Site-Specific Characterization of Oxidative Carbonylation on Recombinant Monoclonal Antibodies

By

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ABSTRACT

In the biotechnology industry, oxidative carbonylation as a post-translational modification of protein pharmaceuticals has not been studied in detail. Using Quality by Design (QbD) principles, understanding the impact of oxidative carbonylation on product quality of protein pharmaceuticals, particularly from a site-specific perspective, is critical. However, comprehensive identification of carbonylation sites has so far remained a very difficult analytical challenge for the industry. In this work, for the first time the identification of specific carbonylation sites on recombinant monoclonal antibodies was reported using a new analytical approach via derivatization with Girard's Reagent T (GRT) and subsequent peptide mapping with high-resolution mass spectrometry. Enhanced ionization efficiency and high quality MS^2 data resulted from GRT derivatization were observed as key benefits of this approach, which enabled direct identification of carbonylation sites without any fractionation or affinity enrichment steps. A simple data filtering process was also incorporated to significantly reduce false positive assignments. Sensitivity and efficiency of this approach were demonstrated by identification of carbonylation sites on both unstressed and oxidized antibody bulk drug substances. The applicability of this approach was further demonstrated by identification of 14 common carbonylation sites on three highly similar IgG1s. This approach represents a significant improvement to the existing analytical methodologies and facilitates extended characterization of oxidative carbonylation on recombinant monoclonal antibodies and potentially other protein pharmaceuticals in the biotechnology industry.

Keywords: oxidative carbonylation, critical quality attributes (CQAs), recombinant monoclonal antibody, quality by design (QbD), metal catalyzed oxidation, post-translational modification

INTRODUCTION

Recombinant monoclonal antibodies (mAbs) are an important class of biotherapeutics, with more than 30 mAb drug products approved for various indications and hundreds in clinical trials.[1] Recently, a Quality-by-Design (QbD) paradigm was introduced by the US Food and Drug Administration (FDA) for the pharmaceutical development of biologics.[2] A key part of the QbD approach is establishing critical quality attributes (CQAs) to help in designing control strategies, setting specifications, and defining a proper manufacturing space.[2] For mAb drug development, CQAs are determined through analytical characterization,[3] clinical studies,[4] and risk assessment.[5] Given the heterogeneous nature of mAbs, characterization of various mAb product variants has proven to be a challenging task.[6],[7],[8] In a recent review article, Beck et al. provided a comprehensive summary of the current state of the art in characterizing a wide range of mAb variants,[9] which illustrates the complexity of mAb molecules and highlights the tremendous analytical efforts required. Moreover, new mAb variants are expected to be discovered in the coming years, further contributing to the already complex landscape of mAb characterization.

In biological systems, protein carbonyls can be formed through the α-amidation pathway during oxidative cleavage of the protein backbone, by Michael addition reaction with α-β-unsaturated aldehydes, during glycation and glycoxidation processes, and by metal catalyzed oxidation (MCO).[10-14] Specifically, for oxidative carbonylation by MCO, side chains of amino acids are attacked by free radicals, such as hydroxyl radicals ($^{\cdot}OH$) generated from reactions catalyzed by metal ions, which result in the formation of glutamic semialdehyde from arginine and proline residues, aminoadipic semialdehyde from lysine residue, and 2-amino-3-ketobutyric acid from threonine residue (Figure 1).[15, 16] Due to their implication in many diseases, such as

Alzheimer's diseases, diabetes, and chronic renal failure, these various types of protein carbonylation have attracted significant interest from the medical research community.[10],[12] Additionally, due to its irreversible and unrepairable nature, protein carbonylation is widely adopted as a biomarker to study oxidative stress in biological systems.[13],[14],[17],[18],[19]

In the biotechnology industry, oxidative carbonylation by metal catalyzed oxidation could be particularly relevant since metals are present throughout the manufacturing process.[20,21],[22],[23] For protein pharmaceuticals, oxidative carbonylation, as a post-translational modification, could have a significant effect on protein physicochemical properties. For example, arginine and lysine carbonylation leads to loss of the positive charge on the respective side chains and increase of the molecule hydrophobicity. Proline carbonylation leads to loss of the steric constraint, an important protein stabilizing force. Furthermore, aldehyde groups resulting from oxidative carbonylation can react with adjacent lysine residues to form a Schiff base, generating intra- or inter- molecule cross-links. These changes in turn can cause loss of function and/or accumulation of high molecular weight aggregates.[13],[14] Therefore, oxidative carbonylation potentially presents a risk to product quality, and should be carefully investigated. However, to date, there have been very few reports investigating oxidative carbonylation in this context. Steinmann et al. reported 0.25 mole of protein carbonyls per mole of protein in oxidized human growth hormone.[24] Bee et al. reported a fourfold increase of protein carbonylation to 0.8 mole of carbonyl per mole of mAb in the aggregates absorbed on a stainless steel surface compared to a control mAb sample and proposed that Fe ions leached from the steel were a likely cause for the oxidative carbonylation in their case.[23]

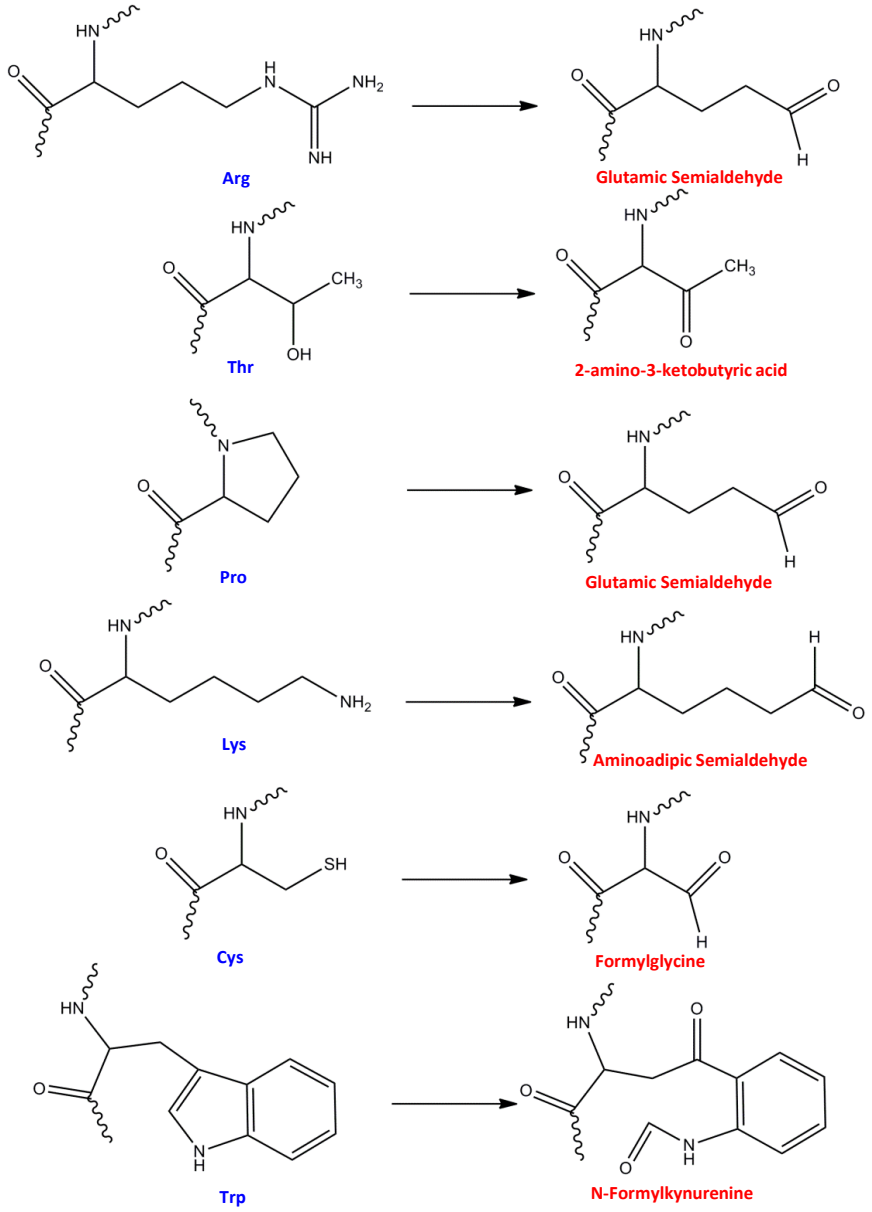

Figure 1. Structures of the oxidative carbonylation products of Arginine, Threonine, Proline, Lysine, Cysteine, and Tryptophan residues.

A critical factor to consider during mAb product quality assessment is immunogenicity, which refers to the ability of the antigen (mAb drug products) to induce the immune responses. The induced immune responses against a mAb product can lead to reduced efficacy of the product and/or cause safety issues such as anaphylaxis.[25] In general, there are two types of the immune responses: the adaptive immunity, which is long lasting and specific, and the non-adaptive immunity, which is generic and does not have high specificity.[26] For the immunogenicity of mAb products, both types of the immune responses may be involved, and the mechanism is quite complex. For example, one route is through stimulation of antigen presenting cells (including the dendritic cells and some B cells) without requiring co-stimulation of the CD4+ helper T cells,[27] while another route is T-cell dependent.[27] In addition, for antigens such as virus coat proteins or aggregates with ordered protein structures or regular repeating pattern, the immune response is typically initiated through activation of marginal zone B cells, which is T-cell independent.[26]

Due to the complex nature of immunogenicity, it is often difficult to evaluate the immunogenicity risk for a monoclonal antibody product candidate.[28] For example, immunogenicity has been observed for even fully humanized recombinant monoclonal antibody products.[29] Furthermore, a wide range of immunogenicity profiles have been observed in the FDA approved antibody therapeutics, with the observed frequency ranging from 0% in Bevacizumab (Avastin) patients to 44% in Abciximab (Reopro) patients.[26] Many factors, such as protein sequence, structural framework, and physiochemical characteristic of the mAb molecule, may contribute to the immunogenicity. While protein sequence and structural framework are typically fixed when a mAb product enters the development stage,

physiochemical characteristic of a mAb product may vary due to variation or changes in the manufacturing process. It is therefore important to perform extensive physiochemical characterization to help evaluate the immunogenicity during mAb product development. As a product quality attribute for the physiochemical characterization, oxidative carbonylation has not been specifically evaluated for its impact on immunogenicity for mAbs. However, existing reports in the literature suggest a link between immunogenicity and various types of carbonylation products, particularly in the autoimmune diseases.[30] For example, Kirkham et al. showed a correlation between the intensity of immune response (reflected by the observed titer of the induced antibody against carbonyl-modified endogenous proteins) and the level of carbonyls in blood and peripheral lung, in both chronic obstructive pulmonary disease patients and ozone-exposed mice.[31] This study supports that the oxidation stress can trigger immune responses against the carbonylated endogenous proteins.[31] In another study, Allison and Fearon demonstrated that a non-immunogenic protein, merozoite surface protein-1, can be effectively converted to an immunogenic antigen by attaching a glycol-aldehyde tag to the protein in mouse model, where the induced immunogenicity was specifically attributed to the aldehyde moiety.[32] Recently, Filipe et al. compared the immunogenicity of mAb samples stressed under different conditions, such as pH shifts, shaking, heating, and metal-catalyzed oxidation, in transgenic and non-transgenic mouse models.[33] Interestingly, the mAb aggregates from the metal-catalyzed oxidation were found to be significantly more immunogenic than the aggregates generated from the other stress conditions.[33] The oxidative carbonylation products, among other oxidation products, were considered a higher immunogenicity risk than other aggregate features.[33]

Given the potential impact to mAb's efficacy, stability, and immunogenicity, oxidative carbonylation, as a product quality attribute, should be thoroughly characterized. At the protein

level, oxidative carbonylation can be quantified by a number of assays.[34],[35] One of the most commonly used assays employs 2,4-dinitrophenyhydrazine (DNPH) as a derivatization reagent. The resulting hydrazone has a strong absorbance at 375 nm, which is the basis for quantitation by UV absorbance spectroscopy of this assay.[35] At the peptide level, identifying and characterizing oxidation carbonylation sites on proteins is particularly challenging.[36] A number of factors contribute to the difficulty. First, carbonylation is normally distributed on multiple sites at low levels.[37],[38] Second, loss of positive charges from lysine and arginine can reduce ionization efficiency and impair detection sensitivity for mass spectrometry analysis. Third, aldehyde groups may react with primary amines during peptide mapping and the sample digestion process, which obscures subsequent analysis. Therefore, chemical derivatization is necessary to protect the carbonyl groups before peptide mapping. Mirzaei and Regnier reported a method using biotin hydrazide as a derivatization reagent to form a hydrazone with protein carbonyl groups, which allows selective enrichment of the biotinylated proteins by avidin affinity chromatography for subsequent tryptic peptide mapping.[39] Applying the biotin hydrazide method, Madian and Regnier identified carbonylated proteins in human plasma;[40] Madian et al. evaluated the effects of antioxidants on carbonylation;[41] Temple et al. identified carbonylation sites on oxidized human serum albumin.[42] Using a similar analytical strategy, Chavez et al. used aldehyde reactive probe (ARP), N'-aminooxymethylcarbonyl hydrazine-D-biotin, for derivatization and affinity capture of carbonylated proteins and identified carbonylation sites on oxidized glyceraldehyde-3-phosphate dehydrogenase and on some mitochondrial proteins.[43] In another report, Mirzaei and Regnier described the method using Girard's P reagent (GRP) for derivatization and strong cation exchange chromatography for enrichment of GRP-peptides after proteolysis.[11] One advantage of the GRP method is that GRP-

derived peptides have increased ionization efficiency with the introduced pyridinium moiety from GRP. Finally, Lee et al. reported a method using a custom-synthesized affinity tag for identification and quantitation of carbonylation sites on human serum albumin.[44]

For biotechnology products, the location of oxidative carbonylation sites on monoclonal antibody drugs will be essential for proper assessment of the impact of this modification on drug product quality. However, site-specific characterization of oxidative carbonylation on therapeutic proteins has yet to be reported. An issue with the current analytical methodologies is that high-yield recovery of chemically labeled oxidatively carbonylated peptides/proteins from affinity capture or strong cation exchange chromatography can be difficult to achieve, which may lead to missed identification of important carbonylation sites on biotherapeutic proteins. Another issue is that some of the chemical tags can interfere with the peptide fragmentation pattern during LC-MS/MS analysis,[11],[45] making data interpretation and site identification difficult.

In this work, a novel analytical method for characterizing oxidative carbonylation on recombinant monoclonal antibodies was reported. Girard Reagent T, a reagent originally used for analysis of a specific nucleoside[46] and steroids,[47] is employed for derivatization of the carbonyl groups on mAbs. Like GRP, Girard Reagent T has a quaternary ammonium moiety which adds a positive charge to the derived peptides for increased ionization efficiency. After derivatization, mAb samples are directly analyzed by peptide mapping without enrichment or fractionation. The high quality of MS/MS spectra of GRT derived peptides allows pinpointing the exact carbonylation sites on mAbs with high confidence by the Proteome Discoverer database search engine. The sensitivity and effectiveness of this method are demonstrated by detecting and identifying multiple carbonylation sites on an unstressed IgG1 bulk drug substance as well as on three different IgG1s stressed by MCO. This method represents a significant improvement over

existing analytical methodologies for characterization of oxidative carbonylation on protein pharmaceuticals and should be readily applicable for characterization of oxidized proteins in other biological systems.

EXPERIMENTAL SECTION

Materials. IgG1A, IgG1B, and IgG1C were manufactured at Genentech (South San Francisco, CA). (Carboxymethyl)trimethylammonium chloride hydrazide (Girard's Reagent T, GRT), dithiothreitol (DTT), iodoacetic acid (IAA), ferrous sulfate, hydrogen peroxide solution (30% in H_2O, w/w), methionine (Met), ethylenediaminetetraacetic acid (EDTA), calcium chloride, tris(hydroxymethyl) aminomethane (Tris), sodium acetate, acetic acid, sodium hydroxide solution (1M in H_2O), and thermolysin from Bacillus were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA), guanidine hydrochloride, tris(2-carboxyethyl) phosphine HCl (TCEP), and BCA protein assay kit were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Acetonitrile (ACN) was purchased from Burdick & Jackson (Muskegon, MI, USA). OxiSelect^{TM} protein carbonyl spectrophotometric assay kit was purchased from Cell Biolabs (San Diego, CA, USA). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA).

Methods. *Oxidation of mAbs.* Antibody samples were subjected to metal catalyzed oxidation. For the oxidation reaction, each antibody sample (5 mg/mL final concentration) was mixed with $FeSO_4$ (2 mM final concentration) and hydrogen peroxide (10 mM final concentration) in a 50 mM sodium succinate buffer, pH 6.5. The reaction mixture was incubated at room temperature for 16 hours for high level oxidation or for 2 hours for medium level oxidation. The oxidation reaction was then stopped by addition of an excess amount of

methionine and EDTA to quench residual hydrogen peroxide and free Fe ions in the reaction mixture.

Determination of Protein Carbonyl Levels of mAbs. Protein carbonylation levels for the unstressed and oxidized IgG1A mAb samples were measured using the OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc., San Diego, CA) following the procedure recommended by the manufacturer. For each sample, three independent measurements were performed. An average value of the protein carbonyl levels from the three independent measurements was then reported for each sample.

Derivatization of mAb samples with GRT. The mAb samples, 16-hour oxidized IgG1A, IgG1B, IgG1C, 2-hour oxidized IgG1A, and unstressed IgG1A, were buffer exchanged into 50 mM sodium acetate buffer, pH 4.5, using Amicon Ultra-15 30 KDa filter units (Millipore, Billerica, MA, USA) to a final protein concentration of 5 mg/mL. The buffer-exchanged mAb samples were then subjected to the GRT labeling reaction. To each sample, GRT was added to a final mAb-to-GRT molar ratio of 1:10,000. After brief mixing, the reaction mixture was incubated at room temperature for 16 hours in the dark. The derivatization reaction was then stopped by freezing at -80 °C. An aliquot of each GRT-derivatized sample was subsequently buffer exchanged into 25 mM sodium phosphate buffer, pH 6.5 using Amicon Ultra-15 30 kDa filter units.

Sodium Borohydride Reduction. GRT-derivatized mAb samples were reduced by sodium borohydride, according to a protocol described by Brady et al.[48] Sodium borohydride solution (100 mM) was prepared fresh in 0.02 M NaOH immediately before its use. An aliquot of 200 µL GRT-derivatized mAb sample (10 mg/mL) was mixed with 160 µL of 400 mM sodium phosphate buffer (pH 7.4) and 40 µL of the sodium borohydride solution. After brief mixing, the

mixture was incubated at room temperature for 1 hour. Subsequently, GRT-derivatized sodium borohydride-reduced mAb samples were buffer exchanged into 25 mM sodium phosphate buffer, pH 6.5 using Amicon Ultra-15 30 kDa filter units.

Molecular Mass Analysis of GRT-derivatized Sodium Borohydride-reduced and TCEP-reduced IgG1A. The GRT-derivatized and sodium borohydride-reduced IgG1A samples (2 mg/mL) were mixed with the TCEP solution (5 mg/ml in H_2O) at 1:1 (volume to volume) ratio. The mixture was incubated at 55 °C for 15 minutes. After incubation, the mixture was cooled to room temperature and diluted with water to a final protein concentration of 0.05 mg/mL and stored at 2-8 °C for subsequent mass analysis. Molecular mass analyses of TCEP-reduced IgG1A samples were carried out on an Agilent 6210 electrospray ionization time-of-flight mass spectrometer (Agilent, Santa Clara, CA). Desalting and separation of the GRT-derivatized Sodium Borohydride-reduced and TCEP-reduced IgG1A samples were performed using an Agilent custom order chip packed with Zorbax 300SB-C8 5µm stationary phase. A linear gradient of 10% to 60% B in 6 minutes at a flow rate of 0.4 µL/min was employed, where mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Approximately 5 ng of the IgG1A material was injected for analysis. The acquired mass spectra were then deconvoluted with the Agilent MassHunter software.

Trypsin and Thermolysin Digestion. GRT-derivatized mAb samples (with and without sodium borohydride reduction) were digested by trypsin and thermolysin. Before enzymatic digestion, the mAb samples were denatured, reduced, and alkylated (S-carboxymethylation) as previously described.[7] Reduced and alkylated mAb samples were then buffer exchanged into a digestion buffer (25 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.2) using a Sephadex G-25M PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For trypsin digestion, the samples

were digested with an enzyme-to-substrate ratio of 1:50 (weight to weight) at 37 °C for 5 hours and the digestion was stopped by addition of TFA to a final concentration of 0.3%. For thermolysin digestion, the samples were digested with an enzyme-to-substrate ratio of 1:100 (weight to weight) at 50 °C for 30 minutes, and stopped by addition of EDTA to a final concentration of 3.75 mM. The trypsin and thermolysin digests were then stored at 2-8 °C prior to subsequent analysis.

LC/ESI-MS/MS Analysis and Proteome Discover Database Search. Trypsin and thermolysin digested mAb samples were analyzed by LC/ESI-MS/MS with an Agilent 1200 HPLC system connected to a Thermo Scientific LTQ OrbiTrap Velos mass spectrometer (San Jose, CA). The digested peptides (35 ug per injection) were separated on an Eclipse XDB-C8 column (2.1x150 mm, 3.5 um). Mobile phase A was 0.1% TFA in water. Mobile phase B was 0.09% TFA in 90% acetonitrile and 10% water (v/v). The peptides were eluted from the column with a step gradient as follows: 0-10% B in 20 minutes and then 10-40% B in 137 minutes, with a flow rate of 0.25 ml/min. Column temperature was controlled at 55 °C. The eluted peptides were analyzed by the OrbiTrap mass spectrometer in the positive ion mode. The electrospray voltage was 4.0 kV. The capillary temperature was 270 °C. The MS data were acquired in a data dependent mode, where a full MS survey scan was followed by MS/MS scans of the 9 most intense ions. The full MS data (m/z 300 to m/z 2000) were acquired in the Orbitrap mass analyzer with a resolution of 60,000. The MS/MS data were acquired in the LTQ by collision induced dissociation, with the normalized collision energy of 35 and the activation time of 10 milliseconds.

The acquired mass spectral data were searched against a custom created database containing only IgG1A, IgG1B, and IgG1C sequences with the Proteome Discover software

using the SEQUEST (V1.20) algorithm. The monoisotopic masses of the modifications of GRT derivatized (and sodium borohydride-reduced) oxidation products of Lys, Arg, Pro, and Thr were manually calculated as shown in Table 1, and were selected as dynamic modifications during the search. Other database search parameters were as follows: precursor mass tolerance of 5 ppm, MS/MS mass tolerance of 0.8 Da, and a fixed modification of carboxymethylation on cysteine. For the trypsin digest, the enzyme specificity was set as full with maximum of two missed cleavages. For the thermolysin digest, the enzyme specificity was set as none. The same database searching parameters as listed above were applied to the samples not treated with sodium borohydride for generating false positive hits.

Residues	**Oxidative Carbonylation**	**GRT Derivatization +$NaBH_4$**	**Delta Mass (Monoisotopic) for Search**
Lysine	$-NH_3$ +O	$+C_5H_{11}N_2O$	$+C_5H_{10}N_2O$ **114.07876**
Arginine	$-CN_3H_5$ +O	$+C_4H_9O$	$+C_4H_8O$ **72. 05697**
Proline	+O	$+C_5H_{14}N_3O$	$+C_5H_{13}N_3O$ **131. 10531**
Threonine	$-H_2$	$+C_5H_{12}N_3$	$+C_5H_{11}N_3$ **113. 09475**

Table 1. Difference in molecular formula and monoisotopic masses for the carbonylation products

RESULTS and DISCUSSION

Analytical Strategy for Extended Characterization of Oxidative Carbonylation on Monoclonal Antibodies. To develop an analytical strategy for extended characterization of monoclonal antibodies, a derivatization strategy that allows direct detection and identification of carbonylated peptides during LC-MS/MS peptide mapping was explored. Specifically, hydrazide reagents that provide enhanced ionization efficiency for MS analysis, and that yield no or little self-fragmentation during collision induced dissociation (CID) experiments are preferred. Previously reported for a specific nucleoside[46] and steroids,[47] Girard's Reagent T (GRT, Figure 2), was considered as the derivatization reagent for this study based on the following features. First, GRT carries a positively charged quaternary ammonium group and has a very high solubility in water. The ionization efficiency of GRT-derivatized peptides should be enhanced because of the positive charge introduced after derivatization and the improved solubility of the resulting peptides. Second, based on the small size and relatively simple chemical structure of GRT, it was speculated that GRT-tagged peptides should be less prone to tag self-fragmentation during the CID experiment than with other more complex hydrazide tags such as ARP.[43] Third, also because of the small molecular size of GRT, more carbonylation sites may be accessible to GRT than to other bulkier hydrazide reagents. In addition, it is worth noting that compared to a similar hydrazide reagent GRP,[11] GRT has a higher solubility in water,[49] which allows higher reagent-to-protein molar ratio for derivatization (given the same protein concentration), and a smaller molecular weight, which potentially provides slightly better site accessibility. Based on these additional considerations, GRT was finally selected as the derivatization reagent in this study.

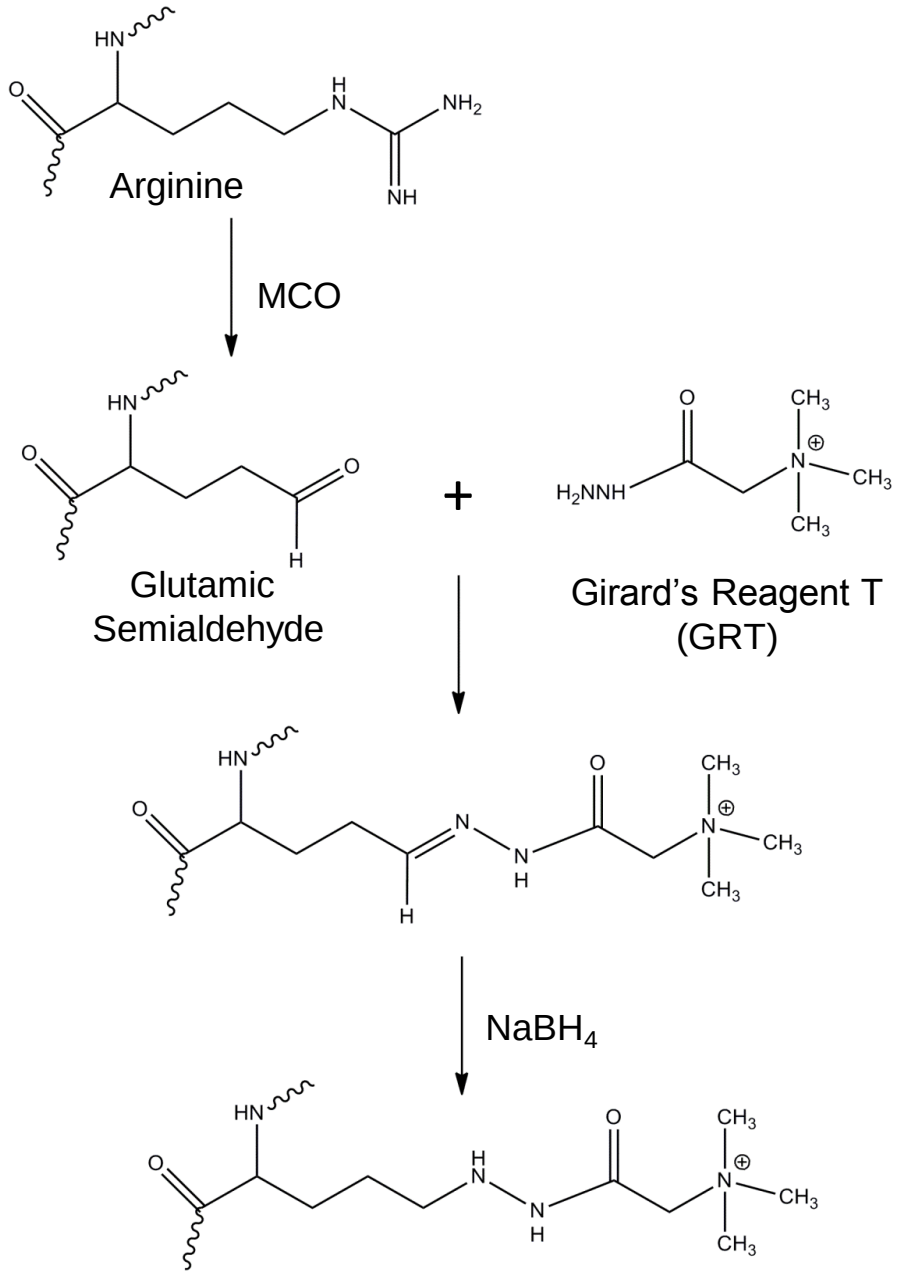

Figure 2. Structure of GRT and the derivatization reaction of GRT with oxidized proteins (showing oxidized arginine residue as an example) to form hydrazone products where the Schiff base is subsequently reduced by $NaBH_4$.

Metal Catalyzed Oxidative Carbonylation of Monoclonal Antibodies. In this study, three full-length IgG1 mAb molecules produced at Genentech were used as model mAbs to investigate oxidative carbonylation. Several factors were considered when selecting industry-relevant stress conditions to generate oxidative carbonylation on the model mAbs. First, among the various metal catalyzed oxidation systems described by Stadtman,[50] the Fe(II)/H_2O_2 system is considered more pharmaceutically relevant since both Fe(III) and H_2O_2 may be commonly present during mAb manufacturing and storage processes, and Fe(III) can be converted to Fe(II) via reaction with H_2O_2.[51] Therefore this oxidation system was selected in this study. Second, the pH for the oxidation reaction was also considered as a critical factor. At basic and neutral pHs, N-terminal amine groups and lysine side chains are increasingly deprotonated and can potentially form Schiff bases with aldehyde groups generated during MCO, thus interfering with subsequent carbonylation analysis. Acidic pHs are therefore preferred. Considering also the pH range of the cell culture environment[21] and the common formulations[52] for mAbs, I selected a pH value of 6.5 for the MCO reaction. Third, I evaluated various buffer systems for an optimal buffer to carry out the oxidation experiment, since interferences with Fe ions or with the oxidation reaction from the buffer components may introduce variations to the oxidation reaction, and thus should be minimized. Many commonly used buffers are not ideal for the Fe(II)/H_2O_2 system. For example, phosphate ions may form insoluble salts with Fe ions; Tris and citrate can act as Fe ion chelators; MES can, in fact, be oxidized by the Fe(II)/H_2O_2 system (unpublished results). Therefore, for this study, sodium succinate buffer was selected for its lack of the solubility and chelating issues with Fe ions, and its capability for buffering at pH 6.5.

Total protein carbonyl levels of the unstressed and oxidized IgG1A samples were determined by the 2,4-dinitrophenylhydrazine (DNPH) colorimetric assay[35] according to the

manufacturer's procedure. The carbonylation levels for the unstressed, 2-hour oxidized, and 16-hour oxidized IgG1A bulk drug substance were 0.05±0.02, 1.3±0.1, and 1.9±0.1 mole of carbonyl per mole of mAb, respectively. These results show that, as expected, significant oxidative carbonylation of mAbs can indeed be induced through metal catalyzed oxidation reactions with the Fe(II)/H_2O_2 system. At such a high protein carbonyl level, it was suspected that oxidative carbonylation could be generated on most if not all potential carbonylation sites on IgG1A with the 16-hour oxidation condition. In contrast, for the unstressed IgG1A, its protein carbonyl level is even lower than the level reported by Bee et al.[23] on an IgG1 bulk drug substance. Therefore the unstressed IgG1A should be a suitable sample to test the sensitivity of the analytical method for this study.

Optimization of the GRT Derivatization Reaction. For the derivatization reaction, the hydrazine group of GRT reacts with carbonyl groups on mAbs to form hydrazones (Figure 2). In this study, the derivatization reaction was carried out in 50 mM sodium acetate buffer at pH 4.5, since hydrazone formation is favored at acidic pHs.[46] The 16-hour oxidized IgG1A sample was used as a model mAb sample to optimize the GRT derivatization condition. First, the mAb-to-GRT molar ratio was optimized by mixing an aliquot of the highly oxidized IgG1A (5 mg/mL in the final reaction mixture) with GRT at 1:500, 1:1,000, 1:2,500, 1:5,000, 1:7,500, 1:10,000, and 1:12,500 mAb-to-GRT molar ratios. The reaction was carried out for 16 hours at room temperature and stopped by freezing at -80 °C. Subsequently, all these samples were treated with sodium borohydride to reduce the Schiff base to a more stable hydrazine product for the mass analysis, enabling the selection of an optimal derivatization condition based on the relative intensities of GRT-derivatized carbonylated species.

In the deconvoluted mass spectra of the reduced IgG1A light chain (LC), as shown in Figure 3, GRT-derivatized carbonylated species were readily observable. The mass species of 23399.1 Da is consistent with GRT-derivatized carbonylated products of threonine/lysine of LC. The mass species of 23383.9, 23415.4, and 23431.7 Da are likely correspondingly Thr/Lys-modified LC mass species of 23269.5, 23301.5, 23317.5 Da, with a consistent mass shift of approximately 113-114 Da, respectively. For the heavy chain (HC), the deconvoluted mass spectra were much more complicated due to the more heterogeneous nature (variations at C-terminal lysine and N-glycosylation[53] of the HC. Nevertheless, the trend of intensities of the derivatized species of HC with increasing GRT-to-mAb molar ratios was generally consistent with that observed for the LC (HC data not shown). To simplify the process for optimizing the derivatization reaction, the relative intensity of the LC product with the mass of 23399.1 Da was used as a surrogate indicator for the extent of GRT derivatization. As shown in Figure 4 and Figure 5 (the raw MS spectra), the mAb-to-GRT molar ratio of 1:10,000 demonstrated an optimal derivatization condition, where the derivatization yields reach a plateau (Figures 6A), and beyond which there was little increase in the relative intensities of the GRT-derived carbonylated species. Similarly, the GRT derivatization reaction time was optimized by evaluating the extent of derivatization for 1, 2, 4, 8, 16, and 24 hours with a mAb-to-GRT molar ratio of 1:10,000. A reaction time of 16 hours demonstrated an optimal derivatization condition (Figure 6B). Therefore the mAb-to-GRT molar ratio of 1:10,000 and the reaction time of 16 hours were selected as a final GRT derivatization condition for the subsequent studies.

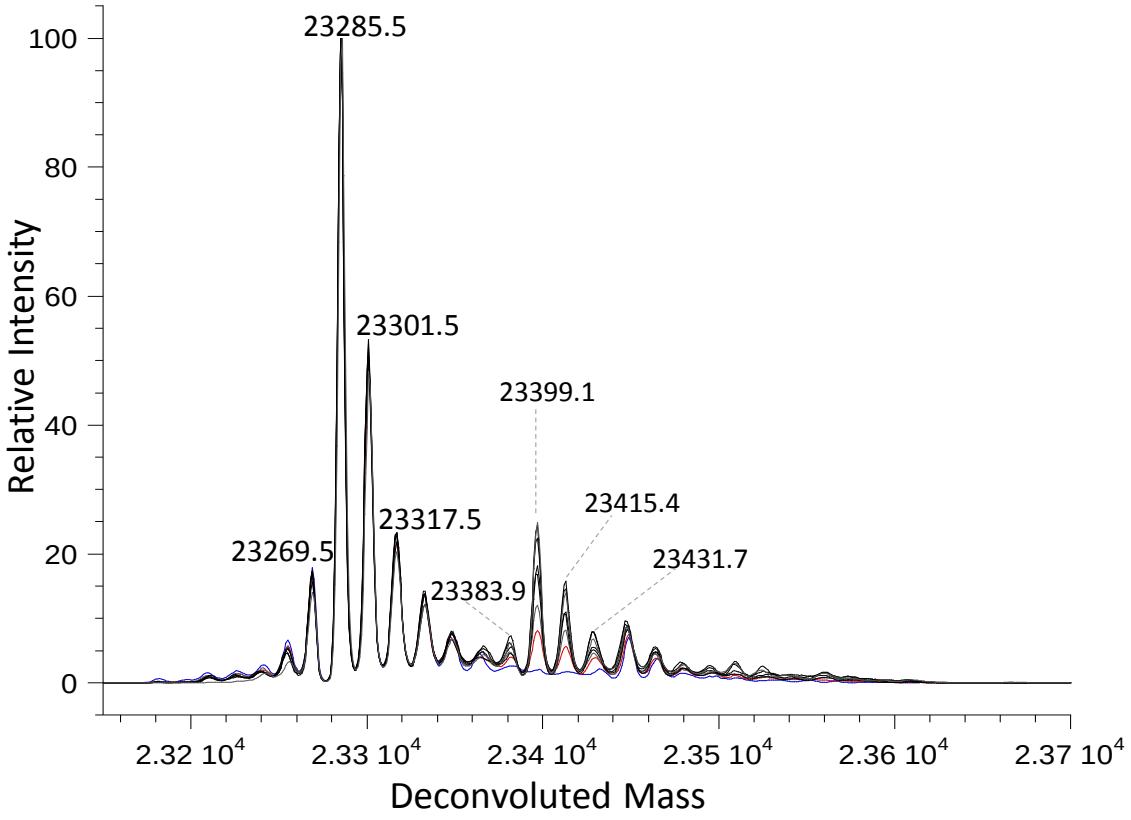

Figure 3. Deconvoluted mass spectra of 16-hour oxidized IgG1A light chains derivatized with GRT in 0 (control), 500, 1,000, 2,500, 5,000, 7,500, 10,000, and 12,500 molar ratios followed by $NaBH_4$ reduction.

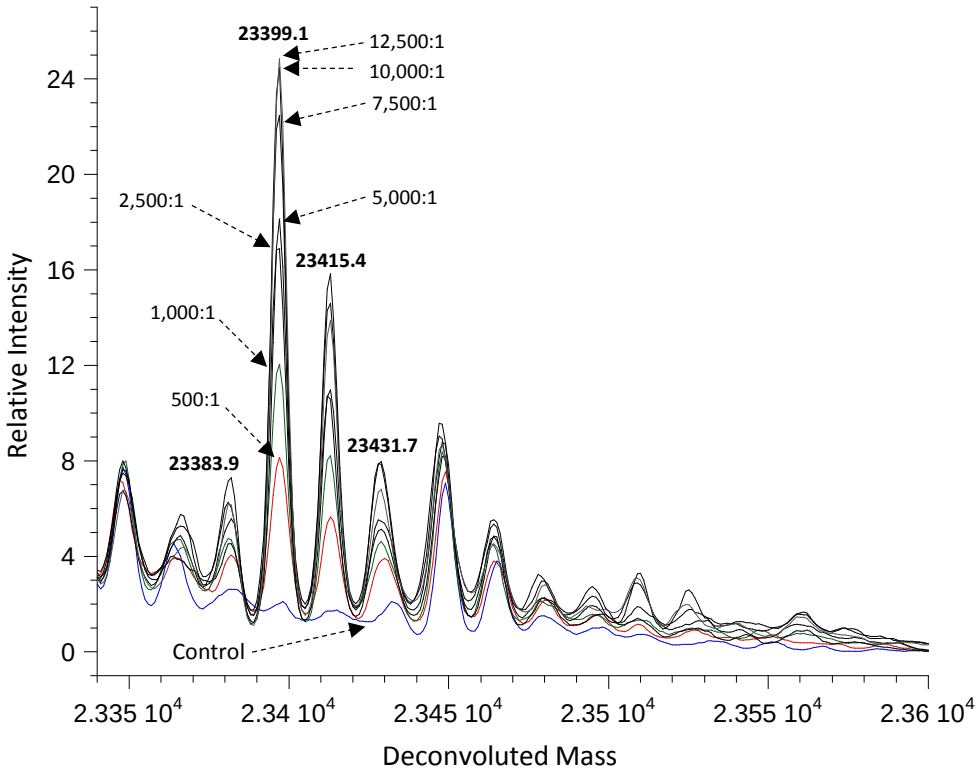

Figure 4. Expanded view of the deconvoluted mass spectra of 16-hour oxidized IgG1A light chains derivatized with GRT in 0 (control), 500, 1,000, 2,500, 5,000, 7,500, 10,000, and 12,500 molar ratios followed by $NaBH_4$ reduction.

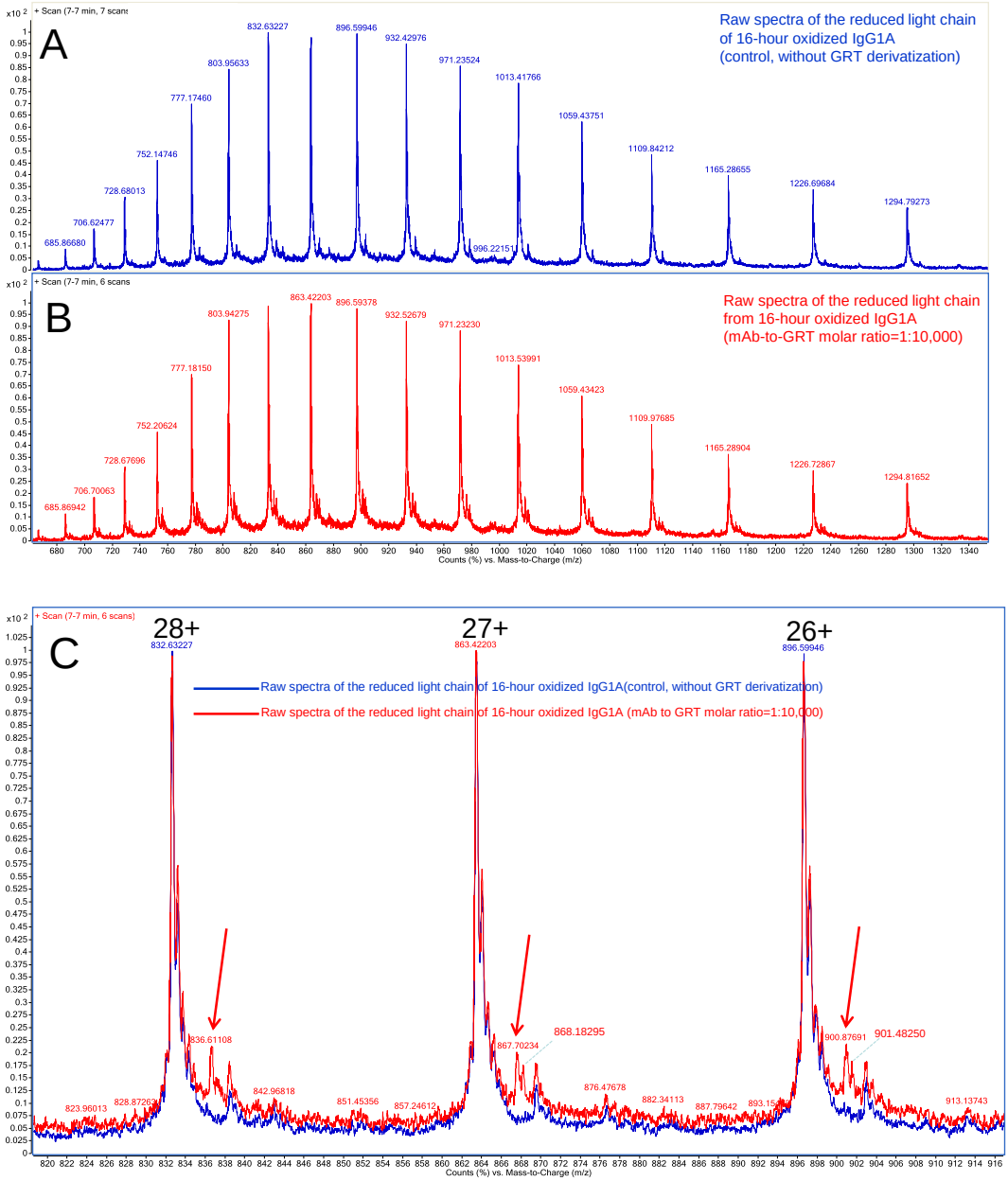

Figure 5. (A) Raw spectra of the reduced light chain of the control sample in the mAb-to-GRT molar ratio optimization experiment. (B) Raw spectra of the reduced light chain of the sample with a mAb-to-GRT molar ratio of 1:10,000 in the mAb-to-GRT molar ratio optimization experiment. (C) Comparison of the raw spectra in an expanded view. GRT derivatization resulted in enhanced ionization efficiency for the carbonylation products (predominant ions resulted from GRT derivatization are pointed out).

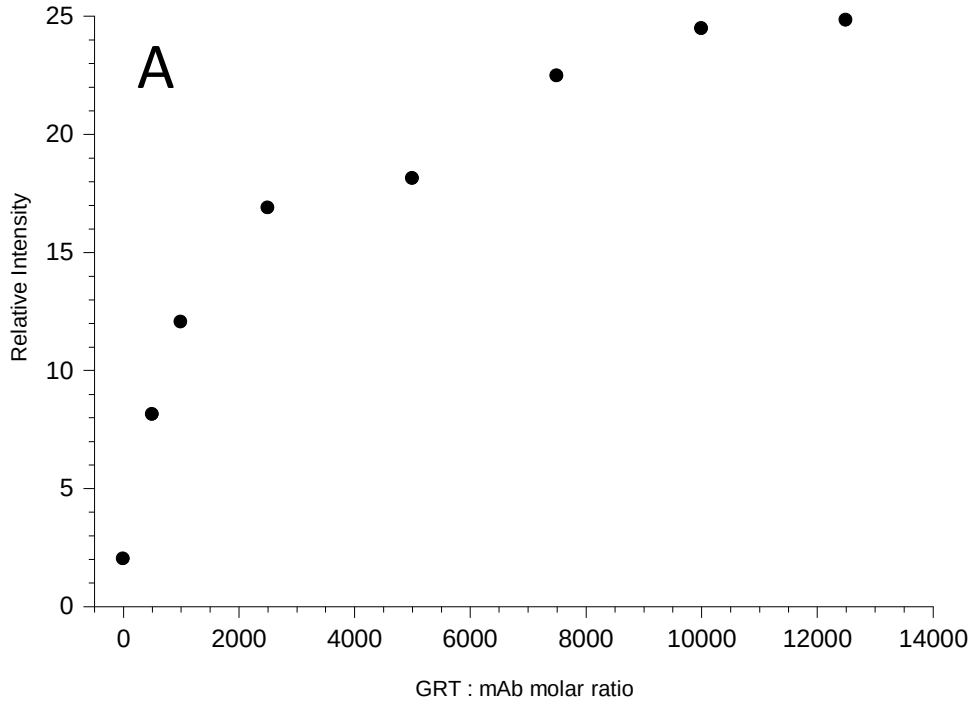

Figure 6A. Molar ratio optimization of GRT derivatization reaction. Oxidized IgG1A sample was mixed with GRT at 0, 500, 1,000, 2,500, 7,500, 10,000, and 12,500 molar ratios and incubated at room temperature for 16 hours. The derivatization reaction reached a plateau at 10,000 molar ratio.

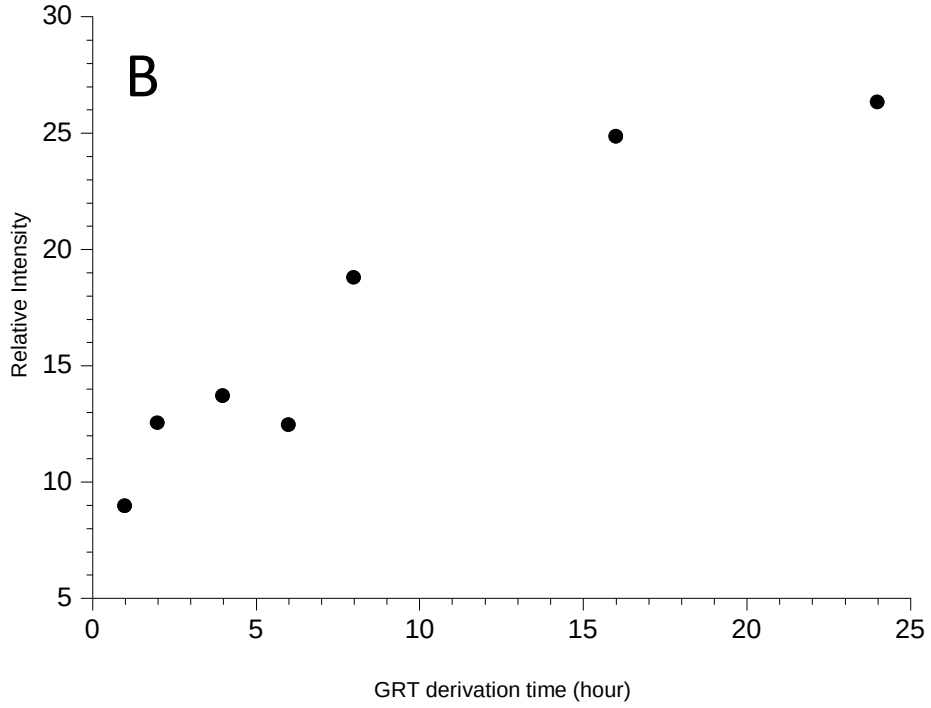

Figure 6B. Time course optimization of GRT derivatization reaction. Derivatization mixtures (molar ratio 1:10,000 of oxidized IgG1A/GRT) were incubated at room temperature, in the dark for 1, 2, 4, 6, 8, 16, and 24 hours. The derivatization reaction reached a plateau at 16 hours.

It is also interesting to note that the relative intensities for all other observed masses remained largely unchanged compared to the intensity of the GRT-derivatized carbonyl species in the mass spectra of reduced IgG1A (Figure 3), which suggests that without GRT derivatization the carbonylated products are either undetectable or present at very low intensities. This observation is consistent with the expectation that derivatization with GRT can enhance ionization efficiency of carbonylated species of mAbs because of the introduced permanent positive charge, thus improving the sensitivity for detecting carbonylation on mAbs.

Proteolysis and LC-MS/MS. For extended characterization of carbonylation sites on mAbs, I carefully considered a proteolysis strategy and LC-MS/MS settings to maximize the success rate of positive identification of carbonylation sites. First, for proteolysis, both trypsin and thermolysin[(54)] digestions were employed, which in combination achieved 100% sequence coverage for all three IgG1s. An additional consideration for using both trypsin and thermolysin is that lysine and arginine carbonylation sites are uncleavable by trypsin; therefore, the resulting tryptic peptides can be very large. As a consequence, the quality of the MS/MS spectra of these large tryptic peptides may be very poor, making identification by database searching difficult. Thermolysin, on the other hand, while not as specific as trypsin, may produce several smaller-sized peptides covering the modified lysine and arginine sites, thereby facilitating identification. Second, for the HPLC condition, to address the concern of the permanent positive charge of GRT on derivatized peptides engaging in a potential strong ionic interaction with surface silanol groups on a silica based reverse phase column,[(55)] a reverse phase column with an end-capped stationary phase was chosen. Third, for MS/MS data acquisition, the collision induced dissociation (CID) mode was selected for the fast scanning rate of the Orbitrap Velos mass

spectrometer in this mode, which allows more low-abundant ions to be selected for fragmentation.

Identification of Carbonylation Sites on Oxidized and Unstressed IgG1A. Identification of carbonylation sites on IgG1A samples was achieved through database search of the MS^2 data acquired from peptide mapping. It should be noted that MCO can generate a large variety of modifications besides carbonylation on proteins.[15, 50] Therefore, false positives may be present in database search results. Additionally, artifacts from enzymatic digestion, such as missed or non-specific cleavages, could also contribute to false positive identification of carbonylation sites. In some cases, these false positives could also have high quality MS^2 data, making them difficult to rule out. Clearly, these issues pose some serious challenges for high-confidence assignment of carbonylation sites on proteins. Surprisingly, there have been very few publications that employed specific strategies to address these false positive issues. In one case, the false positive issues were observed by Mirzaei and Regnier and addressed in their work through the use of stable isotope labeled GRP [56] in a multiplexing fashion.

In this work, a filtering process as illustrated in Figure 7 was employed to reduce false positive identifications. This simple data filtering process was found to be highly effective in reducing false positives. In this process, one aliquot of a GRT-derived mAb sample was reduced by $NaBH_4$ and the other aliquot was not. Subsequently, these two aliquots were subjected to identical peptide mapping and database search processes. After the database search, two lists, A and B, containing identified carbonylation sites from the aliquots with $NaBH_4$ reduction and without $NaBH_4$ reduction, respectively, were generated. Given that the customized modification masses (Table 1) used for database search were calculated including the two additional hydrogen atoms from $NaBH_4$ reduction, all of the carbonylated peptide hits identified in list B are false

positives. Therefore, a carbonylated peptide hit identified in list A can be ruled out as a false positive if this same peptide hit is also identified in list B. As an example to demonstrate the effectiveness of the filtering process, from thermolysin mapping of a 16-hour oxidized IgG1A sample, list A contained 127 peptide hits while list B contained 83 peptides hits. When list A was compared with list B, 67 common peptide hits were found on both lists. These 67 peptide hits were then ruled out as false positives.

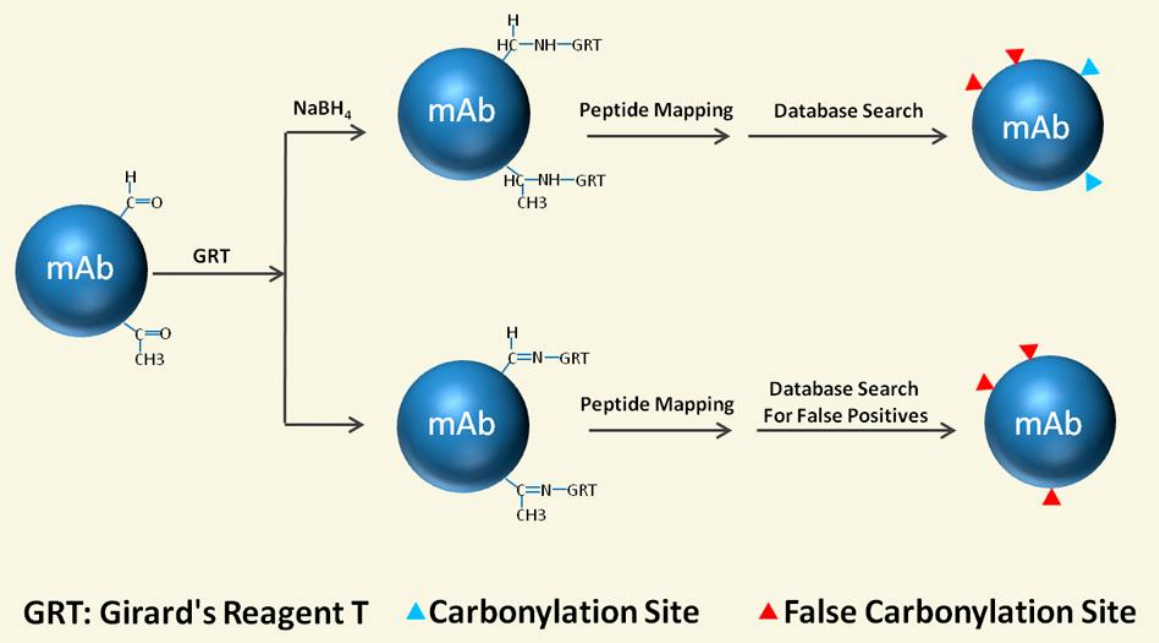

Figure7. The analysis strategy for filtering out false positive identifications.

After the false positives were removed through the filtering process, the remaining MS^2 spectra were further investigate with high-confidence database search scores for positive identification of carbonylation sites. Shown in Figure 8 is an example of collision-induced dissociation (CID) MS^2 spectra for a positively identified carbonylation site of Arg18 on the light chain of IgG1A. Figure 8 illustrates the MS^2 spectrum of a doubly charged GRT-derivatized peptide ion with m/z of 538.80139, corresponding to amino acids 11-20 on the light chain of IgG1A, while the MS^2 spectrum of the doubly charged parent peptide ion with m/z of 502.77255 is shown in the Figure 9. In both MS^2 spectra, a continuous b ion series of b7, b8, and b9, covering the Arg18 site was observed, which allowed us to pinpoint the carbonylation site at Arg18. Additionally, the y ions (y4 to y8) in Figure 8 are consistent with GRT derivatization of carbonylated Arg18 of this peptide. More significantly, in Figure 8, $b9^{2+}$ became a predominant fragment ion. This unique intensity profile is consistent with a positive charge introduced by GRT derivatization into the original carbonyl product derived from Arg18. Overall, the high quality MS^2 data in this example provided an unambiguous assignment of R18 as a carbonylation site on IgG1A.

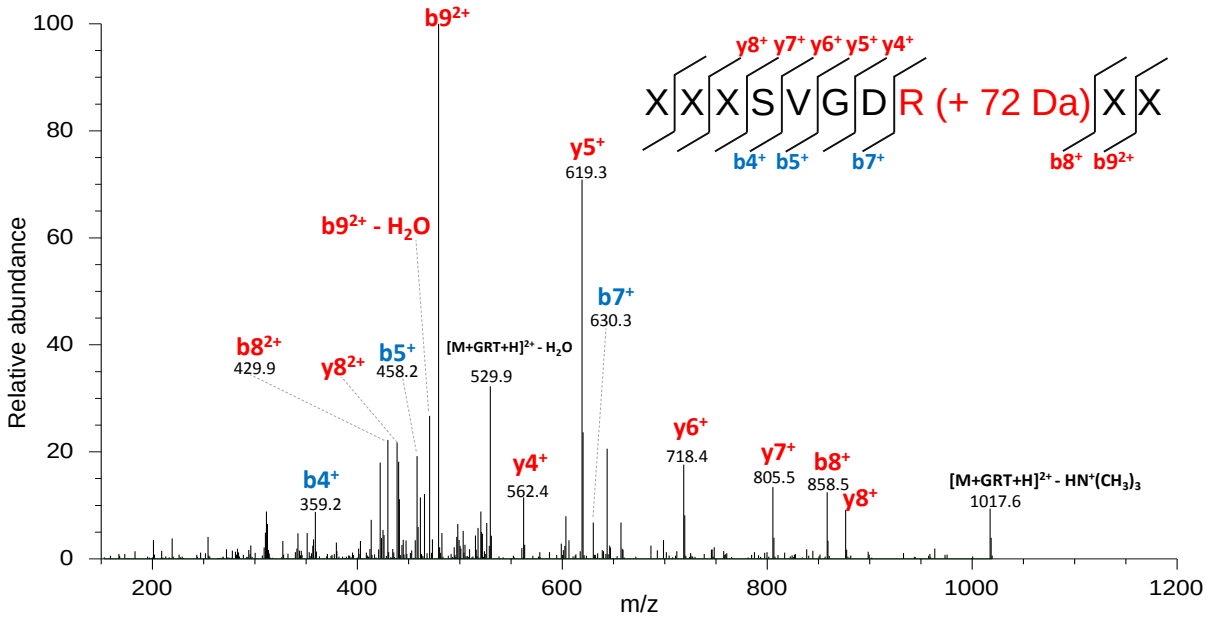

Figure 8. CID MS/MS spectrum of a GRT derivatized carbonylated peptide. R highlighted in red indicates GRT derivatized carbonylated arginine residue.

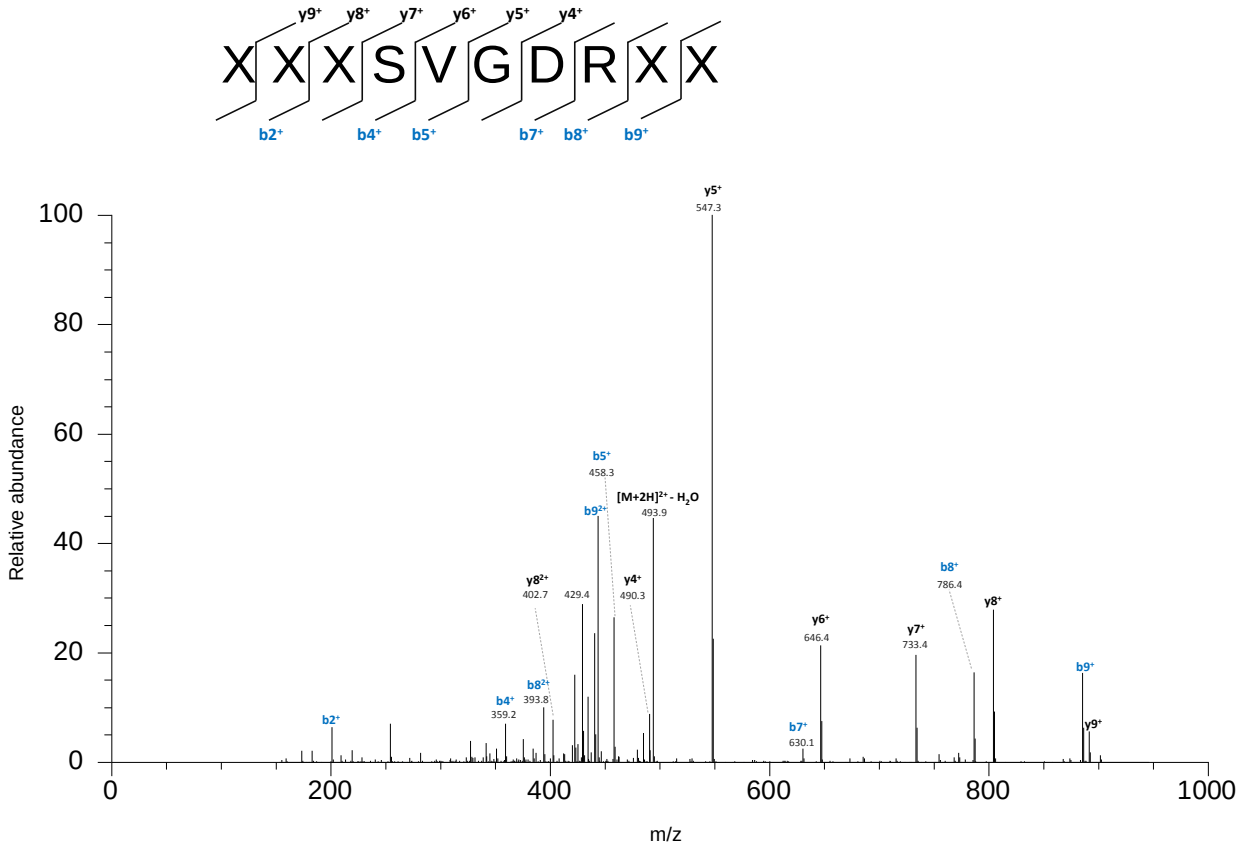

Figure 9. CID MS/MS spectrum of the unmodified R18 peptide XXXSVGDRXX.

As discussed earlier, hydrazide tag-specific fragmentation during CID experiments can be a challenge for identification of carbonylation sites by database search.[36] In most of the cases, GRT self-fragmentation in the MS^2 spectra was not observed. Occasionally, however, GRT self-fragmentation was observed. As an example, the ion with m/z 1017.6 in Figure 8 is a singly charged ion resulting from GRT self-fragmentation, losing the positively charged ammonium moiety, $HN^+ (CH_3)_3$, from the doubly charged GRT-derivatized peptide ion with m/z 538.80139. It is interesting that the GRT self-fragmentation in this case has not affected the database search for the identification of carbonylation sites. This benign effect was likely due to the relatively simple GRT self-fragmentation pattern, as also illustrated in the work on GRT derivatization of a nucleoside,[46] which does not cause the resulting MS^2 spectra to be overly complex. In fact, when the GRT self-fragmentation occurs, the characteristic loss of the ammonium moiety from GRT in the MS^2 spectra can be considered as additional evidence to support positive identification during manual data investigation.

A total of 30 carbonylation sites were identified on the 16-hour oxidized IgG1A, as shown in Table 2. Identification of each of these carbonylation sites passed the four criteria: 1. precursor ion is within 5 ppm of the theoretical mass; 2. identified peptides are present only in the $NaBH_4$ reduced maps (one example shown in Figure 10); 3. database search result is of a high-confidence database search score (Xcorr scores greater than 1.8, 2.3, 3.0, 3.5, for the charge state of 1, 2, 3, 4, respectively); 4. site assignment is further verified by manual investigation of the data. In some cases, carbonylation sites were confirmed on both trypsin and thermolysin maps or from multiple peptides on the thermolysin map, which provided additional support for positive site assignment. It is also interesting to note that threonine was found to be the most frequently oxidized residue on the LC, which is consistent with the deconvoluted light chain mass spectra as shown in Figure 4. Since lysine carbonylation in IgG1A LC was not observed, the 23399.1 mass in Figure 4 likely results from predominantly threonine carbonylation on the LC.

	IgG1 A	IgG1 B	IgG1 C
LC	**T5**$_{Th\ Tr}$, **R18**$_{Th}$, R24$_{Th}$, T69$_{Th}$, T74$_{Th}$, **P80**$_{Th\ Tr}$, T85$_{Th\ Tr}$, T97$_{Th}$, **T109**$_{Tr}$, **T164**$_{Th\ Tr}$, T172$_{Th}$, T180$_{Tr}$, **P204**$_{Tr}$, **T206**$_{Tr}$	**T5**$_{Th\ Tr}$, P8$_{Th\ Tr}$, **R18**$_{Th}$, R24$_{Th}$, R66$_{Th}$, **P80**$_{Th\ Tr}$, **T109**$_{Tr}$, P113$_{Tr}$, P141$_{Tr}$, **T164**$_{Tr}$, T180$_{Th}$, **P204**$_{Tr}$, **T206**$_{Tr}$	**T5**$_{Th\ Tr}$, **R18**$_{Th}$, T20$_{Tr}$, T22$_{Th}$, T69$_{Th\ Tr}$, **P80**$_{Th\ Tr}$, **T109**$_{Tr}$, **T164**$_{Th}$, **P204**$_{Tr}$, **T206**$_{Tr}$
HC	**R19**$_{Th}$, T30$_{Th\ Tr}$, T57$_{Th\ Tr}$, T98$_{Tr}$, T114$_{Th}$, **T120**$_{Th}$, **T135**$_{Tr}$,**T155**$_{Th\ Tr}$, K205$_{Th}$, K214$_{Th}$, T225$_{Th\ Tr}$, **T256**$_{Tr}$, P271$_{Tr}$, **K290**$_{Th}$, **P329**$_{Tr}$, T394$_{Tr}$	**R19**$_{Th}$, T74$_{Th\ Tr}$, K76$_{Th}$, **T123**$_{Tr}$, **T138**$_{Tr}$, **T158**$_{Th}$, T167$_{Th}$, P192$_{Th}$, T198$_{Th}$, R258$_{Th}$, **T259**$_{Th\ Tr}$, **K293**$_{Th,}$ **P332**$_{Th\ Tr}$, P377$_{Tr}$, T396$_{Th}$, P398$_{Tr}$	**R19**$_{Th}$, T74$_{Th\ Tr}$, **T126**$_{Th}$, **T141**$_{Tr}$, **T161**$_{Th}$, T170$_{Th}$, **T262**$_{Th\ Tr}$, P277$_{Tr}$, **K296**$_{Th}$, **P335**$_{Th\ Tr}$, P393$_{Th\ Tr}$

Table 2. Carbonylation sites identified on the 16-hour oxidized IgG1A, IgG1B, and IgG1C (common carbonylation sites are underlined and in **bold;** Th: identified on a Thermolysin map; Tr: identified on a Tryptic map)

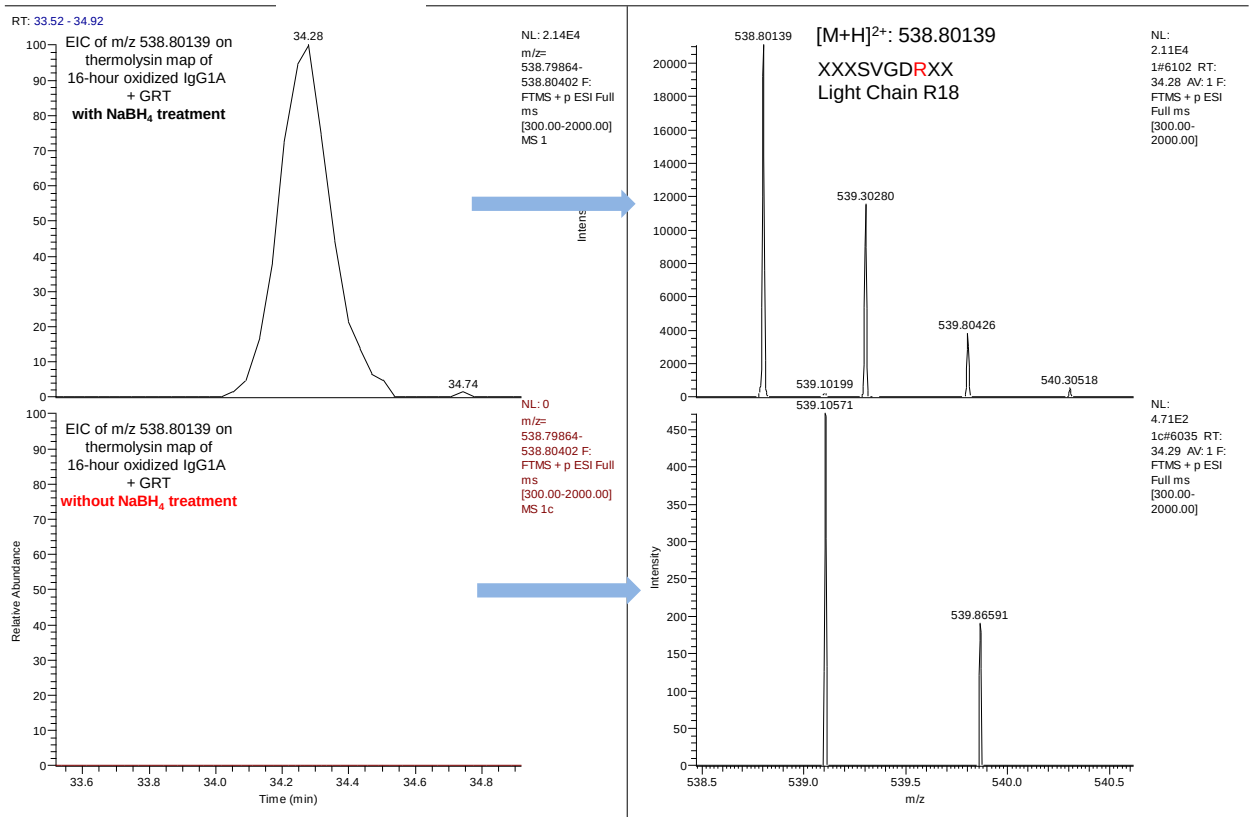

Figure 10. Extracted ion chromatograms (EIC), targeting the $NaBH_4$-reduced GRT derivatized carbonylated R18 peptide (a doubly charged ion of m/z of 538.8013), of the thermolysin maps from the two 16-hour oxidized IgG1A aliquots (the aliquot with $NaBH_4$ reduction was shown in the upper panel; the other aliquot without $NaBH_4$ reduction was shown in the lower panel). Absence of the doubly charged ion of m/z of 538.8013 in the EIC of the aliquot not reduced with $NaBH_4$ (the lower panel) is consistent with positive assignment of R18 as a carbonylation site on IgG1A.

To demonstrate the sensitivity, this approach was applied to the unstressed and 2-hour oxidized (using the Fe(II)/H_2O_2 system) IgG1A, which identified 22 sites on the 2-hour oxidized and 9 sites on the unstressed IgG1A, respectively (Table 3). Identification of the 9 carbonylation sites on the unstressed IgG1A is particularly satisfying as it demonstrates the sensitivity of the method for the identification of site-specific carbonylation on an mAb sample with low protein carbonylation levels without using any affinity or fractionation steps. In addition, this analytical approach was compared with a previously described regular peptide mapping method [(7)] without GRT derivatization for characterization of oxidative carbonylation. It was found that none of the oxidative carbonylated peptides could be identified on the regular peptide maps of both the unstressed and the 16-hour oxidized IgG1A samples (Figure 11A to 11D), which further demonstrated the improved sensitivity of this analytical approach using GRT derivatization.

Light Chain residues (position no.)	Identified in unstressed IgG1A	Identified in 2-hr oxidized IgG1A	Identified in 16-hr oxidized IgG1A
T5	+	+	+
R18		+	+
R24		+	+
T69		+	+
T74	+	+	+
P80		+	+
T85	+	+	+
T97		+	+
T109		+	+
T164	+	+	+
T172		+	+
T180			+
P204			+
T206		+	+
Heavy Chain residues (position no.)			
R19			+
T30	+	+	+
T57		+	+
T98		+	+
T114	+	+	+
T120		+	+
T135		+	+
T155	+	+	+
K205			+
K214			+
T225	+	+	+
T256		+	+
P271			+
K290	+	+	+
P329			+

Table 3.Carbonylation sites identified on oxidized and unstressed bulk drug substances of IgG1A

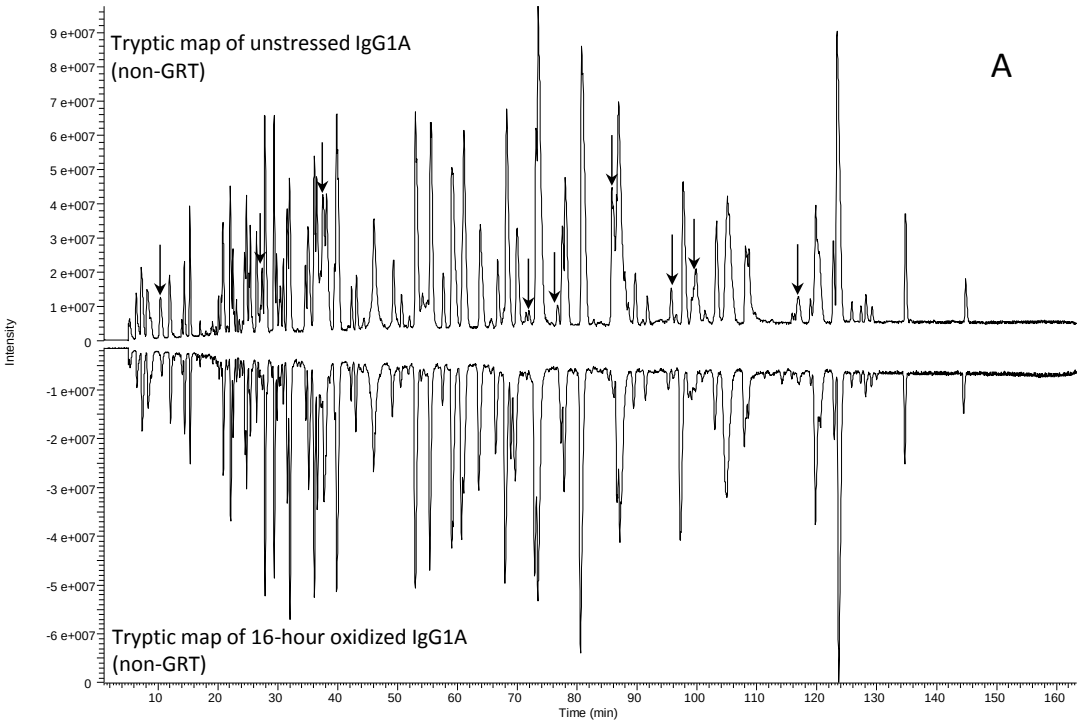

Figure 11. LC-MS base peak chromatograms of (A) Regular (non-GRT treated) tryptic maps of unstressed and 16-hour oxidized IgG1A. Peptide peaks either disappeared or became very small in the oxidized maps were labeled with arrow.

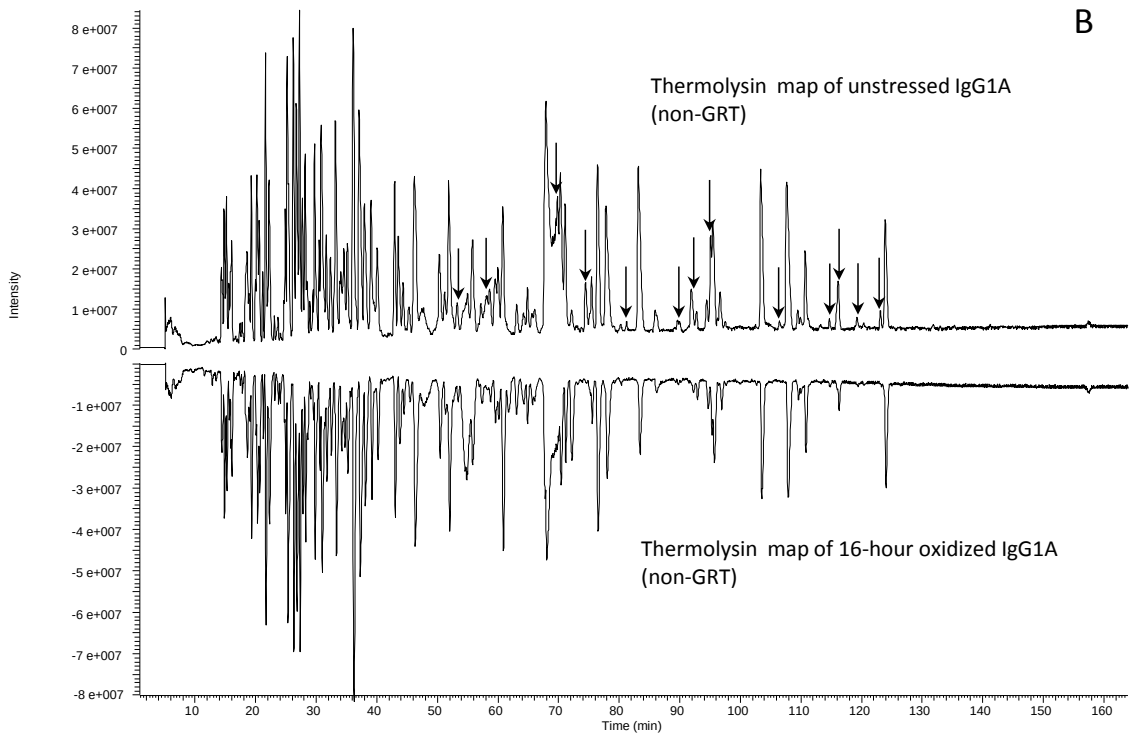

Figure 11. LC-MS base peak chromatograms of (B) Regular (non-GRT treated) thermolysin maps of unstressed and 16-hour oxidized IgG1A. Peptide peaks either disappeared or became very small in the oxidized maps were labeled with arrow.

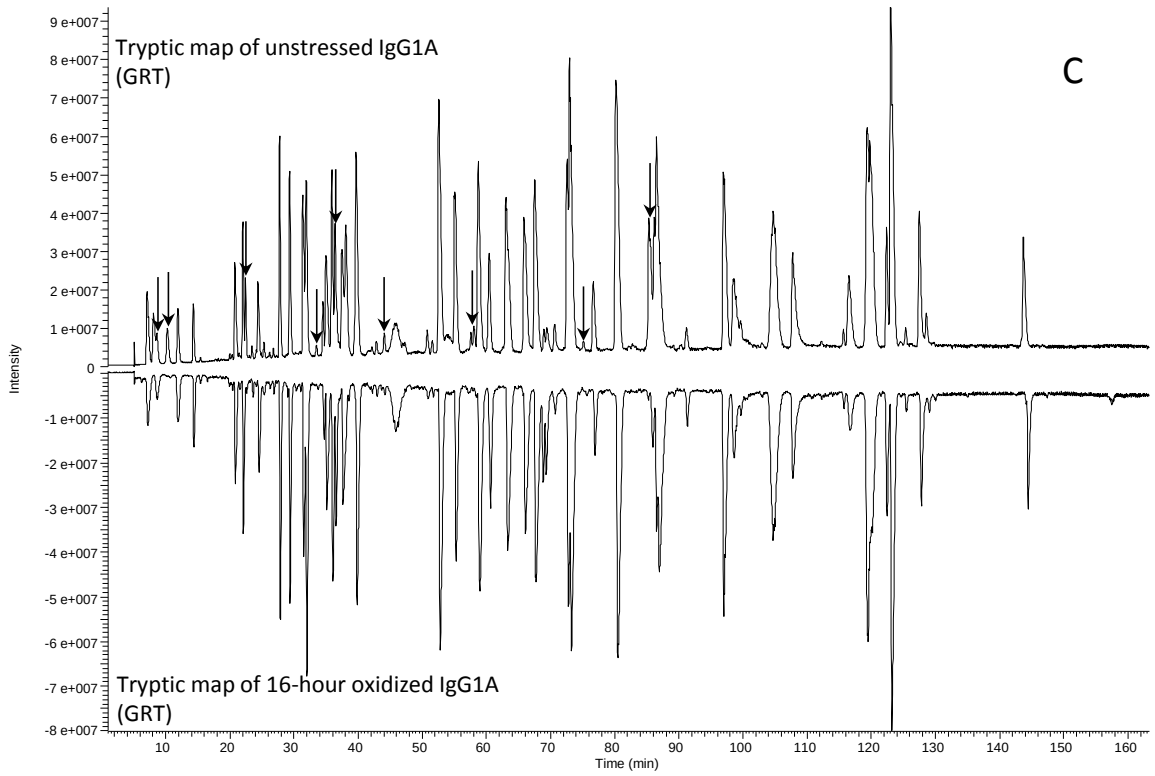

Figure 11. LC-MS base peak chromatograms of (C) GRT-derivatized tryptic maps of unstressed and 16-hour oxidized IgG1A. Peptide peaks either disappeared or became very small in the oxidized maps were labeled with arrow.

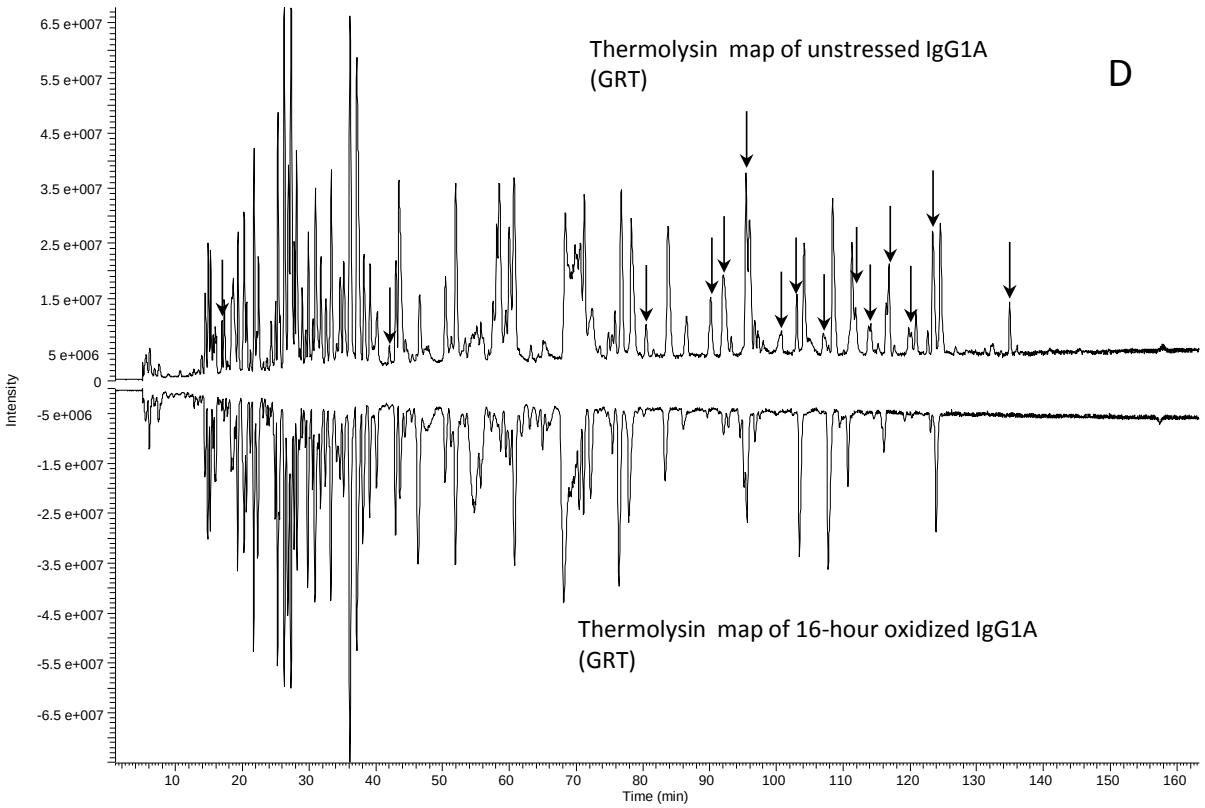

Figure 11. LC-MS base peak chromatograms of (D) GRT-derivatized thermolysin maps of unstressed and 16-hour oxidized IgG1A. Peptide peaks either disappeared or became very small in the oxidized maps were labeled with arrow.

From the carbonylation sites identified on the unstressed, 2-hour, and 16-hour oxidized IgG1A, it was noticed that certain carbonylation sites were only identified on the 16-hour oxidized sample. It is unclear if all the identified target amino acid sites are equally susceptible to MCO-induced carbonylation. Further analysis by quantitative measurement in a site-specific manner will be very helpful to better understand the differences among various carbonylation sites. It should be noted, however, that relative quantitation by extracted ion chromatography in this approach will likely result in over-estimation of site-specific carbonylation levels due to the enhanced ionization efficiency of GRT-derived peptides. For more accurate quantitation, stable isotope-labeled peptide standards may be required, and will be explored in the future studies.

Distribution of Carbonylation Sites on Three Highly Similar IgG1s. The distribution of carbonylation sites within the molecular structure of proteins is an interesting topic for further investigation.[37, 38] An interesting example is that carbonylation sites on human serum albumin (HSA) are clustered while carbonylation sites on bovine serum albumin (BSA) are dispersed,[37] even though HSA and BSA share 75% sequence similarity. To further demonstrate the utility of this approach and to investigate if there are any common carbonylation sites on IgG1s, the oxidative carbonylation sites on three IgG1s sharing 91% sequence similarity were characterized. The oxidative carbonylation sites identified on the 16-hour oxidized IgG1B and IgG1C are summarized in Table 2. Of all the carbonylation sites identified, only 14 sites are common among all three IgG1s (highlighted in **bold** in Table 2). When the 14 common carbonylation sites were mapped to a crystal structure of full-length IgG1 (PDB entry 1HZH) antibody (Figure 12), it was found that all of these common sites are located on the surface and accessible to solvent. It should be interesting for future work to look for any patterns or structural motifs that might emerge to allow prediction of carbonylation sites on IgG1s.

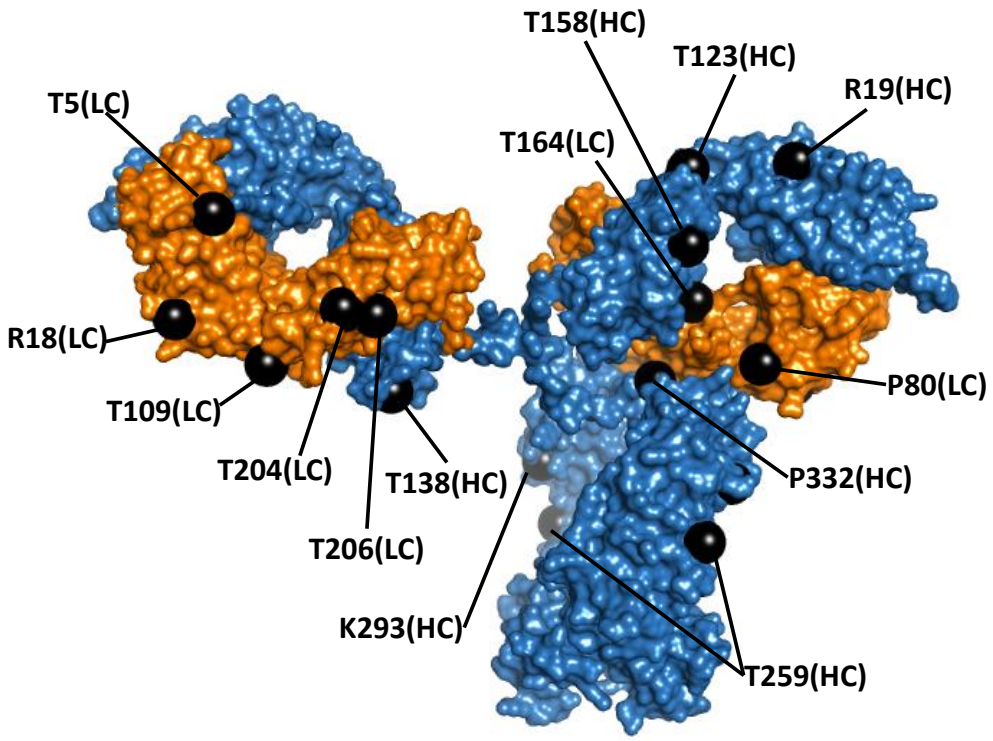

Figure 12. Common carbonylation sites on oxidized IgG1A, IgG1B, and IgG1C displayed on a crystal structure of a full-length IgG1 (PD entry 1HZH) antibody in a surface representation using PyMOL software. Heavy chain is colored in blue and light chain is colored in orange. The carbonylated residues are shown in black spheres.

CONCLUSIONS

Metal catalyzed oxidative carbonylation forms a type of post-translational modification found in therapeutic proteins. Very little is known about this product variant, especially for mAb products. In this report, a novel analytical method that combines derivatization with GRT, peptide mapping with high resolution mass spectrometry, and database searching for high-confidence identification of specific carbonylation sites on mAbs is demonstrated. This method effectively addresses a significant analytical challenge for the biotechnology industry for extended characterization of oxidative carbonylation. Compared with many existing analytical methodologies, this method has a key advantage of identifying carbonylation sites without using any affinity enrichment or fractionation steps. Two favorable features of the method were thought to enable such identification. First, the "charge-tagging" derivatization with GRT introduces a positive charge to the oxidation sites and provides enhanced ionization efficiency. Second, the high quality of CID MS^2 spectra of GRT-derivatized peptides allows identification of carbonylation sites by database search with ease. Additionally, a simple data filtering process was incorporated to reduce false positive identifications, which provides a high degree of confidence in the assignment of carbonylation sites. Overall, this method is highly sensitive and effective as demonstrated by identification of specific carbonylation sites on both unstressed and oxidized mAb drug bulk substances.

To the best of my knowledge, this is the first report of comprehensive identification of oxidative carbonylation sites on recombinant monoclonal antibodies. This method is a valuable tool for the extended characterization of carbonylated product variants on mAbs and potentially other protein pharmaceuticals. Furthermore, this method may be also relevant in the case that oxidative carbonylation is determined to be a critical quality attribute (CQA) and is at levels that

affect safety or efficacy of a mAb, where examination of this variant on different lots may be necessary. Finally, it will be interesting for future studies to investigate the impact of oxidative carbonylation on biological functions of mAbs, although it can be difficult to tease out the specific effect of oxidative carbonylation from that of other concurrent modifications, such as regular methionine/tryptophan oxidations, induced by MCO. Nevertheless, application of this method in future studies will greatly enhance the understanding of this product quality attribute and therefore, conforms well with QbD expectations.

Future Directions

For future studies, I would like to focus on the following three major areas: the degradation mechanism/factors that dictate the specificity and the extent of oxidative carbonylation on monoclonal antibodies, the effect of oxidative carbonylatio on the physical stability of mAbs, and the chemical consequence of the induced carbonyl groups on mAbs during long term storage and during in vitro incubation in plasma.

The mechanism of oxidative carbonylation will be studied by stressing monoclonal antibodies under various oxidation conditions, including different types of metal ions (copper and iron), different concentrations of the metal ions and hydrogen peroxide, different pHs, different buffers/salts/surfactants (PS20, PS80, and Poloxmar), and different temperatures. Subsequently, these oxidized mAbs will be characterized for the total carbonylation levels and the site-specific carbonylation to address these questions: 1. Are metal ions are required for the carbonylation reaction? 2. Do copper and iron ions have different selectivity for the generation of the site-specific carbonylation? 3. Is there any antibody structure/sequence motif for the oxidative carbonylation? 4. Does carbonylation propensity depend on the presence of a neighboring carbonylation site or an adjacent metal binding site? 5. What factors promote or hinder oxidative carbonylation? 6. What are the predominant elements in the oxidation conditions that influence the extent of protein carboxylation? The answers to the above questions will provide valuable information to help design future mAbs with increased chemical stability against oxidative carbonylation. In addition, it will be extremely helpful to the development of the cell culture process or formulations that could minimize oxidative carbonylation during the manufacturing or the storage of mAbs.

The effect of oxidative carbonylation on the physical stability of monoclonal antibodies is another important topic for future studies. This effect can be studied from multiple aspects. One experiment could be performed to measure the melting temperatures (Tm) and the heat capacities of carbonylated mAbs by differential scanning calorimetry (DSC). The DSC experiment, in combination with the site-specific carbonylation information, will be useful to assess the impact of carbonylation on mAb structural stability. In additional, this study could potentially identify the link/correlation (if exists) between site-specific carbonylation and the unfolding of the various domains in Fab and Fc. Other experiments using the conventional biophysical tools, such as 1-anilino-8-naphthalene sulfonate (ANS) binding fluorescence to probe the surface hydrophobicity, far- and near- UV circular dichroism to probe the impact on secondary and tertiary structure, can be helpful to provide further understanding of the impact of carbonylation on mAb physical stability, although proper controls should be introduced in these experiments to tease out the effects from other oxidative products (such as tryptophan and methionine oxidation) that are often co-generated by metal-catalyzed oxidation.

An intriguing hypothesis on the effect of oxidative carbonylation on the physical stability of mAbs is that oxidative carbonylation increases mAb aggregation propensity due to the increased protein hydrophobicity with carbonylation.[23] It will be particularly interesting to investigate this hypothesis by studying the aggregation rates of carbonylated mAbs under accelerated thermal stress conditions, such as at 40 ºC, for 1, 2, and 4 weeks, where the size profiles can be monitored by size exclusion chromatography (SEC) and capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis at each time point. Furthermore, assuming that oxidative carbonylation does drive aggregation, one would expect that certain carbonylation species would be enriched in the aggregate fractions over the time course of the thermal stress

experiment, while the level of the corresponding carbonylation species will likely decrease in the main peak fractions. One experiment can be performed to compare the levels at each carbonylation sites for the main peak fraction and the aggregate fractions using the carbonylation peptide mapping method, which can provide a detailed picture at site-specific level which carbonylation residues may be involved in driving the aggregation.

The chemical consequence (or the chemical instability) of the carbonyl groups on mAbs during storage should be investigated as well in future studies. The aldehyde groups may be susceptible to two chemical pathways: further oxidation to form carboxylic acid and formation of Schiff base with primary amines. For the first chemical pathway, the further oxidation product from the first pathway should be relatively easy to identify by peptide mapping analysis. However, the question is how stable are these aldehyde groups against further oxidation during storage and what factors influence the extent of the further oxidation to carboxylic acid. In addition, since formation of these carboxylic acids makes the mAb molecule more acidic (lower pIs) and more hydrophilic, how would that affect the physical stability of mAbs? For the second chemical pathway, the further degradation products from the second pathway are the intra- and inter- crosslinks between the aldehyde and lysine side chains and the n-terminal amino groups, particularly at higher pHs. Identification of these crosslinks may be challenging and require the use of some advanced analytical methodology. Nevertheless, this study will be necessary to help assess if these crosslinks do happen during manufacturing and storage, since crosslinks may lead to formation of aggregates, which poses an immunogenicity concern for mAbs products.

The physiochemical consequence of the carbonyl groups on mAbs under in vivo conditions (in plasma) may or may not follow the same pathways as that under the storage

condition, since the in vivo environment (plasma) contains various proteins/enzymes and other biomolecules. As shown in the biological systems, intracellular, mildly carbonylated proteins are targeted for turn-over by enzymatic proteolysis, while extensively carbonylated proteins may escape proteolysis by forming high molecular weight species.[13] In addition, there is a thiol-dependent decarbonylation mechanism that cells use to process the carbonylated proteins.[57] In the extracellular compartment (plasma), does the similar decarbonylation/proteolysis mechanism exist? If these mechanisms do exist, would they make carbonylation a less concerning quality attribute for mAb products? Answering these questions will be particularly important to assessing oxidative carbonylation as a critical quality attribute for mAb products, which typically have a half-life of several weeks in blood after being administrated.

One experiment will be performed to compare the physiochemical consequence of carbonylated mAbs incubated in rat plasma and in PBS at 37 °C for 0, 1, 2, 4, 6, and 10 days. At each time point, the mAb samples will be purified using anti-human IgG Fc IgGselect column using the experimental conditions described in Yin et al.'s paper.[58] These purified mAb samples will then be analyzed for their total carbonylation levels and size heterogeneity profiles, which can reveal if the enzymatic proteolysis or the thiol-dependent decarbonylation mechanism also exists in the plasma. In addition, another specific aim of this study is to understand if significant amount of crosslinks may be formed between the carbonylated mAbs and the plasma proteins (via the Schiff-base chemistry), since these cross-linked protein complex can also be an immunogenicity concern.

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