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RESEARCH PAPER

Metal-Mediated Protein Oxidation: Applications of a Modified ELISA-Based Carbonyl Detection Assay for Complex Proteins

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ABSTRACT

Purpose Therapeutic proteins are prone to oxidative modification during manufacturing, processing, and storage that may lead to degradation, aggregation, and immunogenicity. Protein carbonylation is an irreversible oxidative modification and has been identified as a hallmark of severe oxidative stress but not extensively studied for its impact on the stability and activity of therapeutic proteins.

Methods We describe the application of a modified ELISA-based method to quantify global levels of carbonyl modification of complex proteins. We investigated protein oxidation of large protein molecules (transferrin, rabbit IgG, or β-glucosidase) and complex protein samples (human plasma) that were either stored in different buffer formulations, with varying amounts of divalent iron, or under different storage temperatures to determine the impact of different physicochemical stresses on carbonyl modifications.

Results The modified ELISA allows for sensitive and specific carbonyl quantification with measurements that closely match those determined with the conventional spectrophotometric method. The method was useful for complex protein mixtures such as cell lysates without the need for additional procedures to remove DNA and RNA. Our findings demonstrate significant oxidative modification of each of the proteins stored in commonly used buffers and excipients at 37°C, 23°C, and 4°C. The carbonyl levels were further exacerbated with addition of trace amounts of Fe^{2+}. We also measured the extent of protein aggregation under oxidizing conditions.

Conclusions Collectively, our results indicate the importance of better characterizing carbonyl modification of proteins during their storage and use.

KEY WORDS biopharmaceuticals · carbonyl · leachables · polysorbates · stability

INTRODUCTION

Characterization of protein therapeutics elicits challenges that are unique when compared to chemically-synthesized small molecules. Proteins are significantly larger and inherently more complex in structure. In addition to post-translational modifications (PTMs) such as glycosylation, phosphorylation, acetylation, and deamidation that take place *in vivo* (1,2), protein biopharmaceuticals are also susceptible to other modifications such as oxidation and fragmentation during purification, manufacturing, and storage (3–6). PTMs can affect physicochemical characteristics of proteins which can potentially lead to changes in secondary and tertiary structure, solubility, and/or degradation by proteolytic as well as non-proteolytic means. PTMs can also cause loss of function/activity, aggregation, and possibly produce changes in immunogenicity (5,7–14). Monitoring to ensure the quality of pharmaceuticals within the pre-defined physicochemical characteristics is pertinent for stability, safety, and efficacy of the products.

Most amino acid side chains are candidates for oxidative modification by a variety of mechanisms (3,4,15). Oxidative modifications of cysteine and methionine residues have been well characterized. Methionine oxidation to methionine sulfoxide and various other oxidative modifications are reported to be associated with the loss of potency and stability in a number of biopharmaceuticals (16–19). As a consequence of metal-catalyzed oxidation, oxygen radicals, and ionizing radiation, carbonyl covalent modification can be introduced into

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the amino acid side chains of arginine, lysine, proline, and threonine (15,20). Carbonylation involves the formation of a carbonyl (C=O, aldehyde or ketone) moiety on the side chains. Hydroxyl radical-mediated carbonylation can also mediate cleavage of proteins in cells and purified proteins *in vitro* (21–25). Appropriately, the accumulation of carbonyl residues has been used as a hallmark of excessive oxidative stress and protein oxidative modification (4,26,27).

Considering the potentially critical effect of oxidative modification on the stability, efficacy, and safety of therapeutic proteins, it is pertinent to develop sensitive and reliable methodologies to address protein oxidation throughout manufacturing, processing, storage, and use. For examples of carbonylation of proteins related to therapeutic usage, see references (28,29) on studies with human serum albumin and plasma.

Most carbonyl assays rely on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl moiety of amino acid side chains, resulting in a stable covalent bond between DNP (dinitrophenylhydrazone) and the protein (26,27,30,31). DNP is then detected and quantified either spectrophotometrically using the 370 nm absorption, ELISA, or Western blot immunoassay (26,27,31–33). While carbonyl quantification by the currently available ELISA has been shown to exhibit some correlation with the spectrophotometric method, necessity of nucleic acid removal from complex biological samples might limit its wide application. An optimized method with higher correlation with the spectrophotometric method and greater sensitivity to low level of carbonyls without nucleic acid removal is needed to allow the methodology amenable to complex protein samples and medium-throughput applications. In this report, we describe a modified ELISA-based method in which a number of experimental conditions are optimized. We specifically address the correlation between ELISA and standard spectrometric method, as well as evaluate the potential interference of nucleic acid. The results indicate that the modified ELISA combines sensitivity, simplified methodology, and medium-throughput capability for quantification of protein carbonyls in purified protein samples as well as complex biological specimens.

Using this method, we demonstrate oxidation/carbonylation of proteins and human plasma samples that have undergone accelerated oxidation by an iron-mediated Fenton reaction using different formulation buffers and elevated storage temperatures. The results also indicate significant oxidation/carbonylation of samples stored under routine refrigeration. To date, the effects of carbonylation during manufacturing, processing, and storage of large proteins have not been extensively studied. The availability of newer technologies, more sensitive reagents, and the ease of streamlining an ELISA format might allow for a better understanding of oxidative carbonyl modification on protein biopharmaceuticals under manufacturing and storage conditions.

MATERIALS AND METHODS

Chemicals and Antibodies

Unless noted otherwise, all chemicals were from Sigma-Aldrich (St. Louis, MO). Rabbit IgG, almond β-glucosidase, transferrin, lyophilized human plasma, and bovine serum albumin (BSA) were obtained from Sigma. 3,3,5,5-tetramethylbenzidine (TMB Blue Substrate-Chromogen) and HRP-conjugated rabbit anti-goat IgG were purchased from Dako (Carpinteria, CA). Affinity purified goat anti-DNP antibody was from Bethyl Laboratory (Montgomery, TX). The Nunc Maxisorp 96-well plates were obtained from EMD Millipore (Billerica, MA).

Preparation of Oxidized and Reduced Bovine Serum Albumin and Preparation of Standard Solutions

BSA (Cat No. A7906) was dissolved in phosphate buffered saline [PBS (EMD Millipore Cat No. 21–0400)] to a concentration of 10 mg/ml at pH 7.4 and oxidized by incubating with 5 mM NaOCl for 30 min at 23°C followed by extensive dialysis against PBS at 4°C. A 200-fold volume of PBS was used and the buffer was exchanged three times during the dialysis. The carbonyl content was determined spectrophotometrically as described below using an extinction coefficient of DNP $\varepsilon_{370} = 22{,}000\ M^{-1}\ cm^{-1}$ and BSA $\varepsilon_{280} = 44{,}000\ M^{-1}\ cm^{-1}$. The oxidized BSA (oxBSA) preparation used throughout this work contained 23 nmol carbonyl/mg BSA, or 1.5 carbonyl residues per BSA molecule. To prepare the BSA standards for the modified carbonyl ELISA, oxBSA (23 nmol carbonyl/mg) was diluted in PBS containing 0.0125% Triton X-100 to yield 0.1 to 2.5 nmol of carbonyl/ml. No adjustment for protein concentration was made. Standard solutions were stored at −80°C. Reduced BSA was prepared by incubating BSA (10 mg/ml in PBS) with 20 mM $NaBH_4$ for 30 min at 23°C followed by dialysis against dH_2O.

DNPH Derivatization and Determination of Carbonyl Content of Proteins by Spectrophotometer

The carbonyl content of purified or complex samples were determined spectrophotometrically as described by Levine *et al.* (26) with minor modifications to volume of the reaction and concentration of the protein. Briefly, protein in 0.2 ml of buffer (concentration of 3–4 mg/ml) was derivatized by addition of 0.4 ml of 10 mM DNPH in 2 M HCl for 10 min. The

proteins were precipitated by addition of 100% (w/v) trichloroacetic acid (TCA) to a final concentration of 20%. The precipitate was kept on ice for 10 min followed by washing with a 10% TCA solution. The excess residual free DNPH that was co-precipitated with the protein was removed by washing the precipitate three times with ethanol/ethyl acetate (1:1 v/v). The precipitate was then air dried and re-dissolved in 0.1 ml of a solution containing 6 M guanidine and 20 mM potassium phosphate at pH 2.3. The absorbance at 370 nm and 280 nm were then measured by spectrophotometer (Agilent Model 8453) to determine the carbonyl level and protein concentration. The carbonyl content of the protein (nmol/mg protein) was determined as described by Levine *et al.* (26) using 22,000 for the molar extinction coefficient of DNP at 370 nm. Protein concentration was obtained from the equation $[A_{280}-(0.43\times A_{370})]$ / ε_{280}, where ε_{280} is specific to each protein and the $[0.43\times A_{370}]$ subtraction corrects for the DNP contribution to the 280 nm absorbance. Additionally, the free DNPH moiety that co-precipitated with the protein was separated *via* size exclusion chromatography using a Bio-gel P-6 spin column (BioRad, Hercules, CA) and 6 M Gu-HCl solution to address the effectiveness of ethanol/ethyl acetate extraction of DNPH (Supplementary Figure S1).

Accelerated Oxidation of Proteins by Modified Fenton Reaction

Proteins or plasma samples were dissolved in Buffer A (5 mM Na-acetate buffer at pH 5.0, 0.15 M NaCl, and 0.02% Tween 20) or Buffer B (5 mM Na-acetate buffer pH 5.0, 4% mannitol, and 0.02% Tween 20). Accelerated oxidation of protein was achieved by modified Fenton reaction in the presence of 50 μM H_2O_2, 200 μM ascorbic acid, and the indicated concentrations of $FeSO_4$ (see X-axis in Fig. 3). The protein mixtures were kept at 4°C, 37°C, or room temperature for the indicated time and the carbonyl contents were determined. Where indicated, proteins were also oxidized by incubation with 100 μM Fe^{2+} and 25 mM ascorbic acid.

Analysis of Protein Aggregation

Particle formation and turbidity in the protein solution (1 mg/ml) stored under various conditions were initially assessed by measuring 350 nm and 280 nm absorbance. The aggregates were further investigated by dynamic light scattering (Malvern Zetasizer Nano, Malvern Instruments, Westborough, MA) and microfluidic particle imaging analyses (Flowcam, Fluid Imaging, Scarborough, ME). The extent of aggregation was also investigated by binding to a molecular rotor dye, ProteoStat (Catalog no. ENZ-51023-KP002, Enzo Life Sciences, Farmingdale, NY) according to manufacturer instructions. Additionally, we assessed the dimerization/multimerization by gel electrophoresis (4–12% Bis-Tris NuPAGE, Life Technologies, Grand Island, NY).

Modified Carbonyl ELISA Assay

Standard ELISA Conditions

Protein was derivatized by mixing 10 μl protein solution (1 μg/μl), 10 μl 12% SDS (w/v), and 20 μl DNPH (10 mM dissolved in 2 M HCl). After incubation for 10 min at room temperature, 20 μl 2 M Tris-base was added to neutralize the reaction mixture. 3 μl of each DNP-derivatization reaction was mixed with 0.25 ml of adsorption buffer (20 mM $NaHCO_3$, 150 mM NaCl, 0.25% SDS (w/v), pH 8.5) and 0.1 ml of the mix was aliquoted in duplicate into each well of a 96-well Maxisorp plate. Each well contains 0.2 μg derivatized protein. The plate wells were covered with plastic film and incubated overnight at 4°C. The wells were gently rinsed 6–7 times with 0.2–0.3 ml PBST washing buffer (PBS containing 0.05% Tween 20) followed by incubation with 0.2 ml PBST containing 1% BSA at 37°C for 1 h to block non-specific binding sites. After removal of the blocking solution, 0.1 ml goat anti-DNP antibody was added into each well and incubated at room temperature for 1 h (1:2,000 dilution in PBS-T containing 1% BSA). The wells were rinsed gently 6–7 times with 0.2–0.3 ml PBST washing buffer and 0.1 ml rabbit anti–goat IgG antibody, horseradish peroxidase conjugated, was added into each well and incubated at room temperature for 1 h (1:3,000 dilution in PBST containing 1% BSA). After 6–7 rinses with 0.2–0.3 ml PBST washing buffer, the bound secondary antibody was titrated by addition of 0.1 ml TMB substrate equilibrated to room temperature. Typically, sufficient color has developed after 2–5 min of incubation under the experimental conditions described. The reaction was stopped by addition of 0.1 ml stop solution (0.5 M H_2SO_4) followed by measurement of absorbance at 450 nm and 690 nm, a blank reference wavelength. The carbonyl contents of experimental samples were determined from a standard curve generated from the A_{450}-A_{690} reading of oxBSA standard reactions.

RESULTS

Establishing Optimal ELISA Conditions

Standard Reaction Curves for Oxidized BSA and LOD/LOQ of the Assay

We first sought to establish reaction curves for carbonyl content in BSA standard solutions to determine the range and

LOD/LOQ of the ELISA-based assay. The advantage of an ELISA method over other procedures resides in the potential for relatively lower LOD (limit of detection) and LOQ (limit of quantification), and medium throughput applicability. The LOD and LOQ of the assay depend on the difference in color development between the negative controls of the assay and the test samples, as well as the coefficient of variation of the assay. The carbonyl standard solutions are prepared by serially diluting oxidized BSA (oxBSA: oxidized at 23 nmol/mg) in PBS. Under the modified assay condition, the negative controls for the assay included- 1) a DNPH derivatization control where the detection procedure is performed without protein and 2) a negative derivatization control where protein is treated with 2 M HCl instead of DNPH. These negative controls exhibited 0.02–0.05 A_{450}-A_{690} absorbance units above that of a blank well with TMB substrate only.

Figure 1 shows a typical standard curve obtained from analysis of oxBSA. Under the experimental conditions described above, 2–5 min incubation was sufficient to develop a strong signal. Considering the simplified limit of detection (LOD) of an ELISA signal is three times the standard deviation of the negative control, the LOD of the assay is found to be 0.02 pmol/well. Similarly, considering 10 times the standard deviation of the negative control as the limit of quantification (LOQ), this yields 0.05 pmol/well. Figure 1 also shows the dynamic range of the assay where the linear regression curve can be generally applied extends from 0.04 to 0.5 pmol/well or 0.2 to 2.5 nmol/mg protein.

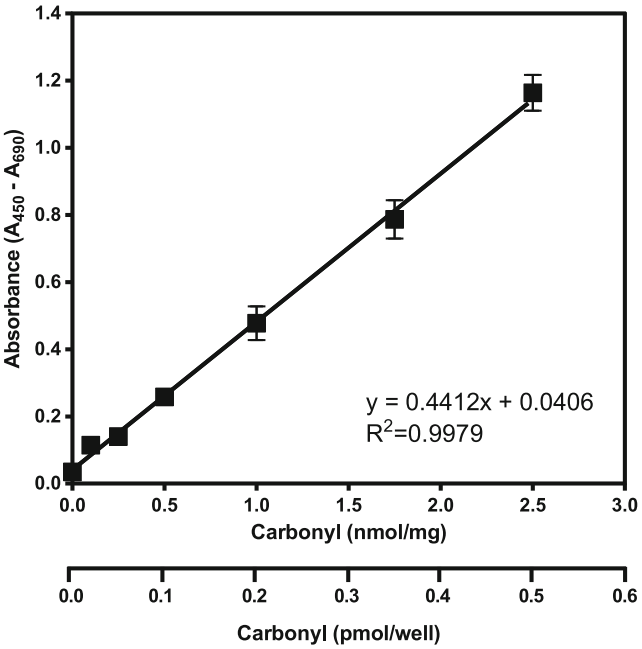


Fig. 1 Standard curve of oxidized BSA. Oxidized BSA standards (0.1, 0.25, 0.5, 1.0, 1.75 and 2.5 nmol carbonyl/ml PBS) were prepared by diluting the stock oxBSA (23 nmol carbonyl/mg protein) in PBS containing 0.0125% Triton X-100. 10 μl of each solution was derivatized and quantified under the standard reaction condition. The values of the Y-axis are averages of duplicate assays. The values on the lower scale bar on the X-axis (pmol/well) indicate DNP (pmol) added to each well. The values of the upper scale bar indicate conversion of this value to carbonyl (nmol/mg) for experimental samples analyzed under the standard assay conditions; for example 0.2 pmol carbonyl/0.2 μg protein/well is equivalent to 1.0 nmol carbonyl/mg protein.

Comparison of the Modified ELISA and Spectrophotometric Assay Formats for Carbonyl Measurement

For carbonyl quantification of purified proteins, spectrophotometric measurement is currently the gold standard to which other detection methodologies are compared (30). Thus, we determined the correlation between carbonyl content of protein obtained by both the modified ELISA and the spectrophotometric methods. Shown in Fig. 2a, the values obtained by the two methods in four protein preparations were very similar and the differences were not statistically significant.

Effect of Nucleic Acid in Complex Samples

Carbonyl moieties in DNA and RNA can also react with DNPH (34). Therefore, carbonyl contents can be overestimated in complex samples such as tissue extract and plasma that may contain nucleic acid. To avoid this potential problem, a streptomycin precipitation of nucleic acid prior to or after derivatization, has been suggested (34,35). To investigate the effect of nucleic acids in protein carbonyl quantification by the modified ELISA, an oxidized BSA sample was spiked with 2 µg (20% w/w) or 0.5 µg (5% w/w) of herring sperm DNA or yeast tRNA, respectively, and the carbonyl contents were measured and compared to un-spiked samples. As shown in Fig. 2b, no significant increase in carbonyl level over control samples was observed, indicating the added nucleic acid (up to 20% w/w) did not interfere with the modified ELISA. Thus, the tested ELISA conditions appear to allow quantification of protein carbonyl without the need to eliminate contaminating nucleic acid.

Effect of Detergents on the Assay

The ELISA method described here utilizes binding of DNP-derivatized proteins to polystyrene surfaces *via* hydrophobic interaction. Because the binding is *via* hydrophobic interaction, detergents might potentially interfere with the binding. We investigated the effect of various detergents on the protein binding by adding them to the adsorption buffer. Commonly used detergents for cell/

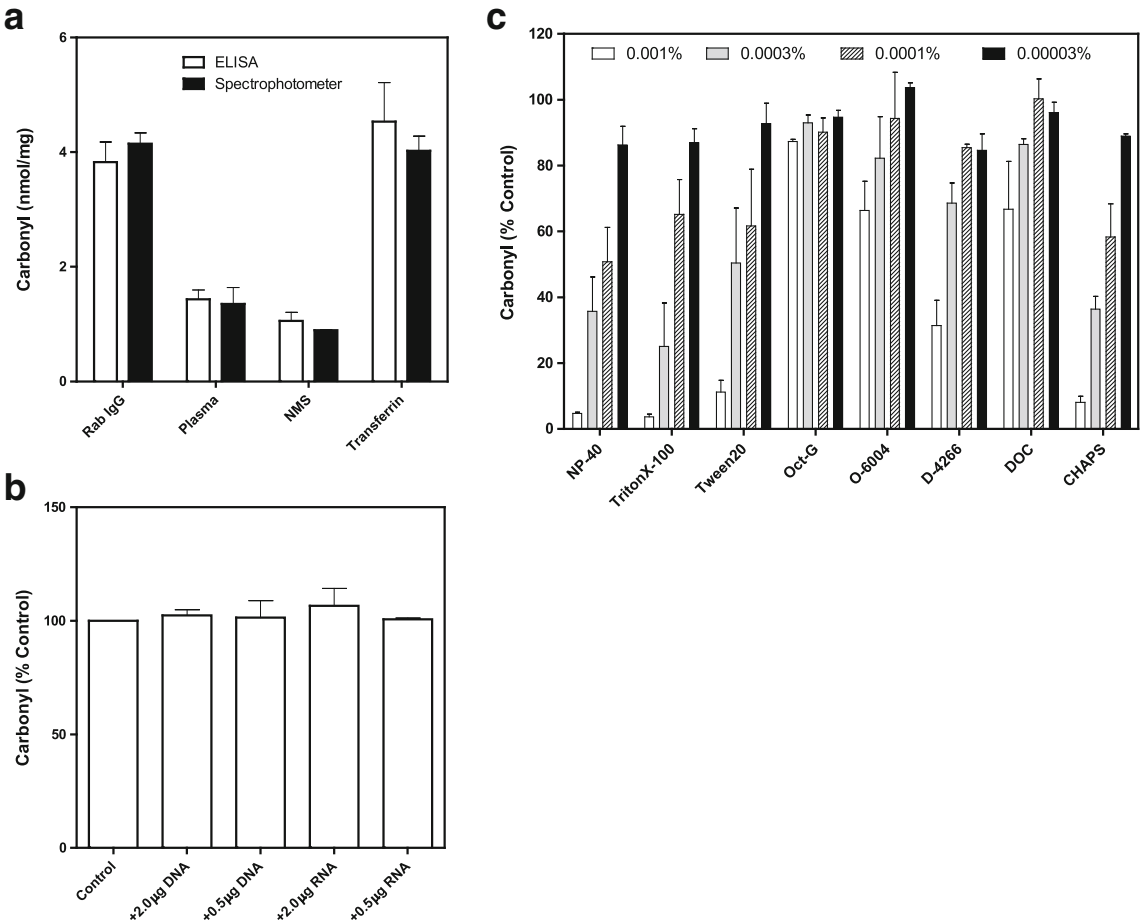


Fig. 2 (**a**) Comparison of ELISA assay and spectrophotometric measurement. Lyophilized human plasma and purified proteins were oxidized with 100 μM Fe^{2+} and 25 mM ascorbic acid and the carbonyl contents were determined by ELISA or spectrophotometric measurement. The effectiveness of free DNPH removal by ethanol/ethyl acetate extraction prior to spectrophotometric measurement was confirmed by comparing to samples where the DNPH was removed by size exclusion utilizing Bio-gel P-6 micro spin column (Supplementary Figure S1). (**b**) Effect of nucleic acid contamination on the protein carbonyl quantitation. The indicated amount of DNA or RNA was added to 10 μg of oxBSA in the standard derivatization reaction, and the carbonyl contents were determined. The Y-axis represents values relative (%) to control without nucleic acid added. (**c**) Effect of detergent on the adsorption and detection of DNPH-derivatized BSA. Oxidized BSA standard at 1 nmol carbonyl/mg protein was derivatized under standard conditions and subjected to ELISA with or without the indicated concentrations (% final) of detergent in the adsorption buffer. ELISA signal (A_{450}-A_{690}) relative to control reaction where no detergent was added was determined. Oct-Glc: Octyl-β-D-glucopyranoside; O-6004: Octyl-β-D-1-thioglucopyranoside; D-4266: 3-(Decyldimethylammonio)propanesulfonate inner salt; DOC: Deoxychorate sodium salt. These data are average of two independent experiments.

tissue lysate preparation with low critical micelle concentration (CMC) including NP-40, Triton X-100, and Tween-20 interfere with the protein adsorption when present in as low as 0.0001% (Fig. 2c). A zwitterionic detergent, CHAPS, was also found to interfere with the assay. In contrast, anionic detergent Deoxycholate (DOC) and non-ionic detergents with high CMC such as octyl-β-D-glucopyranoside and Octyl-β-D-thioglucopyranoside (O-6004) appeared to interfere minimally while 3-(Decyldimethylammonio) propane sulfonate inner salt (at low concentrations) (D-4266) showed interference at approximately 15% at concentrations of 0.0001 and 0.00003%. Thus, our observation suggests that non-ionic detergents with low CMC may interfere with the assay while non-ionic detergents with a high CMC or an anionic detergent might be more compatible with the assay protocol we tested.

Metal-Catalyzed Oxidation of Complex Proteins Under Stressed Conditions

Effect of Iron, Temperature and Excipient on Protein Oxidation/Carbonylation

Trace amount of metals can leach out from various sources such as stainless steel containers, prefilled syringes, and container closure system components that are used during protein manufacturing, storage, shipping, and clinical use (36). Furthermore, polysorbate 20 and 80, nonionic detergents used in biopharmaceutical formulation for preventing aggregation

and promoting stability, may form peroxides at elevated temperatures or upon exposure to light (37). Thus, it is possible that unexpected metal catalyzed oxidation of protein pharmaceuticals could take place under certain stressful conditions (38,39). To demonstrate the potential utility of the carbonyl quantification for monitoring the metal-catalyzed oxidative modification of protein therapeutics, we conducted preliminary studies on iron-mediated protein oxidation.

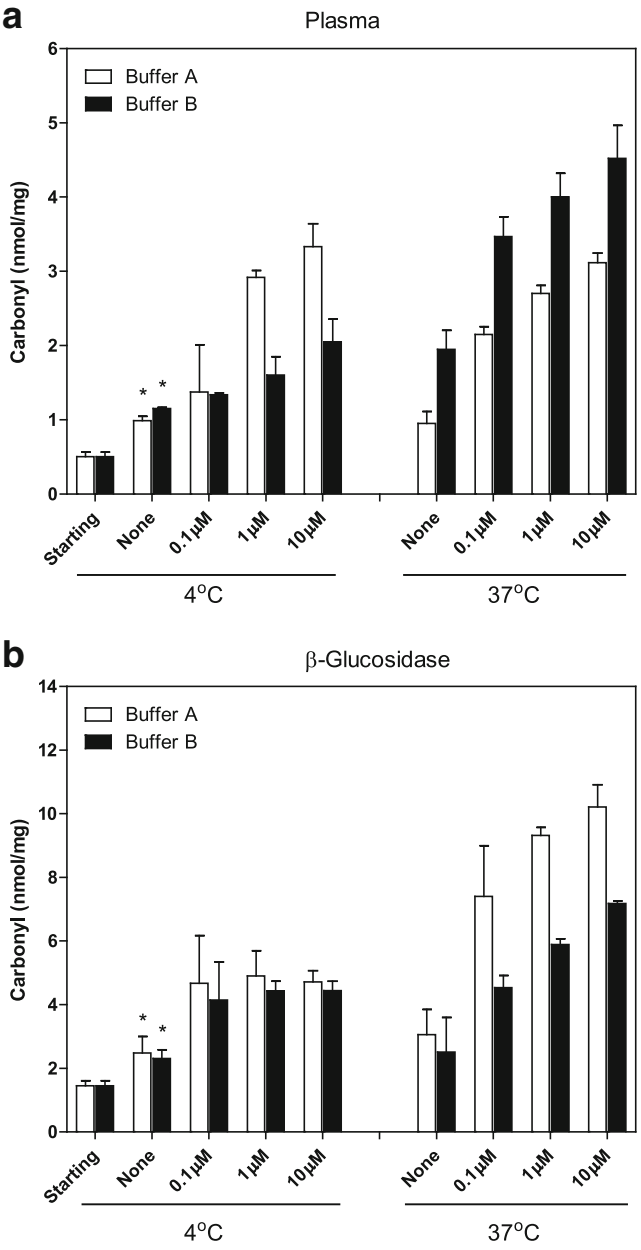
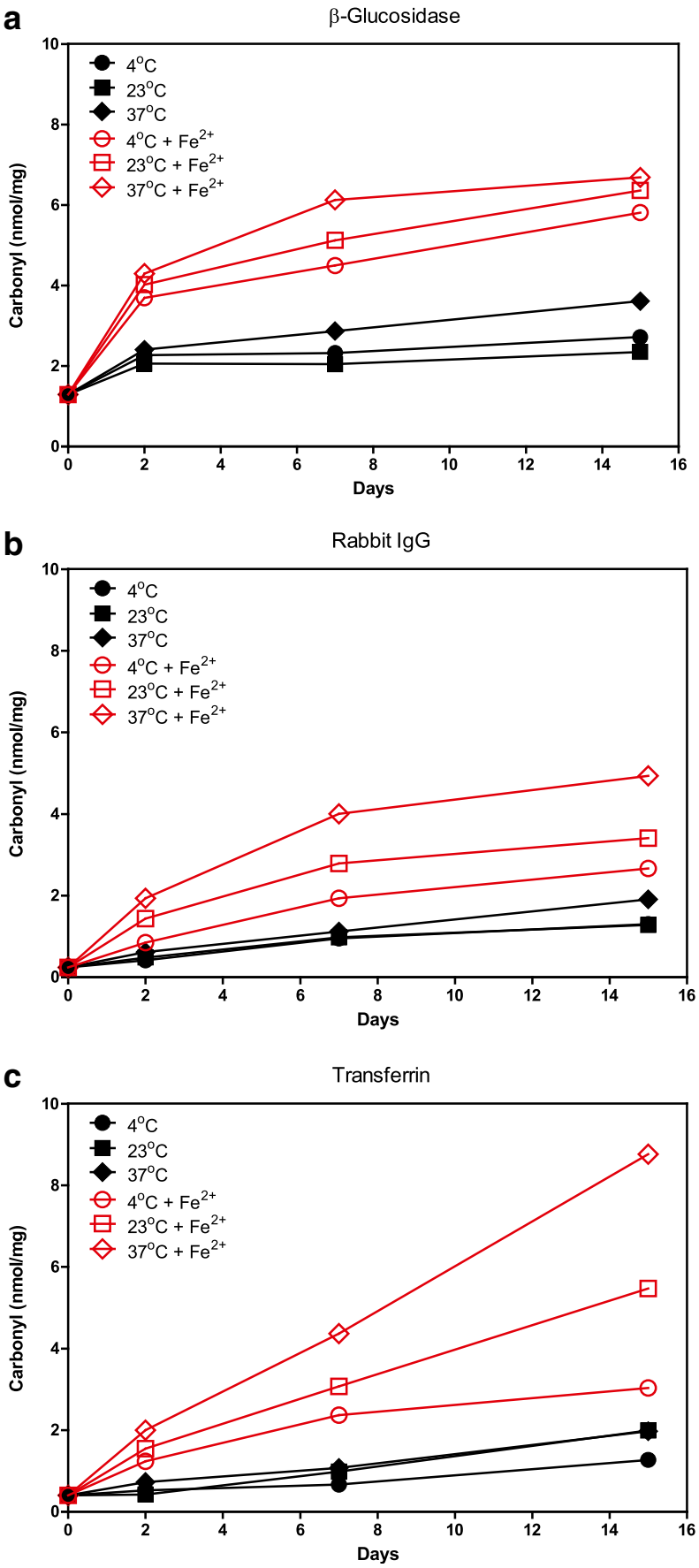


Fig. 3 Effect of iron concentration, storage temperature, and buffer composition on oxidation/carbonylation. Lyophilized human plasma (panel **a**) or almond β-glucosidase (panel **b**) was reconstituted in Buffer A or Buffer B with the indicated concentrations of iron at either 4°C or 37°C. Buffer A contained 5 mM Na-acetate buffer at pH 5.0, 0.15 M NaCl, and 0.02% Tween 20, while Buffer B additionally contained 4% mannitol but did not contain NaCl. These data are an average of two independent experiments. * $p < 0.05$ compared to initial starting preparation (labeled "Starting").

Fig. 4 Time course of protein carbonylation. Samples were prepared in Buffer B with 0.2 μM Fe^{2+}/H_2O_2/ascorbic acid and stored at 4°C, 23°C, or 37°C for up to 2 weeks. β−Glucosidase (panel **a**); Rabbit IgG (panel **b**); or Transferrin (panel **c**) were each tested with or without Fe^{2+}. The data represent an average of two independent experiments.

We first analyzed the effect of Fe^{2+} and storage temperature on the oxidation/carbonylation of lyophilized plasma proteins (Fig. 3a) and almond β-glucosidase (Fig. 3b) prepared in two sodium acetate buffers: Buffer A containing 0.15 M NaCl and 0.02% Tween 20 or Buffer B containing 0.02% Tween 20 and 4% mannitol, an excipient commonly used as a stabilizer in drug formulation (40). A varying amount of Fe^{2+} ranging from 0.1 μM to 10 μM was added to initiate the modified Fenton reaction and samples were stored at either 4°C or 37°C. After 6 days of incubation, the protein carbonyl level was quantified by the modified ELISA. The addition of as little as 0.1 μM Fe^{2+} increased the carbonyl level significantly in both plasma proteins and β-glucosidase as compared to those without added iron (Fig. 3). The extent of carbonylation was Fe^{2+} concentration dependent except β-glucosidase stored at 4°C. Mannitol in the buffer showed complex and opposing effects depending on the protein investigated and storage temperature. The addition of mannitol reduced carbonyl levels in plasma proteins and β-glucosidase stored at 4 and 37°C respectively, while apparently increasing carbonyl levels in plasma sample stored at 37°C. Mannitol had little effect on the carbonylation of β-glucosidase stored at 4°C suggesting a protein-specific response to the presence of mannitol. Notably, carbonyl levels in both plasma proteins and β-glucosidase increased significantly during storage at 4°C without addition of Fe^{2+}. Following 6 days of storage at 4°C the carbonyl content of plasma protein increased from 0.4 nmol/mg in the initial preparations (marked as "Starting" on the X-axes of Fig. 3a) to 1.0 nmol/mg. Similarly, the carbonyl content of β-glucosidase stored for 6 days increased from 1.3 to 2.3 nmol/mg.

We next investigated the time course of oxidation/carbonylation of protein stored at various temperatures in the presence of 0.2 μM Fe^{2+} (Fig. 4). β-glucosidase, rabbit IgG, and transferrin were prepared in Buffer B, and the protein carbonyl levels were monitored during storage over 2 weeks. We chose Buffer B because of the potential stabilizing effect of mannitol during the extended period of incubation. All test proteins revealed an iron-, temperature-, and storage time-dependent increase in carbonylation while each protein exhibited individual levels and kinetics of carbonyl formation (Fig. 4). In the case of rabbit IgG and β-glucosidase with Fe^{2+}/H_2O_2/ascorbic acid, the carbonyl levels reached a plateau after incubation for 2 weeks at 5 nmol/mg (0.75 mol carbonyl/mol protein) and 7 nmol/mg (0.4 mol carbonyl/


a
β-Glucosidase
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4°C
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mol protein), respectively, while the carbonylation of transferrin continued to increase.

Oxidation/Carbonylation and Aggregation

In order to address whether the oxidation/carbonylation is associated with protein aggregation, we first utilized the A_{350}/A_{280} absorbance ratio of the solution as an indicator of light scatter due to protein aggregation (38,39). As shown in Fig. 5a and b, the A_{350}/A_{280} ratio increased significantly for β-glucosidase stored at 37°C with or without added Fe^{2+}/H_2O_2/ascorbic acid, while moderate aggregation was seen in the sample spiked with Fe^{2+}/H_2O_2/ascorbic acid and incubated at 23°C for 2 weeks. No increase was observed in the A_{350}/A_{280} ratio for any of the samples of rabbit IgG or transferrin. This observation was then confirmed by dynamic light scattering and by microfluidic particle imaging analyses using FlowCam. As shown in Fig. 5c, derived count rate in dynamic light scatter measurement indicates the formation of β-glucosidase aggregates but not rabbit IgG or transferrin (Supplementary Figure S2A). The β-glucosidase aggregate is large enough so that supernatant fraction following centrifugation at 13,000 rpm × 10 min shows little dynamic light scattering (see last 2 bars on graph in Fig. 5c). The microfluidic imaging analysis showed the aggregates in β-glucosidase solution stored at 37°C are over 4 μm in diameter and the size increases further with oxidation/carbonylation mediated by Fe^{2+} (Fig. 5d). Next, we tested protein aggregation by using a molecular rotor dye (ProteoStat) that is suggested to fluoresce from the torsional constraint of binding to protein aggregates (41). Consistent with the results obtained from analysis by dynamic light scattering and microfluidic particle imaging, the dye showed increased fluorescence with β-glucosidase stored at 37°C but not rabbit IgG or transferrin (Fig. 5d and Supplementary Figure S2). To examine whether the aggregates are formed through intermolecular disulfide bonds, the protein samples were subjected to denaturing SDS-PAGE analysis in the presence or absence of reducing agent (data not shown). Bands corresponding to dimers or multimers were not observed for any of the samples, indicating the aggregates of β-glucosidase do not undergo covalent aggregation under these conditions.

DISCUSSION

We have developed a modified carbonyl ELISA method with a number of slight modifications to previously published procedures (32,33). The modifications include DNPH reaction conditions, adsorption buffer composition, DNP detection reagents, and the setting of the negative control for the experimental samples. For instance, the procedure reported here uses 0.2 μg of DNP-derivatized protein/well, an amount below the limit of protein binding capacity of the polystyrene well (0.25–0.3 μg/well) *versus* a saturated amount (1.0 μg/well) employed by Buss *et al.* (32). Under saturated conditions, the size, shape, and hydrophobicity of individual proteins can influence adsorption to the polystyrene surface and affect the accessibility of the primary detection antibody. Second, the carbonyl standard solutions we used in this investigation were prepared by serial dilution of oxidized BSA based on carbonyl content and not total protein concentration. This experimental condition was employed based on our observations that protein amounts up to 0.3 μg/well has little effect on adsorption efficiency and that reduced BSA shows low but significant levels of DNPH reactivity. BSA reduced by $NaBH_4$ showed A_{370} equivalent to 0.6–0.8 nmol carbonyl/mg protein and was also detected by ELISA (32). Thus, adjusting protein concentration in BSA standard with reduced BSA, and subtracting the value of reduced BSA as the negative control of the assay may potentially underestimate the carbonyl content of experimental samples (32). The impact of the modifications collectively achieve: 1) sensitive and reliable detection of carbonyl residues using 10 μg of protein, 2) carbonyl values that match closely those determined with the conventional spectrophotometric method, and 3) carbonyl quantification of complex protein samples without the interference of nucleic acid. Close correlation of carbonyl values obtained by the modified carbonyl ELISA and spectrophotometric assay and elimination of the additional nucleic acid removal step greatly enhances the utility of the ELISA format for rapid quantification of oxidation/carbonylation in a wide variety of protein samples.

Under the standard assay conditions, we obtained an LOQ of 0.05 pmol carbonyl/well, indicating the assay can quantify an increase of 0.25 nmol/mg protein carbonylation (equivalent to 0.017 mol/mol of MW 50 kDa protein). This sensitivity represents a significant advantage over the spectrophotometric method (26,27) and roughly translates to being able to detect a change of 0.006 A_{370} units for every 1.0 A_{280} unit (assuming the A_{280} of 1 mg/ml protein is 1.0).

This initial study applying the methodology demonstrates the potential importance of monitoring protein oxidation/carbonylation. Results shown in Fig. 3 indicate: 1) proteins can be oxidized even during storage at 4°C, 2) presence of very low concentrations of added Fe^{2+} increase the oxidation/carbonylation, and 3) mannitol, a common excipient, exerts differing effects on carbonylation depending on the protein, storage temperature, and buffer composition. The exact relationship between the presence of mannitol and the physicochemical characteristics of a protein is not known and will require focused studies on the higher-order structure of individual proteins and their mechanisms for metal-catalyzed oxidation. The iron concentrations used in our investigation (0.1 and 0.2 μM) are similar to those observed by Zhou *et al.*

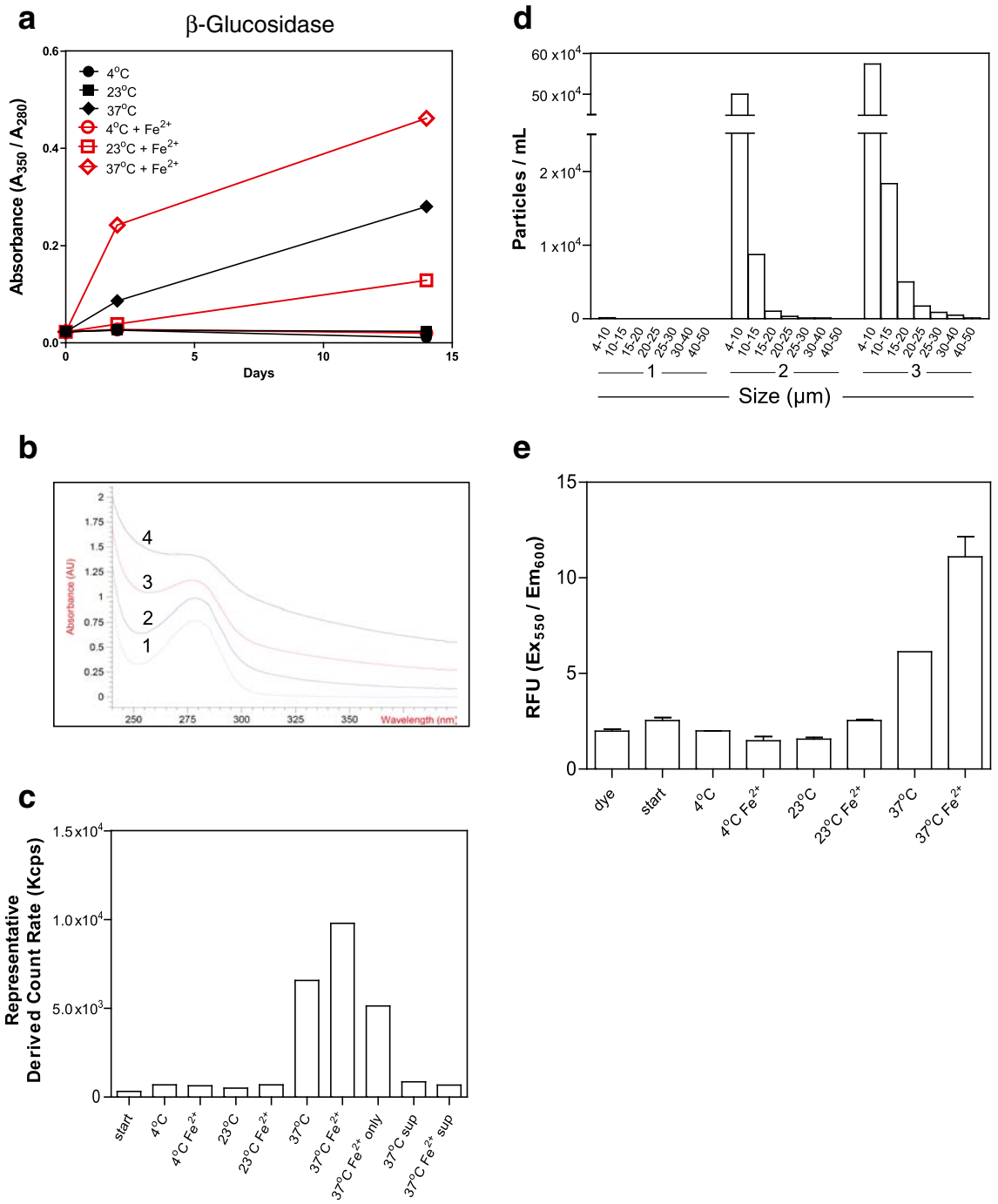

Fig. 5 Analysis of β-glucosidase aggregation. (**a**) The A_{350}/A_{280} ratio of β-glucosidase solution stored for 2 or 14 days was measured with or without Fe^{2+}. The ratio indicates light scattering caused by protein aggregates. (**b**) Representative spectrogram of β-glucosidase stored for 14 days under various conditions. The samples are labeled as follows: (*line 1*) at 4°C, (*line 2*) at 23°C with Fe^{2+}/H_2O_2/ascorbic acid, (*line 3*) at 37°C, (*line 4*) at 37°C with Fe^{2+}/H_2O_2/ascorbic acid. (**c**) Analysis of β-glucosidase aggregation using Zetasizer dynamic light scattering. β-glucosidase was stored in Buffer B for 7 days with or without Fe^{2+}/H_2O_2/ascorbic acid at various temperatures. Y-axis shows representative derived count rates (Kcps: Kilo counts per second). Starting refers to the starting β-glucosidase solution before storage. 37°C- sup or 37°C+ Fe^{2+} sup denote supernatant fraction after centrifugation at 13,000 × 10 min of corresponding sample. (**d**) Distribution of the aggregate size analyzed by microfluidic particle imaging. The X-axis indicates the representative size distribution of aggregates in micron. (*1*) β-glucosidase stored at 4°C without Fe^{2+}/H_2O_2/ascorbic acid; (*2*) β-glucosidase stored at 37°C without Fe^{2+}/H_2O_2/ascorbic acid; (*3*) β-glucosidase stored at 37°C with Fe^{2+}/H_2O_2/ascorbic acid. (**e**) Binding of a molecular rotator dye to β-glucosidase. 25 μg of β-glucosidase was mixed with dye as described in the methods section. Y-axis denotes relative fluorescence emission (excitation 550 nm/emission 600 nm). Dye only control had no added protein, Starting refers to the starting β-glucosidase solution before storage.

where the authors measured iron leachate at 20–30 ppb concentration in a buffer using stainless steel coupons immersed for extended periods of time (36). Therefore, while the results shown here are set to induce protein oxidation at an accelerated rate, it is theoretically plausible that Fe^{2+} mediated protein oxidation/carbonylation can take place under routine storage conditions.

The differential effect of Fe^{2+} mediated protein oxidation/carbonylation on physicochemical characteristics was further demonstrated by analysis of aggregation. As shown in Fig. 5, large aggregates were formed in β-glucosidase solution after 7 days of storage at 37°C and this aggregation was further exacerbated with addition of Fe^{2+}/H_2O_2/ascorbic acid. No aggregation was observed with β-glucosidase stored at 23°C with or without Fe^{2+}/H_2O_2/ascorbic acid. Collectively, these findings indicate that storage temperature might be a primary triggering factor for aggregation of β-glucosidase and suggest that oxidation/carbonylation exacerbates this aggregation.

Taken together, these results demonstrate the validity of carbonyl quantitation to address protein oxidation that can take place under normal as well as stressed conditions. Clearly, the results shown here are snapshots of protein oxidation/carbonylation under narrow, specified set of stressed conditions, and more studies are needed to dissect the exact dynamics and kinetics of carbonylation for a protein of interest and to determine its criticality for therapeutic proteins. Multiple factors such as pH, buffer composition, storage temperature, agitation, in-use conditions, excipients, and presence of trace amounts of metal may likely affect oxidation in a protein-specific manner (19,42,43). The unique response displayed by each protein to Fe^{2+} mediated oxidation/carbonylation, with or without mannitol, emphasizes the importance of formulation optimization specific to each protein drug product.

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REFERENCES

1. Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol. 2006;24(10):1241–52.
2. Mann M, Jensen ON. Proteomic analysis of post-translational modifications. Nat Biotechnol. 2003;21(3):255–61.
3. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J. 1997;324(Pt 1):1–18.
4. Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev. 2000;32(3–4):307–26.
5. Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri AM, *et al.* Protein oxidation and cellular homeostasis: emphasis on metabolism. Biochim Biophys Acta. 2007;1773(2):93–104.
6. Torosantucci R, Schoneich C, Jiskoot W. Oxidation of therapeutic proteins and peptides: structural and biological consequences. Pharm Res. 2014;31(3):541–53.
7. Sola RJ, Griebenow K. Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci. 2009;98(4):1223–45.
8. Jenkins N, Murphy L, Tyther R. Post-translational modifications of recombinant proteins: significance for biopharmaceuticals. Mol Biotechnol. 2008;39(2):113–8.
9. Tovey MG, Legrand J, Lallemand C. Overcoming immunogenicity associated with the use of biopharmaceuticals. Expert Rev Clin Pharmacol. 2011;4(5):623–31.
10. Torosantucci R, Sharov VS, van Beers M, Brinks V, Schoneich C, Jiskoot W. Identification of oxidation sites and covalent cross-links in metal catalyzed oxidized interferon Beta-1a: potential implications for protein aggregation and immunogenicity. Mol Pharm. 2013;10(6):2311–22.
11. Purohit VS, Middaugh CR, Balasubramanian SV. Influence of aggregation on immunogenicity of recombinant human Factor VIII in hemophilia A mice. J Pharm Sci. 2006;95(2):358–71.
12. Kappos L, Clanet M, Sandberg-Wollheim M, Radue EW, Hartung HP, Hohlfeld R, *et al.* Neutralizing antibodies and efficacy of interferon beta-1a: a 4-year controlled study. Neurology. 2005;65(1):40–7.
13. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian J-J, Martin-Dupont P, *et al.* Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N Engl J Med. 2002;346(7):469–75.
14. Reipert BM, van Helden PMW, Schwarz H-P, Hausl C. Mechanisms of action of immune tolerance induction against factor VIII in patients with congenital haemophilia A and factor VIII inhibitors. Br J Haematol. 2007;136(1):12–25.
15. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem. 1997;272(33):20313–6.
16. Lu HS, Fausset PR, Narhi LO, Horan T, Shinagawa K, Shimamoto G, *et al.* Chemical modification and site-directed mutagenesis of methionine residues in recombinant human granulocyte colony-stimulating factor: effect on stability and biological activity. Arch Biochem Biophys. 1999;362(1):1–11.
17. Pan B, Abel J, Ricci MS, Brems DN, Wang DIC, Trout BL. Comparative oxidation studies of methionine residues reflect a structural effect on chemical kinetics in rhG-CSF. Biochemistry. 2006;45(51):15430–43.
18. Mulinacci F, Capelle MAH, Gurny R, Drake AF, Arvinte T. Stability of human growth hormone: influence of methionine oxidation on thermal folding. J Pharm Sci. 2011;100(2):451–63.
19. Yin J, Chu J-W, Ricci MS, Brems DN, Wang DIC, Trout BL. Effects of excipients on the hydrogen peroxide-induced oxidation of methionine residues in granulocyte colony-stimulating factor. Pharm Res. 2005;22(1):141–7.

20. Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med. 1990;9(4):315–25.
21. Xu G, Chance MR. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. Chem Rev. 2007;107(8):3514–43.
22. Aryal B, Jeong J, Rao VA. Doxorubicin-induced carbonylation and degradation of cardiac myosin binding protein C promote cardiotoxicity. Proc Natl Acad Sci U S A. 2014;111(5):2011–6.
23. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem. 2008;283(32):21837–41.
24. Castro-Acosta RM, Rodriguez-Limas WA, Valderrama B, Ramirez OT, Palomares LA. Effect of metal catalyzed oxidation in recombinant viral protein assemblies. Microb Cell Factories. 2014;13(1):25.
25. Shao CH, Capek HL, Patel KP, Wang M, Tang K, DeSouza C, *et al.* Carbonylation contributes to SERCA2a activity loss and diastolic dysfunction in a rat model of type 1 diabetes. Diabetes. 2011;60(3):947–59.
26. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 1994;233:346–57.
27. Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 1994;233:357–63.
28. Kouno Y, Anraku M, Yamasaki K, Okayama Y, Iohara D, Ishima Y, *et al.* N-acetyl-l-methionine is a superior protectant of human serum albumin against photo-oxidation and reactive oxygen species compared to N-acetyl-l-tryptophan. Biochim Biophys Acta (BBA) Gen Subj. 2014;1840:2806–12.
29. Madian AG, Diaz-Maldonado N, Gao Q, Regnier FE. Oxidative stress induced carbonylation in human plasma. J Proteome. 2011;74(11):2395–416.
30. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta. 2003;329(1–2):23–38.
31. Shacter E, Williams JA, Lim M, Levine RL. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Radic Biol Med. 1994;17(5):429–37.
32. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. Free Radic Biol Med. 1997;23(3):361–6.
33. Winterbourn CC, Buss IH. Protein carbonyl measurement by enzyme-linked immunosorbent assay. Methods Enzymol. 1999;300:106–11.
34. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, *et al.* Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 1990;186:464–78.
35. Luo S, Wehr NB. Protein carbonylation: avoiding pitfalls in the 2,4-dinitrophenylhydrazine assay. Redox Rep. 2009;14(4):159–66.
36. Zhou S, Schoneich C, Singh SK. Biologics formulation factors affecting metal leachables from stainless steel. AAPS PharmSciTech. 2011;12(1):411–21.
37. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate 80 and protein stability. J Pharm Sci. 2002;91(10):2252–64.
38. Zhao F, Ghezzo-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(14):9019–29.
39. 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(1):58–69.
40. Jorgensen L, Hostrup S, Moeller EH, Grohganz H. Recent trends in stabilising peptides and proteins in pharmaceutical formulation—considerations in the choice of excipients. Expert Opin Drug Deliv. 2009;6(11):1219–30.
41. Shen D, Coleman J, Chan E, Nicholson TP, Dai L, Sheppard PW, *et al.* Novel cell- and tissue-based assays for detecting misfolded and aggregated protein accumulation within aggresomes and inclusion bodies. Cell Biochem Biophys. 2011;60(3):173–85.
42. 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(10):2543–55.
43. Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. J Pharm Sci. 1997;86(11):1250–5.