 in the chain A is 66% solvent exposed and the pK_a value (6.35) is slightly shifted. The calculated pI of hRLx molecule from its sequence is 6.9, but in a structure based calculation the pI of hRLx was found to be 9. The pK_a and other calculations were performed using the protein properties module in MOE2012.10. Figure 1 shows a completed structural model of hRLx. The hydrophobic and hydrophilic surface patches, computed using the above mentioned software, are also shown along with a potential aggregation prone region (APR) capable of forming the cross-β steric zipper motif [53]

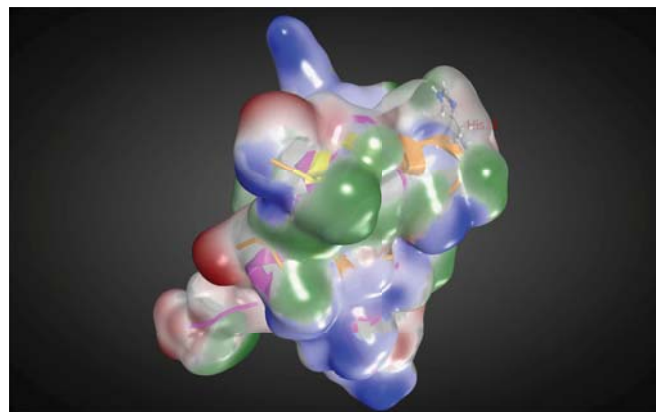


Fig. (1). A structural model of human Relaxin (hRLx) (derived from PDB entry 6RLX) is shown in ribbon form along with surface exposed hydrophobic (green), positively charged (blue) and negatively charged (red) surface patches. The ribbons for chains A and B are shown in saffron and magenta respectively. The portion of Chain A ribbon that contains an aggregation prone region (APR) is shown in yellow. The chain A residue, His12, where MCO leads to destabilization and aggregation of hRLx, is shown in ball and stick representation.

in the chain A. The potential APR (2-LYSAL-6) was detected using a combination of TANGO [54] and PAGE [55], as described by Wang *et al.* [56]. It is found in Chain A of hRLx and lies in an α -helix that also contains His12 at its C-terminal. Besides this, His12 also falls in structural vicinity of hydrophobic patches (shown in green color in Fig. 1). These observations help us rationalize how destabilization of hRLx via MCO of His12 could potentially push it into an aggregation competent state, especially at higher pH values where the calculated net charge on the molecule is also close to zero.

Another direct consequence of oxidation is inter-molecular protein cross-linking and dimerization. For example, oxidation of free Cys residues leads to dimerization of recombinant human Ciliary neurotrophic factor (rhCNTF) [44]. MCO triggered cross linking has been associated with the formation of di-tyrosine also. Bovine eye lens proteins were found to crosslink via di-tyrosine in [H₂O₂/Cu] but not in other MCO systems (e.g. H₂O₂/Fe/EDTA, Ascorbate/Cu, Ascorbate/Fe/EDTA) [57]. The cross-linking was suppressed by reducing agents Glutathione or Ascorbic acid through quenching of the tyrosyl radicals. This specificity of Cu was related to binding of Cu and not of the chelated Fe ion. Liu *et al.* [58] show the possibility of a His-Lys crosslink in the case of MCO [Ascorbic acid/Cu(II)] using surrogates for His and Lys in protein-free model systems. The initial oxidation of the His side chain to 2-imidazolone would further oxidize in the presence of a nearby Lys side chain to form an imidazole-amine adduct. Based on their investigations, the authors proposed that His-Lys cross linking could lead to formation of higher molecular weight species in proteins.

Curiously, oxidation has also been reported to stabilize the structure and prevent aggregation. In the case of the intrinsically disordered protein α -synuclein, oxidation of all four of its Met residues, by H₂O₂, results in an increased degree of disorder, but also eliminates its ability to form amyloid-fibrils [59]. Furthermore, Met-ox α -synuclein added to un-modified α -synuclein inhibited fibrillation at ratios $\geq 4:1$. Similarly, oxidation of its single Met residue enhanced the physical stability and reduced the aggregation rate of human calcitonin [60]. However, these protein oxidations are not metal catalyzed. In case of α -synuclein, MCO leads to opposite observations (described below).

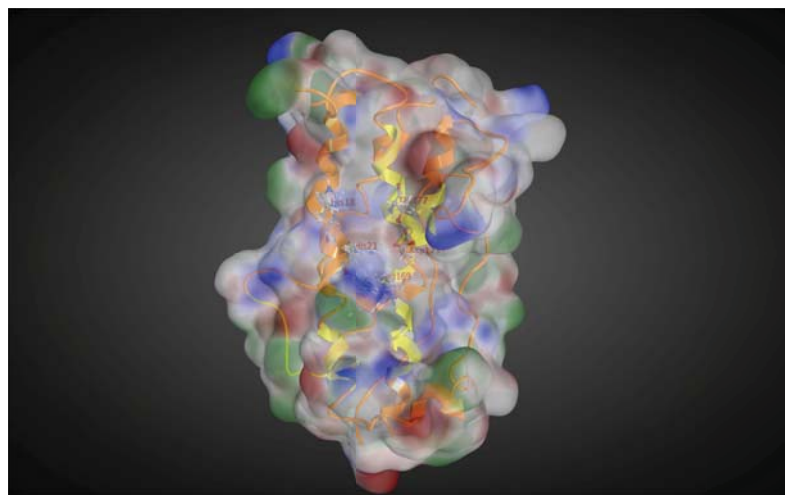
IMPACT OF METAL ION BINDING ON PROTEIN STRUCTURAL INTEGRITY

Metal ions can also impact structural integrity of a protein without any prior chemical degradation. Even proteins, not known to require metal binding for enzymatic or other functional activities, contain several residues that can bind metal ions. Residues may also be arrayed in a spatial pattern that may enable stronger binding than by a single residue (e.g. His) alone. Such metal-binding sites can be generally described as consisting of a central shell of hydrophilic ligands to chelate the ion, with a surrounding shell of hydrophobic residues. Common biologically important metals (e.g., Mg^{2+} , Zn^{2+} , Ca^{2+} , Mn^{2+}) frequently bind proteins selectively in different geometric configurations, utilizing different ligand atoms, mainly oxygen, nitrogen, and sulfur from side-chain groups, and oxygen from main-chain carbonyls [61]. Environmental conditions such as pH, ionic strength, and competing ions can impact the nature and extent of the interaction.

The impact of metal ion binding may become immediately apparent as a change in primary, secondary or higher order structures but may also manifest itself by impacting on long-term storage stability, which is a particular concern for biotherapeutics. Note that detection of changes in protein structure and stability may be limited by sensitivity of the experimental methods used. Furthermore, long-term storage stability structural impact may be confounded by chemical changes such as oxidation induced by the metal ion. Cu(II) ions oxidize His18 and His21 in human growth hormone (hGH) located in the metal-binding pocket [62]. However, Cu(II) binding also reduces the thermodynamic stability (free energy of unfolding) of this protein [63] and, therefore, increases hGH aggregation-propensity during storage. A complete structural model of mature hGH (residues 27-217) was derived using the PDB entry

1HGU and is shown in (Fig. 2a). The two copper binding His-residues, His18 and His21, lie in close proximity of one another and are solvent exposed. ASA of His18 is 33% and that for His21 is 23%. The pKa values for these residues are also significantly shifted, being 3.59 for His18 and 3.35 for His21. This observation indicates that the two His residues may lie in a charged environment. Consistently, most of the neighboring residues for His18 and His21 are either charged or polar. In particular, His21 is part of a hydrogen bond network. It forms hydrogen bonds with Arg169 and Asp173, which is in turn hydrogen bonded with Thr177. The observations indicate Cu(II) ions binding with His18 and His21 would perturb electrostatic balance in the surrounding region, potentially leading to lower thermodynamic stability for hGH [63]. Fig. 2b presents sequence locations of the potential APRs in hGH sequence identified using the TANGO/PAGE combination [64] and the amyloidogenic hexapeptide patterns described by Lopez de la Paz and Serrano [65]. These regions are shown in yellow color in the (Fig. 2a). The region 172-KVETFLRIV-180, which is in contact with both His18 and His21, is predicted to contain a TANGO/PAGE APR (176-FLRIV-180) and an amyloidogenic hexapeptide pattern (172-KVETFL-177) for acidic pH conditions. All residues except Arg178 in the above mentioned region are solvent inaccessible in the native state of hGH. The destabilization caused by Cu(II) binding with His18 and His 21 could potentially solvent expose the 172-KVETFLRIV-180 region, leading to increased aggregation propensity of hGH as observed in the experiments [63].

The impact of interaction of a metal ion on protein structure will generally be metal-ion identity and concentration dependent. For example, a derivative of GLP-1 showed changes in secondary structure (detected by FTIR and intrinsic fluorescence) with addition of Al^{3+} and Zn^{2+} but not with K^{+} and Mg^{2+} . An increase in β -



2a

>Human Growth Hormone (hGH)

```
FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIP  
SNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQ  
TLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIV  
QCRSVEGSCGF
```

2b

Fig. (2). Metal catalyzed oxidation of human growth hormone (hGH). (a) A structural model of hGH (derived from the PDB entry 1HGU) is shown in ribbon format (safron) along with the surface exposed hydrophobic (green), positively charged (blue) and negatively charged (red) surface patches. Portions of the molecule that contain predicted APRs are shown in yellow. The MCO sites, His18 and His21, are shown in ball and stick representation. His21 is part of a four member hydrogen-bond network. This network contains Arg169, Asp173 and Thr177 besides His 21. These residues are also shown in ball and stick representation. (b) Amino acid sequence of hGH is shown. The two MCO sites, His18 and His21, are shown in bigger bold red font. The TANGO/ PAGE predicted APRs are shown in the yellow background and the regions detected using an amyloidogenic hexapeptide acidic pH pattern are shown in turquoise background. Several residues in the predicted APRs and amyloidogenic patterns overlap. Such residues are also shown in the yellow background. Note that all residues that fall within TANGO/PAGE APRs and amyloidogenic patterns are shown in yellow colored ribbons in (Fig. 2a).

sheet at the expense of α -helical content was apparent above 25 ppm, although this is likely to be impacted by sensitivity of the method. Accelerated storage stability studies with the same ions also showed an increase in fibrillation kinetics as detected by ThT fluorescence [66].

Di- and tri-valent metal ions have also been shown to cause fibrillation of the intrinsically unstructured protein α -synuclein [7]. Aluminum was the most effective, along with Cu^{2+} , Fe^{3+} , Co^{3+} and Mn^{2+} (concentration ranges below 25 mM); efficacy was correlated to charge density of the ion and to the amount of secondary structure induced by the ion. Binding of cations to the negatively charged α -synuclein results in charge neutralization and rapid formation of a more compact and structured conformation. Polyvalent cations also serve to cross-link or bridge between two or more carboxylates, thus increasing the efficiency of fibrillation. Mature fibrils examined after pelleting and extensive washing were found to contain the metal cation, indicating tight binding to the fibrils. Interestingly, metals such as Al^{3+} , Zn^{2+} , Pb^{2+} and Ti^{3+} were able to overcome the above described Met-oxidation induced stabilization of α -synuclein [67]. Similarly, small amounts of Al^{3+} (10 – 100 μM) were found to result in enhanced aggregation rate of rhFVIII [68]. Only minor perturbations were detectable in the secondary and tertiary structure using spectroscopic techniques. However these changes were clearly sufficient to alter the quaternary structure of rhFVIII to an aggregation-competent state.

Spectral changes reflective of secondary and tertiary structural changes were seen with addition of Al^{3+} into BSA solution at neutral pH [69]. A shift from α -helical to β -rich structure was seen even below a concentration deemed the critical induced-aggregation concentration of the aluminum ion (~ 0.08 mM for 2.5×10^{-6} M BSA). Structural perturbations were thus apparent at low levels of the ion before the changes became significant enough to cause aggregation.

Zhou *et al.* [3] also reported subtle changes to the tertiary structure of an IgG2 mAb by added Fe(III) ions which resulted in increased physical and chemical degradation of the mAb over time. Titration calorimetry was used to show weak binding ($K_d = 0.18$ mM) of approximately 20 iron ions/mAb molecule. To further understand the impact of iron binding on physico-chemical properties of this mAb, a homology based structural model for the full length mAb was built and analyzed for spatial proximity between predicted iron binding sites and aggregation prone regions. The iron binding sites were predicted using a procedure adapted from Goyal and Mande [26]. Several potential iron ion binding residue clusters could be identified throughout the structure of the mAb, including in the CDRs (complementary determining region), hinge regions and $\text{C}_\text{H}2$: $\text{C}_\text{H}3$ domain interfaces (Fig. 3). In particular, the CDRs and adjoining framework regions of this mAb contain several predicted APRs that lie in close spatial proximity with the predicted iron binding residues. These observations help rationalize the experimental observation of increasing oxidation and aggregation of this IgG2 of mAb with increasing iron concentration (unpublished data).

Binding of metals can lead to impact on protein structure that is specific to the metal. For example, iron (and copper) induce structural changes in α -synuclein that differs from that induced by magnesium, as detected by changes in the tyrosine fluorescence spectrum – iron lowers the peaks whereas magnesium increases them. It was found that magnesium inhibits the spontaneous as well as iron-induced aggregation of α -synuclein. The binding site of Mg^{2+} is likely different from that of Fe^{2+} . It is hypothesized while the binding of Mg^{2+} does not inhibit the binding of Fe^{2+} , it does prevent the formation of an aggregation competent conformer [70]. Along the same lines, it was reported that Cu(II) ions (up to a certain ratio with respect to protein) can inhibit the Zn^{2+} induced aggregation of

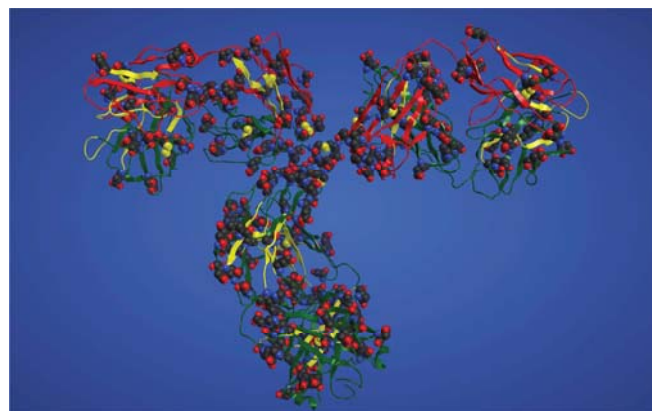


Fig. (3). A homology based molecular model of a full length IgG2 mAb is shown. The green and red ribbons indicate heavy and light chains respectively. The portions of the ribbons that contain TANGO/PAGE predicted APRs are shown in the yellow color. All potential iron binding residue clusters are shown in ball and stick representation. Note that an IgG2 mAb is considerably larger than hRLx and hGH and contains about 20 potential iron binding sites.

$\text{A}\beta$ -peptide. Zn^{2+} induces $\text{A}\beta$ aggregation by binding to His residues via the N_ϵ atom and cross-linking of the peptides. This occurs over a wide pH range ($\text{pH} > 6.0$). On the other hand, Cu(II) binding to $\text{A}\beta$ is pH dependent. At mildly acidic pH (6.0), Cu(II) binds in a mode similar to Zn^{2+} and forms insoluble aggregates ($6.0 - < 7.0$). However, under neutral pH, Cu(II) binds to the N_ϵ atom of His along with other main chain amide-nitrogens and produces soluble complexes instead. Thus Cu(II) induced aggregation of $\text{A}\beta$ is strongly pH dependent. Furthermore, Cu(II)- $\text{A}\beta$ complex at neutral pH prevents Zn^{2+} from binding to the His, and thus drastically Zn^{2+} induced aggregation [71, 72]. Similar behavior was observed in interaction of Cu(II) with the octapeptide (PHGGGWGQ) which occurs near the N-terminus of prion protein [73]. It is possible that the pH-sensitivity of Cu(II)-protein interaction is quite general. However the generality of the effect of Cu(II) inducing aggregation only under mildly acidic conditions is not known.

Tungsten ions have been found to leach into liquid products in pre-filled syringes (PFS), arising from residues of the pin used to make the needle-hole during the syringe forming process. A number of publications have analyzed the impact of tungsten ions on biotherapeutics. Jiang *et al.* [74] found significant aggregation and precipitation in an alpha-helix rich protein and in an Fc-Fusion protein. The extent of aggregation was higher at lower pH (4 vs 6.3). In these cases, no impact on secondary or tertiary structures of the proteins was detectable by CD, FTIR and Raman spectroscopy. The cause of aggregation was attributed to electrostatic interaction between the proteins (positively charged) and polytungstate anions ($[\text{H}_2\text{W}_{12}\text{O}_{40}]^{6-}$, $[\text{HW}_{12}\text{O}_{40}]^{7-}$; other possibilities such as $[\text{W}_7\text{O}_{24}]^{6-}$, $[\text{H}_2\text{W}_{12}\text{O}_{42}]^{10-}$ have been suggested) formed at low pHs. It is possible that the polytungstate ions either simply neutralize charge or electrostatically cross-link multiple protein molecules, leading to loss of solubility or aggregation. Bee *et al* (2009) in their study with a mAb, termed this phenomenon as tungsten induced coagulation. However, Seidl *et al.* [12] found that sodium polytungstate bound strongly but reversibly to EPO (epoetin alfa) and resulted in rapid unfolding and loss of secondary structure. Aggregates were induced, including a proportion of covalent disulfide-linked dimers.

Interactions of the polytungstate anion (the polyoxometalate $[\text{H}_2\text{W}_{12}\text{O}_{40}]^{6-}$) with human serum albumin (HSA) were shown to be electrostatically driven to a specific binding site. Binding of the polyoxometalate $[\text{NaP}_5\text{W}_{30}\text{O}_{110}]^{14-}$ to HSA was also electrostatically driven but potentially with up to five binding sites, one of them competitive [75]. Accommodation of the larger size of $[\text{NaP}_5\text{W}_{30}\text{O}_{110}]^{14-}$ required a disruption in the folding structure unlike that for

binding pocket for $[H_2W_{12}O_{40}]^{6-}$. Discounting detection sensitivity, the differences in the reports between presence or lack of structural impact may have some specificity with the protein in question. It is likely that in all cases, the electrostatic component is the driver for the interaction. The impact of the interaction is then dependent on the species of tungsten interacting, the amount and the site of interaction on the protein.

CONCLUSION

This article has presented a broad overview of potential impact of metal ions arising as leachates, on the physico-chemical stability of the biotherapeutic drug products. Understanding the mechanisms behind metal-induced degradation routes is essential to devise rational strategies for their prevention, as well as to bring forward the next generation candidates that are less susceptible to these vulnerabilities. MCO tends to be site-specific and the impact is also determined by the structure and flexibility / rigidity of the binding-site. As illustrative case studies, we have used molecular sequence and structural information available for hRlx, hGH and an IgG2 mAb to rationalize the experimental observations. In each example, potential APRs and surface hydrophobic patches could be identified in close structural proximity with the residues whose binding with metal ions leads to protein oxidation, destabilization and aggregation. This link between site-specific MCO and aggregation is highly undesirable from the perspective of pharmaceutical development of biotherapeutic drug products, especially when delivery requires high concentration for the active ingredient as in subcutaneous administration. However from rational molecular design perspective, it offers exciting opportunities towards achieving multiple goals via single point mutations. While it is not feasible to mutate away all potential metal binding sites in a biotherapeutic candidate, disruption of the APRs spatially proximal to these sites can possibly help mitigate the impact of metal-ions as well as improving solubility of the candidate.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Declared none.

ABBREVIATIONS

MCO	=	Metal Catalyzed Oxidation
ROS	=	Reactive Oxygen Species
PFS	=	Pre-filled syringe
hRlx	=	Human Relaxin
hGH	=	Human Growth Hormone
IgG2	=	Immunoglobulin γ of isotype 2
EDTA	=	Ethylenediaminetetraacetate
DTPA	=	Diethylenetriaminepentaacetate
NTA	=	Nitrilotriacetate
EDDA	=	Ethylenediaminediacetate
EGTA	=	Ethylenebis(oxyethylenenitrilo)tetraacetate

REFERENCES

- Allain L WQ. Impact of package leachables on the stability of pharmaceutical products. *Am Pharm Rev* 2007; 10: 38-44.
- Fliszar KA, Walker D, Allain L. Profiling of metal ions leached from pharmaceutical packaging materials. *PDA J Pharm Sci Technol* 2006; 60: 337-42.
- Zhou S, Zhang B, Sturm E, *et al.* Comparative evaluation of disodium edetate and diethylenetriaminepentaacetic acid as iron chelators to prevent metal-catalyzed destabilization of a therapeutic monoclonal antibody. *J Pharm Sci* 2010; 99: 4239-50.
- Marx G, Chevion M. Site-specific modification of albumin by free radicals. Reaction with copper(II) and ascorbate. *Biochem J* 1986; 236: 397-400.
- Uchida K, Kawakishi S. Identification of oxidized histidine generated at the active site of Cu,Zn-superoxide dismutase exposed to H₂O₂. Selective generation of 2-oxo-histidine at the histidine 118. *J Biol Chem* 1994; 269: 2405-10.
- Li S, Nguyen TH, Schoneich C, Borchardt RT. Aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. *Biochemistry* 1995; 34: 5762-72.
- Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. *J Biol Chem* 2001; 276: 44284-96.
- Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. Precipitation of a monoclonal antibody by soluble tungsten. *J Pharm Sci* 2009; 98: 3290-301.
- Farber JM, Levine RL. Sequence of a peptide susceptible to mixed-function oxidation. Probable cation binding site in glutamine synthetase. *J Biol Chem* 1986; 261: 4574-8.
- Cleland JL PM, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit Rev Ther Drug Carrier Syst* 1993; 10: 307-77.
- Markovic I. Challenges associated with extractables and/or leachables substances in therapeutic biologic protein products. *American Pharmaceutical Review* 2006; 9: 20-27.
- Seidl A, Hainzl O, Richter M, *et al.* Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity. *Pharm Res* 2012; 29: 1454-67.
- Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. *J Pharm Sci* 1997; 86: 1250-5.
- Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM. Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. *Pharm Res* 2003; 20: 1952-60.
- Li N, Osborne B, Singh SK, Wang W. Metal-leachate-induced conjugate protein instability. *J Pharm Sci* 2012; 101: 2733-43.
- Duffield DR, Wilson GS, Glass RS, Schoneich C. Selective site-specific fenton oxidation of methionine in model peptides: evidence for a metal-bound oxidant. *J Pharm Sci* 2004; 93: 1122-30.
- Hovorka SW, Hong J, Cleland JL, Schoneich C. Metal-catalyzed oxidation of human growth hormone: modulation by solvent-induced changes of protein conformation. *J Pharm Sci* 2001; 90: 58-69.
- Li S, Schoneich C, Borchardt RT. Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization. *Biotechnol Bioeng* 1995; 48: 490-500.
- Li S, Schoneich C, Borchardt RT. Chemical pathways of peptide degradation. VIII. Oxidation of methionine in small model peptides by prooxidant/transition metal ion systems: influence of selective scavengers for reactive oxygen intermediates. *Pharm Res* 1995; 12: 348-55.
- Hovorka S, Schoneich C. Oxidative degradation of pharmaceuticals: theory, mechanisms and inhibition. *J Pharm Sci* 2001; 90: 253-69.
- Ji JA, Zhang B, Cheng W, Wang YJ. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization. *J Pharm Sci* 2009; 98: 4485-500.
- Stadtman ER, Oliver CN. Metal-catalyzed oxidation of proteins. Physiological consequences. *J Biol Chem* 1991; 266: 2005-8.
- Creighton TE. *The Physical and Chemical Basis of Molecular Biology*. Helvetian Press 2010.
- Schoneich C. Mechanisms of metal-catalyzed oxidation of histidine to 2-oxo-histidine in peptides and proteins. *J Pharm Biomed Anal* 2000; 21: 1093-7.
- Sarkar B. Metal protein interactions. *Prog Food Nutr Sci* 1987; 11: 363-400.
- Goyal K, Mande SC. Exploiting 3D structural templates for detection of metal-binding sites in protein structures. *Proteins* 2008; 70: 1206-18.

- [27] Levine RL. Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J Biol Chem* 1983; 258: 11828-33.
- [28] Levine RL. Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J Biol Chem* 1983; 258: 11823-7.
- [29] Nguyen TH, Burnier J, Meng W. The kinetics of relaxin oxidation by hydrogen peroxide. *Pharm Res* 1993; 10: 1563-71.
- [30] Rose PW, Bi C, Bluhm WF, *et al.* The RCSB Protein Data Bank: new resources for research and education. *Nucleic Acids Res* 2013; 41: D475-82.
- [31] Kamerzell TJ, Middaugh CR. The complex inter-relationships between protein flexibility and stability. *J Pharm Sci* 2008; 97: 3494-517.
- [32] Yuan Z, Bailey TL, Teasdale RD. Prediction of protein B-factor profiles. *Proteins* 2005; 58: 905-12.
- [33] Halle B. Flexibility and packing in proteins. *Proc Natl Acad Sci USA* 2002; 99: 1274-9.
- [34] Schlessinger A, Rost B. Protein flexibility and rigidity predicted from sequence. *Proteins* 2005; 61: 115-26.
- [35] Parthasarathy S, Murthy MR. Protein thermal stability: insights from atomic displacement parameters (B values). *Protein Eng* 2000; 13: 9-13.
- [36] Lam XM, Lai WG, Chan EK, Ling V, Hsu CC. Site-specific tryptophan oxidation induced by autocatalytic reaction of polysorbate 20 in protein formulation. *Pharm Res* 2011; 28: 2543-55.
- [37] Li S, Schoneich C, Wilson GS, Borchardt RT. Chemical pathways of peptide degradation. V. Ascorbic acid promotes rather than inhibits the oxidation of methionine to methionine sulfoxide in small model peptides. *Pharm Res* 1993; 10: 1572-9.
- [38] Schoneich C, Zhao F, Wilson GS, Borchardt RT. Iron-thiolate induced oxidation of methionine to methionine sulfoxide in small model peptides. Intramolecular catalysis by histidine. *Biochim Biophys Acta* 1993; 1158: 307-22.
- [39] Sadineni V, Schoneich C. Selective oxidation of Zn2+ -insulin catalyzed by Cu2+. *J Pharm Sci* 2007; 96: 1844-7.
- [40] Kocha T, Yamaguchi M, Ohtaki H, Fukuda T, Aoyagi T. Hydrogen peroxide-mediated degradation of protein: different oxidation modes of copper- and iron-dependent hydroxyl radicals on the degradation of albumin. *Biochim Biophys Acta* 1997; 1337: 319-26.
- [41] Uchida KE, Itakura N, Kawakishi KS. The Hydroxyl Radical Generated by an Iron(II)/EDTA/Ascorbate System Preferentially Attacks Tryptophan Residues of the Protein. *Agric Biol Chem* 1989; 53: 3285-92.
- [42] Uchida KK, S. Ascorbate-Mediated Specific Modification of Histidine-Containing Peptides. *J Agric Food Chem* 1989; 37: 897-901.
- [43] Stadtman ER, Berlett BS. Fenton chemistry. Amino acid oxidation. *J Biol Chem* 1991; 266: 17201-11.
- [44] Knepp VM, Whatley JL, Muchnik A, Calderwood TS. Identification of antioxidants for prevention of peroxide-mediated oxidation of recombinant human ciliary neurotrophic factor and recombinant human nerve growth factor. *PDA J Pharm Sci Technol* 1996; 50: 163-71.
- [45] Jadhav PD, Shelke DN, Bhohe RA. Mixed ligand chelates of Nickel ion with some aminocarboxylic, dicarboxylic and phenolic acids. *J Inorg Nucl Chem* 1978; 40: 572-4.
- [46] Halliwell B, Gutteridge JM. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* 1986; 246: 501-14.
- [47] Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* 1993; 62: 797-821.
- [48] Zhao F, Yang J, Schoneich C. Effects of polyaminocarboxylate metal chelators on iron-thiolate induced oxidation of methionine- and histidine-containing peptides. *Pharm Res* 1996; 13: 931-8.
- [49] Kim YH, Berry AH, Spencer DS, Stites WE. Comparing the effect on protein stability of methionine oxidation versus mutagenesis: steps toward engineering oxidative resistance in proteins. *Protein Eng* 2001; 14: 343-7.
- [50] Bava KA, Gromiha MM, Uedaira H, Kitajima K, Sarai A. ProTherm, version 4.0: thermodynamic database for proteins and mutants. *Nucleic Acids Res* 2004; 32: D120-1.
- [51] Vogt W. Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med* 1995; 18: 93-105.
- [52] Khossravi M, Shire SJ, Borchardt RT. Evidence for the involvement of histidine A(12) in the aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. *Biochemistry* 2000; 39: 5876-85.
- [53] Sawaya MR, Sambashivan S, Nelson R, *et al.* Atomic structures of amyloid cross-beta spines reveal varied steric zippers. *Nature* 2007; 447: 453-7.
- [54] Fernandez-Escamilla AM, Rousseau F, Schymkowitz J, Serrano L. Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins. *Nat Biotechnol* 2004; 22: 1302-6.
- [55] Tartaglia GG, Cavalli A, Pellarin R, Caflisch A. Prediction of aggregation rate and aggregation-prone segments in polypeptide sequences. *Protein Sci* 2005; 14: 2723-34.
- [56] Wang X, Singh SK, Kumar S. Potential aggregation-prone regions in complementarity-determining regions of antibodies and their contribution towards antigen recognition: a computational analysis. *Pharm Res* 2010; 27: 1512-29.
- [57] Kato Y, Kitamoto N, Kawai Y, Osawa T. The hydrogen peroxide/copper ion system, but not other metal-catalyzed oxidation systems, produces protein-bound dityrosine. *Free Radic Biol Med* 2001; 31: 624-32.
- [58] Liu Y, Sun G, David A, Sayre LM. Model studies on the metal-catalyzed protein oxidation: structure of a possible His-Lys cross-link. *Chem Res Toxicol* 2004; 17: 110-8.
- [59] Uversky VN, Yamin G, Souillac PO, Goers J, Glaser CB, Fink AL. Methionine oxidation inhibits fibrillation of human alpha-synuclein *in vitro*. *FEBS Lett* 2002; 517: 239-44.
- [60] Mulinacci F, Poirier E, Capelle MA, Gurny R, Arvinte T. Enhanced physical stability of human calcitonin after methionine oxidation. *Eur J Pharm Biopharm* 2011; 78: 229-38.
- [61] Kirberger M, Wang X, Deng H, Yang W, Chen G, Yang JJ. Statistical analysis of structural characteristics of protein Ca2+-binding sites. *J Biol Inorg Chem* 2008; 13: 1169-81.
- [62] Zhao F, Ghezzi-Schoneich E, Aced GI, Hong J, Milby T, Schoneich C. Metal-catalyzed oxidation of histidine in human growth hormone. Mechanism, isotope effects, and inhibition by a mild denaturing alcohol. *J Biol Chem* 1997; 272: 9019-29.
- [63] Saboury AA, Atri MS, Sanati MH, Moosavi-Movahedi AA, Haghbeen K. Effects of calcium binding on the structure and stability of human growth hormone. *Int J Biol Macromol* 2005; 36: 305-9.
- [64] Wang W, Vlasak J, Li Y, *et al.* Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Mol Immunol* 2011; 48: 860-6.
- [65] Lopez de la Paz M, Serrano L. Sequence determinants of amyloid fibril formation. *Proc Natl Acad Sci USA* 2004; 101: 87-92.
- [66] Christensen S, Moeller EH, Bonde C, Lilleoere AM. Preliminary studies of the physical stability of a glucagon-like peptide-1 derivative in the presence of metal ions. *Eur J Pharm Biopharm* 2007; 66: 366-71.
- [67] Yamin G, Glaser CB, Uversky VN, Fink AL. Certain metals trigger fibrillation of methionine-oxidized alpha-synuclein. *J Biol Chem* 2003; 278: 27630-5.
- [68] Derrick TS, Kashi RS, Durrani M, Jhingan A, Middaugh CR. Effect of metal cations on the conformation and inactivation of recombinant human factor VIII. *J Pharm Sci* 2004; 93: 2549-57.
- [69] Long X, Zhang C, Cheng J, Bi S. A novel method for study of the aggregation of protein induced by metal ion aluminum(III) using resonance Rayleigh scattering technique. *Spectrochim Acta A Mol Biomol Spectrosc* 2008; 69: 71-7.
- [70] Golts N, Snyder H, Frasier M, Theisler C, Choi P, Wolozin B. Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. *J Biol Chem* 2002; 277: 16116-23.
- [71] Miura T, Suzuki K, Kohata N, Takeuchi H. Metal binding modes of Alzheimer's amyloid beta-peptide in insoluble aggregates and soluble complexes. *Biochemistry* 2000; 39: 7024-31.

- [72] Suzuki K, Miura T, Takeuchi H. Inhibitory effect of copper(II) on zinc(II)-induced aggregation of amyloid beta-peptide. *Biochem Biophys Res Commun* 2001; 285: 991-6.
- [73] Miura T, Hori-i A, Mototani H, Takeuchi H. Raman spectroscopic study on the copper(II) binding mode of prion octapeptide and its pH dependence. *Biochemistry* 1999; 38: 11560-9.
- [74] Jiang Y, Nashed-Samuel Y, Li C, *et al.* Tungsten-induced protein aggregation: solution behavior. *J Pharm Sci* 2009; 98: 4695-710.
- [75] Zhang G, Keita B, Craescu CT, Miron S, de Oliveira P, Nadjio L. Polyoxometalate binding to human serum albumin: a thermodynamic and spectroscopic approach. *J Phys Chem B* 2007; 111: 11253-9.

Received: April 17, 2013

Accepted: May 21, 2013