Discovery of Unknown Modifications in Recombinant Monoclonal Antibodies

By Chris Chumsae
B.A. Chemistry, Assumption College
M.S. Chemistry, Northeastern University

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Zhaohui Sunny Zhou
Associate Professor of Chemistry and Chemical Biology
Recombinant monoclonal antibodies have become one of the most important classes of biotherapeutics today. Due to their complexity, they must be produced by cellular expression. They are subject to various posttranslational modifications during manufacture, formulation and storage. Occasionally, the antibody may encounter a reactive molecule which in turn, results in a new variant. The application of modern analytical strategies including charge-based approaches and mass spectrometry in a defined workflow can effectively elucidate the presence of new species and help to understand the chemistry and underlying root cause. This dissertation will discuss the discovery of three novel chemical modifications in a recombinant monoclonal antibody therapeutic. First, methylglyoxal from cellular metabolism was found to react and modify the side chains of susceptible arginine residues. The adduction increased the mass by 54 or 72 daltons and caused the antibody to elute earlier in weak cation exchange chromatography (WCX). Second, the N-terminus of the antibody was determined to be modified by citric acid used as an excipient in the formulation buffer during storage. The modification increased the mass by 156 or 174 daltons and also resulted in an earlier elution of the modified species by WCX. Lastly, the antibody was modified by the addition of vitamin C (ascorbic acid) added to the cell culture as a supplement. It was determined that ascorbic acid degraded to xylosone which in turn reacted with primary amines in the antibody. The modification increased the molecular weight by 130 or 148 daltons and once again caused the antibody to elute earlier. These results were confirmed using stable isotope labeled ascorbic acid. All of these variants were initially observed as minor peaks in the chromatogram but the application of mass spectrometry strategies and spiking studies proved that such unassuming changes are worth further investigation.
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<th>Description</th>
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<tr>
<td>$^{13}$C</td>
<td>$^{13}$C labeled carbon</td>
</tr>
<tr>
<td>1.3-BPG</td>
<td>3-bisphosphoglycerate</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>C18</td>
<td>octadecylsilane</td>
</tr>
<tr>
<td>CDR1</td>
<td>complementary determining region 1</td>
</tr>
<tr>
<td>CDR2</td>
<td>complementary determining region 2</td>
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<td>CDR3</td>
<td>complementary determining region 3</td>
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<tr>
<td>CH1</td>
<td>constant heavy domain 1</td>
</tr>
<tr>
<td>CH2</td>
<td>constant heavy domain 2</td>
</tr>
<tr>
<td>CH3</td>
<td>constant heavy domain 3</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DDA</td>
<td>data dependent acquisition</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxy acetone phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EIC</td>
<td>extracted ion chromatogram</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
</tr>
<tr>
<td>G-3-P</td>
<td>glyceraldehyde-3-phosphate</td>
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<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally regarded as safe</td>
</tr>
<tr>
<td>G0F</td>
<td>core fucosylated biantennary glycan with 0 terminal galactose</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HCD</td>
<td>hard collision dissociation</td>
</tr>
<tr>
<td>IgG1</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>isoD</td>
<td>isoaspartic acid</td>
</tr>
<tr>
<td>isoAsp</td>
<td>isoaspartic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>Lys-0</td>
<td>antibody with 0 C-terminal lysine</td>
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<tr>
<td>Lys-1</td>
<td>antibody with 1 C-terminal lysine</td>
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<tr>
<td>Lys-2</td>
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<td>endoproteinase Lys-C</td>
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<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>mL</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MaxEnt</td>
<td>Maximum Entropy</td>
</tr>
<tr>
<td>MGO</td>
<td>methylglyoxal</td>
</tr>
<tr>
<td>MOLD</td>
<td>methylglyoxal lysine dimer</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
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<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>nanoMoles</td>
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<td>pgk</td>
<td>3-phosphglycerol lysine</td>
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<td>PNGaseF</td>
<td>protein N-glycanase</td>
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<td>PTM</td>
<td>posttranslational modification</td>
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<td>R</td>
<td>arginine</td>
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<tr>
<td>Q1</td>
<td>quadrupole 1</td>
</tr>
<tr>
<td>Q2</td>
<td>quadrupole 2 (collision cell)</td>
</tr>
<tr>
<td>Q-Tof</td>
<td>quadrupole-time of flight mass spectrometer</td>
</tr>
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<td>reactive oxygen species</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion current</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UPLC</td>
<td>ultra high pressure liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VH</td>
<td>variable heavy domain</td>
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<tr>
<td>WCX</td>
<td>weak cation exchange chromatography</td>
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<td>XIC</td>
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Chapter 1: Introduction and Background

1.1 Introduction

Recombinant monoclonal antibodies have become one of the most important classes of biotherapeutics due to their target specificity and the ease of manufacture using standard protocols\textsuperscript{1-2}. Thus, they have been widely studied due to their tremendous growth and applicability across broad therapeutic areas\textsuperscript{3}. As a consequence, the field of bioanalytical chemistry as it applies to the analysis and characterization of these proteins has evolved significantly over the last twenty years. In particular, techniques which delve into the primary, secondary, tertiary and quaternary structure have uncovered many chemical changes which occur due to multiple factors.

There are four sub-classes of immunoglobulin G, however, the most common sub-class used in the generation of antibody drugs is IgG1. Recombinant monoclonal antibodies are comprised of two heavy chains and two light chains which are assembled together by four interchain disulfide bonds. The heavy chain is comprised of four immunoglobulin domains stabilized by intrachain disulfide bonds. Three of these domains (CH1, CH2 and CH3) are conserved and the binding domain is variable (VH) with the greatest variability occurring in the binding regions known as the complementary determining regions or CDR1, CDR2 and CDR3. In addition, the heavy chain has a glycosylation site in the CH2 domain. The presence of this glycosylation serves to provide stability to the domain and may influence the domain conformation\textsuperscript{4}. The light chain consists of a variable and constant domain, the VL and CL, respectively. The heavy chain is approximately 450 amino acids in length and the light chain is
approximately 215 amino acids in length. The structure of a recombinant monoclonal antibody is depicted in Figure 1-1.

**Figure 1-1:** The structure of a recombinant monoclonal antibody
Recombinant monoclonal antibodies are far more complicated than small molecule drugs due to the sheer size plus additional posttranslational modifications. Such modifications may be driven enzymatically or chemically and increase the molecular heterogeneity of recombinant mAbs. These are discussed in detail in several recent reviews\textsuperscript{5-8} and include but are not limited to deamidation\textsuperscript{9-14}, glycation\textsuperscript{15-20}, incomplete C-terminal lysine processing\textsuperscript{21-25}, N-linked glycosylation\textsuperscript{26-34} and O-linked glycosylation\textsuperscript{35-40}, C-terminal amidation\textsuperscript{41}, oxidation\textsuperscript{42-45} and N-terminal pyroglutamate formation\textsuperscript{5-8,13,46-51}. Representative posttranslational modifications are depicted in Figure 1-2.

Recent reports have elucidated a new classification of posttranslational modification by endogenous reactive molecules. In general, these are far less studied with respect to protein analysis of recombinant biotherapeutics. Such species may be reactive metabolites, unreactive species capable of forming a reactive intermediate or molecules which may degrade into reactive species. In addition, such posttranslational modifications may be the result of the intracellular conditions such as those which lead to reactive oxygen species (ROS).
Figure 1-2: Representation of some common post-translational modifications which may occur in recombinant monoclonal antibodies.
1.2 Reactive Species

1.2.1 Free Thiols

One of the most familiar classes of reactive molecules is the free thiols$^{52-53}$. Reduced thiols are very potent nucleophiles which are capable of forming disulfide bonds with other free thiols in proteins or with intact disulfide bonds thereby disrupting the intended cysteine pairing and inducing a scrambling event$^{52-54}$. Examples of products of free thiols include cysteinylation$^{55-57}$ and glutathionylation$^{58-59}$. Common free thiols are shown in Figure 1-3. The reaction of one of these species with a catalytic cysteine resulting in a disulfide bond will ablate protein function$^{56,60-61}$. In addition, free thiols may promote disulfide bond scrambling which has the potential of destabilizing the protein or altering its structure$^{62-63}$. In all of these cases, an increase in molecular weight will be observed imparted by the addition of these reactive species.
I. Reactive free thiols in cell culture

$$R = A, B \text{ or } C$$

A. 
\[
\begin{array}{c}
\text{HS} \\
\text{NH}_2 \\
\text{O}
\end{array}
\]
\[
\begin{array}{c}
\text{SH} \\
\text{NH}_2 \\
\text{O}
\end{array}
\]
cysteine

B. 
\[
\begin{array}{c}
\text{HS} \\
\text{NH}_2 \\
\text{O}
\end{array}
\]
\[
\begin{array}{c}
\text{SH} \\
\text{NH}_2 \\
\text{O}
\end{array}
\]
homocysteine

C. 
\[
\begin{array}{c}
\text{HO} \\
\text{NH}_2 \\
\text{O}
\end{array}
\]
\[
\begin{array}{c}
\text{SH} \\
\text{NH} \\
\text{N}
\end{array}
\]
\[
\begin{array}{c}
\text{O} \\
\text{N}
\end{array}
\]
glutathione

II. Free thiol reactivity on proteins

$$R$$

$$\text{SH}$$

$$\text{R}$$

$$\text{SH}$$

Figure 1-3: Structures of free thiols found in biological systems including recombinant cell culture.
1.2.2 Homocysteine thiolactone and N-Homocysteinylation

A precursor to cysteine biosynthesis is homocysteine which may either form methionine following methylation or cystathionine, the precursor of cysteine, following a vitamin B6 mediated complex with serine\textsuperscript{64}. In addition to its potential to react with free cysteines within the primary structure of a protein\textsuperscript{65}, it may also be transformed into homocysteine thiolactone through the action of methyonyl tRNA synthetase\textsuperscript{66-67} (see Figure 1-4). Homocysteine thiolactone may then react with primary amines in a protein, specifically lysine, resulting in N-homocysteinylation which modifies the side chains of lysine and increases the mass by 117 daltons\textsuperscript{66-67}. 
**Figure 1-4**: Formation of homocysteine thiolactone and the subsequent product with susceptible lysine residues.
1.2.3 Glycation

Carbohydrates may react with proteins when their reducing end (electrophilic aldehyde) encounters a reactive primary amine to form a Schiff base (aldimine) as shown in Figure 1-5\textsuperscript{15-16}. An Amidori rearrangement will form a stable ketoamine exhibiting a mass increase of 162 daltons\textsuperscript{16,68}. Although some level of glycation is unavoidable because glucose is added to the cell culture as a primary energy source, the tertiary structure of the protein may facilitate the Amidori rearrangement and lead to significant levels of glycation\textsuperscript{15}. To this point, reports of histidine residues\textsuperscript{16} and acidic residues\textsuperscript{15} facilitating glycation suggest their side chains may promote the abstraction of a proton from the glucose C2 atom which initiates a rearrangement of the aldimine to a more stable ketoamine.

Glycation has also been reported when glucose was used in the formulation of a biotherapeutic molecule\textsuperscript{69}. More surprisingly, glycation was associated with a protein drug which was formulated in sucrose\textsuperscript{70-71}. In these reports, the glycosydic bond between the fructose and glucose sub-units was hydrolyzed during storage thus freeing the reducing end of glucose and enabling glycation to occur. Thus, glycation resulted from an unexpected source.
**Figure 1-5:** Mechanism of glycation. The epsilon primary amine of lysine attacks the reducing end of glucose forming a Schiff base following dehydration. An Amidori rearrangement produces the more stable ketoamine.
1.2.4 Advanced Glycation Endproducts

Advanced glycation end products (AGE) are another set of reactive molecules derived from glucose which may contribute to molecular heterogeneity. Advanced glycation end-products have their analytical roots based in the analysis of blood samples from patients with diabetes. High glucose levels facilitate their formation. An increase in the oxidative stress exerted on the cell may promote the formation of AGE’s as this may retard the glyoxylase I/II pathway due to reduced glutathione. Common AGEs are shown in Figure 1-5 (I).

Specifically, methylglyoxal and glyoxal have been associated with chemical modifications of proteins. Methylglyoxal is a by-product of glycolysis. The two glycolytic intermediates of the catalysis of fructose bisphosphate aldolase are glyceraldehyde-3-phosphate (G-3-P) and dihydroxy acetone phosphate (DHAP). If DHAP loses its phosphate group before the catalysis of triose phosphate isomerase can occur then methylglyoxal will form. In addition to arginine residues, MGO has been shown to have reactivity with lysine and cysteine residues albeit to a lesser extent. Advanced glycation end products have been implicated in protein cross-linking between arginine and lysine residues resulting in a pentosidine or between two lysine residues forming a methylglyoxal lysine dimer (MOLD). Representative products of AGEs and protein are shown in Figure 1-6 (II).
I. Advanced glycation end products (AGE) capable of reacting with proteins

- **Glyoxal**

- **Methylglyoxal**

- **3-deoxyglucosone**

II. Representative protein products of AGEs

- **Carboxymethyl-lysine**

- **Carboxyethyl-lysine**

- **MG-arginine**

- **Pentosidine**

- **MOLD**

**Figure I-6:** I. Common advanced glycation end products derived from glucose. II. Representative products of AGE’s with nucleophilic amino acid side chains.
1.2.5 1,3-Bisphosphoglycerate

The possibility that other reactive intermediates or metabolites prevalent in biological systems may contribute to new posttranslational modifications is an intriguing one. Interestingly, 1,3-bisphosphoglycerate (1.3-BPG), the glycolytic intermediate generated from the catalysis of glyceraldehyde-3-phosphate dehydrogenase has been reported as another example of a reactive metabolite\textsuperscript{81}. 1,3-BPG is a strong electrophile due to its acylphosphate moiety\textsuperscript{81-82}. Specifically, 1,3-BPG has been shown to react with the lysine primary amine forming 3-phosphoglycerol lysine (pgk) as shown in Figure 1-7.
Figure 1-7: The product of a reaction with 1,3-bisphosphoglycerate and the epsilon primary amine of lysine.
1.2.6 Peroxides and Lipid Peroxidation

Other examples of reactive electrophiles in biological systems are the products of lipid peroxidation\textsuperscript{83}. Oxidative stress may lead to an increase in reactive oxygen species. Consequently, peroxides have been shown to react with unsaturated fats forming a variety of reactive electrophiles\textsuperscript{84-85}. The nucleophilic side chains of cysteine, histidine and lysine have been reported to react with these species through Michael addition resulting in carbonylation\textsuperscript{86-87}. Specifically, protein modifications by reactions with 4-hydroxynonenal (4-HNE), malondialdehyde and oxononenal (see Figure 1-8) have been reported\textsuperscript{88-92}. Such modifications may be far more ubiquitous following oxidative stress than previously thought. Recombinant monoclonal antibodies which recognize protein adducts formed by these reactive electrophiles have proven to be a valuable tool\textsuperscript{93-94}. 

I. Reactive electrophiles formed from lipid peroxidation

![Chemical structure of 4-hydroxy-2-nonenal (4-HNE)]

II. Product of carbonylation of a cysteine residue by 4-HNE

![Chemical structure of the product of cysteine residue attack on 4-HNE]

**Figure 1-8:** I. The structures of reactive electrophiles following lipid peroxidation. These species may react with the side chains of lysine, cysteine and histidine. II. The product of cysteine side chain attack on the electrophilic center of 4-HNE.
1.2.7 Peroxide-mediated Sulfenylation

Related to oxidative stress and the production of reactive oxygen species is peroxide-mediated sulfenylation of free cysteine residues\textsuperscript{95}. Hydrogen peroxide may react with free sulfhydryls to form Cys-sulfenic and Cys-sulfinic acid\textsuperscript{96-97} (see Figure 1-9). The formation of these posttranslational modifications has emerged as another signaling mechanism available to biological systems analogous to phosphorylation\textsuperscript{95}. These species have proven to be labile and thus difficult to measure using today’s analytical approaches\textsuperscript{98}, thereby requiring the implementation of selective trapping reactions for their detection. Specifically, trapping of sulfenylated products may be achieved by stabilization with dimedone or iododimedone\textsuperscript{99}. The stabilized products have subsequently been analyzed by proteomics workflows. These labile PTM’s remind us that

1.2.8 The complexity of a biological system

Biological systems are extremely complicated with countless chemical processes in constant flux. In addition, changes in the external environment can induce major shifts in the cellular metabolic flow. It would be a Herculean feat to catalogue all of these processes and in turn identify a comprehensive list of all reactive molecules and species. What is possible, however, is to gain an understanding of general chemical reactivity that may occur between proteins and chemical functionalities. By taking a diligent approach and open mind towards seemingly insignificant changes in standard analytical assessments, analytical protein scientists can continue to expand the list of these variants and their underlying chemistry. The discovery of chemical modification which may impact the function of the therapeutic or increase the risk of
immunogenicity can mitigate potential risks to patients and ensure that biopharmaceutical companies release drugs which meet the highest product quality.
Figure 1-9: The structure of cysteine, cys-sulfenic acid, cys-sulfinic acid and cys-sulfonic acid.
1.2.9 Intracellular influences and Reactive Oxygen Species

The intracellular environment of CHO cells has been implicated in the formation of reactive oxygen species (ROS). Reactive oxygen species are normal by-products of cellular metabolism\textsuperscript{100}. Specifically, the formation of superoxide in the mitochondria has been linked to the presence of enzymes that can donate electrons to O\textsubscript{2} thus forming O\textsubscript{2}\textsuperscript{-} (superoxide)\textsuperscript{101}. For such transformations, the ratio of NADH/NAD\textsuperscript{+} is elevated facilitating an increase in the abundance of enzymes reacting with O\textsubscript{2}. During periods of cellular stress, the levels of ROS may increase dramatically\textsuperscript{102}. Reactive oxygen species have been associated with damage to DNA, RNA, and proteins\textsuperscript{103}. In addition, reactive oxygen species have been linked to lipid peroxidation and advanced glycation end products\textsuperscript{104-105}.

At high ROS levels, cells produce more glutathione and thioredoxin that can act to scavenge reactive oxygen species\textsuperscript{106}. These mechanisms are regulated by the intracellular redox. Specifically, glutathione disulfide and thioredoxin cannot control ROS accumulation, thereby further raising ROS levels that lead to cellular damage. Upon oxidative stress, the cell will attempt to convert GS-SG to GSH to increase antioxidant reserves. Enzymes such as glutathione reductase facilitate this transformation\textsuperscript{107}.

Cysteine starvation has been linked to glutathione deficiency\textsuperscript{108}. The cysteine moiety is the functional residue within glutathione making it a critical component. A lack of intracellular cysteine due to cellular starvation thus leads to a depletion in glutathione. As a result, the levels of intracellular ROS may increase.

Both glutathione depletion induced by high levels of reactive oxygen species and cysteine starvation resulted in a similar cell culture phenotype. Both have a clear role with respect to the accumulation of reactive oxygen species. In addition, both of these events may be directly
related where lower cysteine levels but not necessarily achieving complete starvation coupled to increased demand of glutathione due to ROS can lead to a depletion of cysteine, glutathione or both. As a result of these factors, the glyoxylase I/II pathway will be down regulated thereby increasing cellular levels of methylglyoxyl\textsuperscript{109}.

The implementation of a chemically defined media, which is discussed in Chapter 2, could play a role in either of these scenarios. Cellular starvation and cysteine depletion induced by insufficient media components in a chemically defined media may explain depletion of glutathione and thus the retardation in the glyoxylase I/II pathway. In addition, chemically defined media has been shown to induce higher cell densities thus increasing the overall culture metabolic demands. In these cases, the culture exhibited signs of oxidative stress and increased reactive oxygen species. The formation of ROS may have been a result of cellular starvation or may have directly induced through a futile attempt to arrest the oxidative stress leading to depletion of reduced glutathione and cysteine. More studies would be necessary to better understand how either of these scenarios contributes to the formation of methylglyoxal.

1.2.10 Detecting the Unexpected

All of these examples remind us that reactive molecules may arise from an unexpected source and facilitate an increase in unwanted protein modifications. Accordingly, the chemical modification of a recombinant monoclonal antibody presents new challenges to the biotechnology industry. These therapeutic ‘magic bullets’ are destined for administration to patients therefore their subsequent product quality is held to a high bar. Even low levels of heterogeneity may be problematic to the patient due to loss of potency, increased risk of immunogenicity, or both. Chemical modifications to the complementary determining regions
can ablate antibody function. In addition, these chemical modifications are not limited to the expressed biotherapeutic, albeit, they will exist as the most abundant protein in the cell culture. Key host cell proteins may also be affected by modification of these reactive molecules. Such events can affect the overall health and expression levels of the cell culture. It is therefore important to understand the underlying product quality and to extrapolate root cause from the analytical observations.
1.3   Analytical Strategies to Discover the Unexpected

1.3.1   Analytical Detective Work

Today’s analytical strategies are well suited to perform deep characterization of proteins and protein drugs. Technologies such as mass spectrometry, liquid chromatography and capillary electrophoresis are capable of measuring very subtle differences in the primary structure of a recombinant monoclonal antibody. Applied work flows consisting of complimentary methodologies can be used to uncover molecular heterogeneity and root cause through a stepwise approach. Specifically, chromatographic approaches can separate a recombinant monoclonal antibody with a chemical modification from the native antibody. Subsequently, analytical tools may be applied to understand the nature of the variant. Of all these tools, mass spectrometry has emerged as the most powerful analytical tool enabling the assessment of the entire primary structure of a recombinant monoclonal antibody. Discrete changes can be determined at the residue level facilitating the identification of new discoveries in this important class of biotherapeutics.

1.3.2   Weak Cation Exchange Chromatography

Proteins have a global charge which is due to the cumulative effects of its protonated basic residues and its deprotonated acidic residues in the primary structure and is represented as the isoelectric point. The pH and local environment can influence these charges. Additionally, one must distinguish between those charged residues which are located on the surface of the protein and thus exposed. In addition to local environment, chemical modifications can influence the surface charge of the protein. For instance, if a basic amino acid is covalently modified, the side chain pKa may become depressed potentially leading to a deprotonation at
that site$^{16,66}$. A protein in this scenario would exhibit a reduced number of basic charges on its surface.

A very powerful technique in discerning changes in a recombinant monoclonal antibody primary structure is weak cation exchange chromatography$^{21,25}$. Weak cation exchange columns contain polymeric beads coated with carboxylate groups$^{110}$. It is the electrostatic interaction between protonated basic residues on the protein surface (lysine, arginine, histidine) and these carboxylates which cause the protein to bind to the column. At first glance, it appears to be a low resolution technique as compared to today’s modern mass spectrometry workflows. However, it is remarkably sensitive to any site specific changes in the number of surface charges on the antibody. For instance, it has been shown that a single site of glycation on a hyper-susceptible lysine in a recombinant monoclonal antibody resulted in a decrease in pI presumably due to the loss of this protonation site$^{15}$.

The interpretation of low levels of a chemical modification is hampered by the presence of the acidic species which comprise a highly heterogeneous population$^{5-6,8,19,21}$. Contributors include terminal sialylation$^{29,111-113}$, asparagine deamidation$^{13-14}$, and glycation$^{15-16}$ just to name a few. The appearance of a new species may exist as a slight increase in the UV profile which may be difficult to discern from the already complex unresolved chromatographic region. In addition, it may be challenging to differentiate an unresolved low abundance chromatographic peak from differences due to fluctuations in the chromatographic performance.

Often, a variant may only exist in trace amount making the discovery and analysis of the variant difficult. Isolating minor peaks or regions of subtle changes in the chromatographic separation has proven to be a valuable strategy$^{43,114}$. A specific region which may be changing can be isolated for subsequent analysis by mass spectrometry. In this way, the overall
complexity will be reduced and the variant which maybe present can be enriched. It is important to note that other endogenous contributors within this region may also be present therefore it is prudent to compare LC/MS spectra against a control. Additionally, the shift in the retention time may provide clues about the distribution of a modification on a recombinant monoclonal antibody. For example, if more than one peak is isolated, the relative retention time shifts and corresponding levels of a variant may begin to establish the effects that multiple modifications may have on the surface charge.

1.3.2 Reduced Liquid Chromatography/Mass Spectrometry

Reduced LC/MS analysis is a key step in determining root cause of differences observed in the weak cation exchange chromatogram. The antibody sample is treated with a reducing agent such as 10 mM DTT which breaks the interchain disulfide bridges between the heavy and light chains. The sample is introduced into the mass spectrometer using reversed phase chromatography which will resolve the light chain from the heavy chain. Within the electrospray source, the sample will become desolvated and stripped of negative counter ions to enhance ionization. A quadrupole-time of flight mass spectrometer (Q-Tof) used in MS mode will measure a distribution of multiply charged species corresponding to the eluting light chain or heavy chain. The spectral pattern is deconvoluted using the Maximum Entropy (MaxEnt) algorithm which reconstructs the single charged spectrum.

The technique provides the molecular weight of the recombinant monoclonal antibody light chain and heavy chain as well as any other molecular weights due to the modifications associated with the primary structure. For example, mass shifts of +16 daltons or +162 daltons can be attributed to the formation of methionine sulfoxide or glycation, respectively. In
addition, there are databases which exist and have extensive lists of changes in mass and a reported root cause. Two such databases are Deltamass (http://www.abrf.org/index.cfm/dm.home) and Unimod (http://www.unimod.org). It is possible to make an initial assignment to an observed variant based on mass shift especially if the analysis was performed on a high mass accuracy instrument.

1.3.4 Spiking Studies with Putative Reactive Species

From a quote; “analytical scientist are good at finding what they know”. This statement is quite true. In other words, it is easy to find something if you know what to look for. However, should the observed mass shift in the recombinant protein drug be unfamiliar and not easily recognized, then identifying the underlying change to the primary structure can be quite challenging. It is now up to the analytical scientist to perform some detective work and try to pinpoint specific differences which may have occurred in the recombinant monoclonal antibody during cell culture or long term storage.

One must keep an open mind when it comes to such challenges. If a potential candidate, i.e., reactive species, can be identified from what is known about the changes in molecular weight and the conditions from the cell culture, storage, etc, then a spiking study of the candidate or panel of candidates may be performed. Specifically, if the reactive molecule or its stable precursor is available, it can be spiked into a pure sample of the recombinant monoclonal antibody. In practice, the ideal species of this “pure sample” will be the major peak which typically consists of the recombinant monoclonal antibody without C-terminal lysine on both of its heavy chains and devoid of the acidic species. Following an appropriate incubation with the
spiked agent, the sample may be analyzed by weak cation exchange chromatography and by reduced LC/MS. The results can be compared to the initial observations. In addition, any shifts in the chromatographic profile may be directly attributed to changes in the primary structure induced by the presence of the spiked agent. If the retention times between the initial sample which showed subtle changes and the stressed sample are in good agreement, the possibility increases that this reactive molecule is the root cause.

### 1.3.5 Peptide Mapping with Mass Spectrometry

A peptide map is often referred to as the protein fingerprint. The digestion of a reduced and alkylated recombinant monoclonal antibody with a highly specific protease will generate a predictable and reproducible peptide map. Trypsin is commonly used to digest the reduced and alkylated protein at the C-terminus of lysine and arginine residues generating peptides which can be separated on a reversed phase C18 (octadecylsilane) column. High efficiency separations may be achieved by using small particle size (1.7 µ) and the application of ultra-high pressure liquid chromatography (UPLC). The peptides will be separated based on their relative hydrophobicity as the percent of organic mobile phase increases. Eluting peptides may be detected by UV absorbance at 214 nm before being introduced into the electrospray source.

Q-TOF and LTQ-Orbitrap mass spectrometers may be used to perform peptide mapping. Both of these instruments are capable of performing MS and MS/MS. In MS full scan mode, Q-TOF type instruments direct the ion flow through the first quadrupole and collision cell to a pusher where the ions are cooled. The pusher then repells the ion packet into the time of flight tube where following a refocusing in the reflectrons, they are directed to the detector. The
software measures the time the ions were in flight to determine the mass using the formula: $KE = \frac{1}{2}mv^2$. In MS/MS mode, typically data dependent acquisition (DDA) is used. A defined number of the most abundant ions (e.g. 5) are each isolated by the quadrupole or Q1 region. The isolated ion passes to the collision cell or Q2 where it encounters inert argon. Collisions with the inert argon cause the peptides to lose residues from the C-terminus or N-terminus resulting in b ions or y ions, respectively. Energy may be applied to accelerate the ions through this region to induce greater fragmentation. The fragmented peptide is once again detected in the TOF. The fragmentation pattern may be compared to a predicted pattern either manually or with processing software, which can determine the amino acid sequence of the peptide. The same process will be applied to the second most intense ion and so forth until all five have been measured. These ions may be excluded from the determination of the next 5 most abundant ions in order to evaluate less abundant peptides.

Using an LTQ-Orbitrap platform in a high resolution mode, the ions are passed through the linear ion trap and accumulate in the C-trap. The ions are then directed into the orbitrap where the ions orbit and oscillate around a central electrode. Once the ions have achieved the specific orbit based on their mass to charge ratio, the ions are directed to the detector. A fourier transformation is applied to the data which is used to construct the peptide mass spectra. For MS/MS, data dependent acquisition may also be applied. One at a time, the most abundant peptides are isolated sequentially using the linear ion trap. Each ion is then fragmented in the ion trap by increasing the applied energy resulting in collisionally induced dissociation. The fragments are then directed into the C-trap for subsequent entry into the orbitrap. The fragments are separated and detected. This mode of MS/MS sometimes provides better b ion coverage that with the use of a collision cell but suffers from the one third rule. To this point, the LTQ-
Orbitrap Velos has an additional collision cell analogous to that discussed with the Q-TOF and fragments parent ions by collision with inert gas molecules (denoted as hard collisionally induced dissociation by the manufacturer). Furthermore both of the activation modes may be used in a single analysis. In this configuration, one could perform alternating CID and HCD fragmentation so as to have complimentary fragmentation patterns significantly improving the depth of the data. Of course, the drawback of this approach is that it reduces the number of MS/MS scans which may be acquired over a given time frame.

The peptide mapping data may be examined chromatographically by evaluating the UV and TIC traces for obvious differences. Of course, evaluating the mass data provides the most pertinent information with regards to changes in the primary structure of the recombinant monoclonal antibody, however, often necessitates a detailed search of the suspected mass shift. The data may be submitted to a search algorithm which seeks to measure and assign amino acid sequences to the fragmentation pattern. In addition, if part of the primary structure is evident in a pattern but there is a mass shift, the software will attempt to assign a defined variant to a specific residue in the peptide. Carboxymethylation of cysteine residues is a typical example of this. The one limitation to using this strategy to assign variants is that you must A priori assign what potential variants you may expect to see. Assigning oxidation to methionine, deamidation to asparagine, or glycation to lysine are some common applications of this approach. The big challenge is that if you are working with an unknown variant, you do not know its mass and you do not know the susceptible residues in the primary structure.

To the previous point, localizing the target residue of the modification may be the most critical step in determining the source of the observed heterogeneity. Many of the computational search engines require that you set a target residue and the expected mass shift. If the analytical
scientist has a good idea of what the adduction may be then speculating the target residue or residues based on nucleophilicity and other favorable chemical properties may be possible. Performing a non-specific search will be quite computationally intensive and often provides so many false positives that the data loses value.

Sometimes it is prudent to perform a manual search for peptides which may exhibit the observed mass shift from previous analytics. This can take time, but if identification can be made, it results in significant progress to solving the source of the modification. Once the site of modification is determined, the chemistry of the candidate reactive molecule and residue can be hypothesized. The chemical modification will likely increase the overall size of the target residue. If lysine or arginine are modified the reactive molecule, the steric effects of the molecule will likely occlude the residue from the trypsin active site. Therefore, if lysine or arginine residues are suspected as the site of modification, miscleaved tryptic peptides must also be considered. In addition, alternate proteases may be employed with different specificity than trypsin resulting in fully cleaved peptides. Being able to make a direct comparison between the native and modified peptide is vital to gaining any quantitative information. This is not possible when trying to compare a modified miscleaved peptide to fully cleaved unmodified peptides.

1.3.6 Agreement between the spiked agent and the initial findings

Once the site of the unknown modification has been determined and a likely candidate has been identified, it is important to apply the analytical strategies to determine if all of the generated data is in good agreement. Of course, all of these techniques should be applied to both the spiking studies. The generation of a more pronounced peak in the weak cation exchange
chromatogram which has high similarity to the initial observations provides evidence the spiked agent has the same influences on charge as the unknown species.

The mass spectral data will be of particular value in assessing the similarities between the unknown and the forced product. Specifically, the MS and tandem MS data should be compared between the two with an emphasis on mass accuracy and spectral profile. For instance, highly similar fragmentation profiles between the same peptide from both conditions suggest that not only do two samples have the same primary structure but that both have the same site of modification. In addition, the modification may influence the fragmentation and ionization of specific ion series. Specifically, modification of arginine residues by malondialdehyde resulted in a fragmentation pattern that was rich in B ions due to the influence the adduction had on the peptide backbone\textsuperscript{115}. The generation of equivalent MS and MS/MS spectra provides solid evidence that the spiked material is the reactive molecule which resulted in the variant.

1.3.7 Stable Isotope Labels

The use of a stable isotope labeled analog of the spiked agent can help to identify conclusively the source of a chemical modification in the recombinant monoclonal antibody. The incorporation of the heavy label serves to unambiguously assign a specific molecule as the source of the adduction. This technique can be particularly powerful because the labeled molecule may co-elute with the reactive species due to their equivalent chemical properties but can easily be discerned by a mass spectrometer. Stable isotope labeling has proven valuable in the identification of a glycation site using heavy labeled glucose\textsuperscript{116}. In addition, cross-linked peptides were identified apriori by using the incorporation of \textsuperscript{18}O during the digestion process\textsuperscript{117}.  

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1.3.8 **Interpretation of all of the Findings**

The final things to consider pertain to the origination of the reactive species and the influence the cell culture conditions, storage conditions, etc. may have had on its appearance, reactivity or both. Understanding the chemistry of the reactive molecule is a must. The influence of redox and pKa on the reactive molecule and the recombinant monoclonal antibody can not only help to elucidate the mechanism but may also provide clues about conditions which can prevent the modification.

Understanding the global prevalence of the modification will not only add to the overall knowledge of the reactive molecule but will also provide a better understanding to which residues may be the most susceptible, ie reactive in the recombinant monoclonal antibody on a broad basis. Such information can be applied to future studies as potential hotspots. Of particular importance is the consideration of the Structure-Function relationship as it pertains to the chemical modification. Variants which exist in the complimentary determining regions (CDR’s) may ablate recognition of the epitope creating the most significant functional liabilities therefore particular attention should be taken discerning the fidelity of these regions\textsuperscript{118-119}.

1.3.9 **Summary**

Altogether, a comprehensive analytical approach which utilizes techniques capable of discerning minor changes in the primary structure along with state of the art mass spectrometry techniques can provide an effective strategy in determining whether and unexpected change to
the primary structure has occurred. Enhanced understanding of the biology of the expression system can provide for better guided hypotheses to what may be the underlying reasons for the observed changes. The implementation of high resolution mass detectors, peptide mapping, spiking studies, sample enrichment and stable isotope labeling can provide conclusive support for the nature of an unknown variant. Lastly, communication of this information to the scientific community will build the overall knowledge within the field and facilitate the identification of these unwanted variants.

This dissertation presents the discovery of three unique chemical modifications which occurred in a recombinant monoclonal antibody. These variants are all reported for the first time with respect to recombinant protein drugs. In all three cases, the observed mass change was not a familiar one, at least in the literature compiled for recombinant protein drugs. In addition, the previously described analytical workflows were necessary to determine the nature and prevalence of each of the specific variants. This work serves to expand the knowledge base of protein modifications which can occur in protein drugs. In addition, it validates these analytical strategies as viable approaches for assigning identities to ambiguous molecular weight variances. Lastly, it underscores the utility of weak cation exchange chromatography. Although it is not a high resolution method, its ability to discern minor changes in the protein drug is quite valuable and an important first step in determining the true nature of changes to the primary structure.
1.4 References


Chapter 2  Arginine Modifications by Methylglyoxal: Discovery in a Recombinant Monoclonal Antibody and Contribution to Acidic Species

This chapter is based on a published paper with the same title

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2.1 Abstract:

Heterogeneity is common among protein therapeutics. For example, the so-called acidic species (charge variants) are typically observed when recombinant monoclonal antibodies (mAbs) are analyzed by weak-cation exchange chromatography (WCX). Several protein posttranslational modifications have been established as contributors, but still cannot completely account for all heterogeneity. As reported herein, an unexpected modification by methylglyoxal (MGO) was identified, for the first time, in a recombinant monoclonal antibody expressed in Chinese hamster ovary (CHO) cells. Modifications of arginine residues by methylglyoxal lead to two adducts (dihydroxyimidazolidine and hydroimidazolone) with increase of molecular weights of 72 and 54 Daltons, respectively. In addition, the modification by methylglyoxal causes the antibody to elute earlier in the weak cation exchange chromatogram. Consequently, the extent to which an antibody was modified at multiple sites corresponds to the degree of shift in elution time. Furthermore, cell culture parameters also affected the extent of modifications by methylglyoxal, a highly reactive metabolite that can be generated from glucose or lipids or other metabolic pathways. Our findings again highlight the impact that cell culture conditions can have on the product quality of recombinant protein pharmaceuticals.
2.2 Introduction

Recombinant biotherapeutics are associated with an inherently increased level of structural complexity as compared to traditional small molecule drugs. Various protein post-translational modifications (PTM’s) have been well documented as major contributors to heterogeneity in recombinant monoclonal antibodies. Some of these processes occur during fermentation, such as glycosylation and sialic acid incorporation, while others can occur through purification, storage and even sample preparation, such as oxidation and disulfide bond scrambling. Yet the known modifications still cannot explain all the variants.

A group of extensively studied charge variants include the so-called acidic species that are observed when recombinant monoclonal antibodies are analyzed by weak-cation exchange (WCX) chromatography, see Figure 1. One major contributing factor is the removal of the C-terminal lysine of the heavy chain by cell-produced carboxypeptidase, reducing the overall positive charge; these variants are commonly referred to as Lys-0, Lys-1 and Lys-2 species. C-Terminal amidation is another enzymatic process during fermentation. Spontaneous non-enzymatic transformations include the formation of pyroglutamate (Pyro-Glu) from an N-terminal glutamine (Gln) that removes the positive charge of the free N-terminus, and the deamidation of asparagine (Asn) to aspartic (Asp) or isoaspartic acid (isoAsp or isoD) that introduces negatively charged carboxylic acids. Other modifications without altering the formal charges can shift the retention time of an antibody on weak cation exchange chromatography, likely due to perturbation of local charge and conformation, such as incomplete glycosylation and the presence of free cysteinyl thiols instead of disulfide bond scrambling. It is worth noting that some modifications are imparted by metabolites, such as glycation by glucose, methionine oxidation by reactive oxygen species (ROS), cysteinylation by cysteine, and N-
homocysteinylataion by homocysteine thiolactone$^{26-27}$. Again, it is interesting to note that although many modifications have been reported; the observed heterogeneity of recombinant monoclonal antibodies on weak cation exchange chromatography still cannot be explained completely, suggesting more modifications are yet to be identified.

As report herein, we observed two well-defined acidic species under certain cell culture conditions. Detailed analyses have revealed that several arginine (Arg) residues were modified by methylglyoxal (MGO), further confirmed by comparing native antibody treated with authentic MGO. As illustrated in Scheme 1, the resulting dihydroxyimidazolidine and hydroimidazolone adducts increase molecular weights by 54 and 72 Daltons, respectively; these modifications cause the antibody to elute earlier in the weak cation exchange chromatogram. Consequently, the extent to which an antibody was modified at multiple sites corresponds to the degree of shift elution time. While protein modification by MGO is known in biology, our discovery is the first for a recombinant protein product. Furthermore, cell culture parameters also affect the extent of modifications by methylglyoxal, a highly reactive metabolite that can be generated from glucose, lipids or other metabolic pathways.
Scheme 2-1. Chemical modification of arginine by methylglyoxal (MGO). The guanidine of the arginine side chain reacts with the dicarbonyls to form a dihydroxyimidazolidine with a mass increase of 72 Da, and the subsequent loss of water leads to a hydroimidazolone with a mass increase of 54 Da.
2.3 Materials and Methods

2.3.1 Materials

The recombinant monoclonal antibody was produced by stably transfected Chinese hamster ovary (CHO) cells cultured in a bioreactor and purified at AbbVie Bioresearch Center (Worcester, MA). Dithiothreitol (DTT) was from Sigma (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were from J.T.Baker (Phillipsburg, NJ). Formic acid (FA) was from EMD (Gibbstown, NJ). Trypsin was from Worthington (Lakewood, NJ). Endoproteinase Lys-C was from Roche (Indianapolis, IN). Methylglyoxal was from MP Biomedicals (Solon, OH). Guanidine-HCl was from Thermo Scientific (Rockford, IL).

2.3.2 Weak cation exchange (WCX) chromatography

The antibody in low salt buffer was loaded onto a ProPac 4 x 250 mm WCX-10 column (Dionex, Sunnyvale, CA) at 94% mobile phase A (10 mM sodium phosphate, pH 7.5) and 6% mobile phase B (10 mM sodium phosphate and 500 mM sodium chloride, pH 5.5) at a flow-rate of 1 mL/min. The percentage of mobile phase B was increased from 6% to 16% over 20 min to elute the antibody monitored by UV absorbance at 280 nm. The column was then washed using 100% mobile phase B and then equilibrated using 6% mobile phase B for 9 minutes between injections. Fractionation was performed across the WCX-10 chromatogram. Distinct peaks were collected as individual fractions. The collected fractions were concentrated using Amicon Ultra-15 centrifugal filter with a molecular weight cut-off of 10 kDa (Millipore, Billerica, MA) and a table-top centrifuge operated at 4 °C.

2.3.3 LC-MS analysis of light and heavy chains of antibody
Light chain and heavy chain of the antibody from different fractions were analyzed using an HPLC (Agilent 1260, Santa Clara, CA) with a reversed phase column (Vydac, C4, 1 x 150 mm i.d., 5µ particle size) coupled to a Q-TOF mass spectrometer (Agilent, 6510). Antibody was reduced using DTT (10 mM final concentration). Ten microliters of each sample was loaded at 95% mobile phase A (0.08% formic acid and 0.02% TFA in Milli-Q water) and 5% mobile phase B (0.08% formic acid and 0.02% TFA in acetonitrile) and then eluted using a gradient from 5% mobile phase B to 35% mobile phase B in 20 min. The column was washed using 90% mobile phase B and equilibrated using 5% mobile phase B for 10 min. The flow rate was 50 µL/min and column oven temperature was 60 ºC. The mass spectrometer was operated in positive ion mode with a scan range from m/z 600 to 3200. Ion spray voltage was 4500 volts and the source temperature was 350 ºC.

2.3.4 Tryptic and Lys-C digestion

Protein factions from WCX were denatured using 6 M guanidine hydrochloride in 100 mM Tris, pH 8.0 and reduced using 10 mM DTT at 37 ºC for 30 min. Alkylation was performed using 25 mM iodoacetic acid at 37 ºC for 30 min. The samples were buffer exchanged to 10 mM Tris pH 8.0 using NAP-5 columns (GE Healthcare, Piscataway, NJ). The samples were digested either with trypsin or Lys-C at 1:20 (w:w, enzyme:antibody) and incubated at 37 ºC for 4 hours.

2.3.5 LC-MS analysis of peptides

A UPLC (Acquity, Waters, Milford, MA) equipped with a UPLC C18 reversed phase column (Waters, 1 x 150 mm i.d., 1.7µ particle size) and a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, West Palm Beach, FL) were used to analyze peptide
samples. Forty µL of each sample was loaded at 98% mobile phase A (0.08% formic acid and 0.02% TFA in Milli-Q water) and 2% mobile phase B (0.08% formic acid and 0.02% TFA in acetonitrile) and then eluted using a gradient from 2% mobile phase B to 35% mobile phase B in 80 min. The column was washed using 90% mobile phase B and equilibrated using 2% mobile phase B for 10 min. The flow rate was 50 µL/min and column oven temperature was 60 ºC. The mass spectrometer was operated in positive ion mode with a scan range from m/z 400 to 2000 with alternating collision induced dissociation (CID) and hard collision dissociation (HCD) of the three most intense parent masses. Ion spray voltage was set at 4500 volts and the source temperature was set at 350 ºC. The data was were analyzed by searching extracted mass traces of cleaved and subsequently miscleaved tryptic peptides containing peptides with mass increases of 54 and 72 Daltons. The data were also searched against the theoretical primary structure using the Sequest algorithm (Thermo Scientific, West Palm Beach, FL).

2.3.6 Methylglyoxal reaction with monoclonal antibody

Authentic methylglyoxal (MP Biomedical, Solon, OH) was incubated with the isoform of the recombinant monoclonal antibody without C-terminal Lys (denoted as Lys-0), collected from the WCX-10 chromatography. Lys-0 antibody at 1 mg/mL in 10 mM sodium phosphate at pH 7.0 was incubated with 2.8 mM methylglyoxal at 35 ºC for 0, 1, 2, 3, 4 and 5 hours. Lys-0 antibody incubated under the same conditions without methylglyoxal was used as a control. The samples were analyzed by WCX as described in the previous section. Peaks that appeared in the acidic region of the chromatogram in the sample treated with methylglyoxal for 2 hours were fractionated for further analysis as described for the samples from cell culture, and the data from the MGO spiking fractions and the cell culture fractions were compared.
2.3.7 Global Analysis of MGO Modification

Methylglyoxal can modify arginine, lysine and cysteine residues\textsuperscript{28-29}. To this point, pertinent structures for potential modifications were searched for both manually and with the Sequest Algorithm (Thermo Scientific, West Palm Beach, FL) to understand the degree to which the antibody has been modified. Both lysine and arginine must be considered to result in mis-cleavages when trypsin is used as the digest agent. In contrast, modified cysteine residues will lie within a tryptic peptide, but its reactivity is hampered by the fact that all cysteines in a recombinant monoclonal antibody should be involved in disulfide bonds. Structures considered in the analysis are adducts of arginine (dihydroxyimidazolidine, hydroimidazolone and argpyrimidine), lysine (N-epsilon (1-carboxyethyl) lysine\textsuperscript{28}, and cysteine (carboxyethyl cysteine)\textsuperscript{28-29}.

2.3.8 Quantification of Modified Peptides

Quantification of MGO modified peptides was performed on endoprotease Lys-C generated peptides. Comparison of Lys-C peptides eliminates MGO-induced mis-cleavages allowing for direct quantification between the matching modified and unmodified peptides. These data were generated using the parameters outlined in the LC/MS analysis of peptides section discussed previously. Extracted mass chromatograms corresponding to Lys-C peptides encompassing identified MGO sites from the tryptic mapping experiments were compared for the native peptide and +54 and +72 Dalton increases. The integrated areas of the confirmed extracted ion chromatogram (EIC) peaks of the modified and native Lys-C peptides were used to quantify the percent of methylglyoxal modification at each of the susceptible sites.
2.4 Results and Discussion

2.4.1 New acidic species induced by changes in cell culture

Under culture conditions M (modified), an increase in acidic species occurred during protein expression. In Figure 2-1, the weak cation exchange chromatogram shows two well-defined peaks with considerable shorter retention time than that of the main peak (Lys-0 isoform), which were absent under conditions N (normal). Although, it was determined later by a spiking study that after reduction, light chain with 2% modification of the 54 Da species can be detected and 5% modification can be unambiguously assigned, fractions were collected to enrich materials with such modifications. Each fraction was reduced to generate heavy chain and light chains, which were analyzed by LC/MS; the resulting mass spectra are shown in Figure 2-2. The molecular weight of 23408.24 Da for the major peak was in agreement with the theoretical molecular weight of 23408.13 Da for the light chain (LC) based on its amino acid sequence. In addition, two distinct peaks with molecular weights of 23462.44 Da and 23480.26 Da were also observed, corresponding to increases of approximately 54 Da and 72 Da over that for the native light chain. Moreover, a ladder of additional peaks with mass increase of 54 or 72 Da increments, albeit with lower intensity, was also observed, likely due to the same modifications occurring at multiple sites. A similar pattern of modifications was also observed for the heavy chain as shown in Figure 2-2. The major peak with a molecular weight of 50637.95 Da is in agreement with the theoretical molecular weight (50636.78 Da) of the heavy chain without the C-terminal Lys (Lys-0) and with a core-fucosylated biantenary complex oligosaccharide without the terminal galactose (G0F). Similar to the light chain, two additional peaks with molecular weights of 50692.06 Da and 50709.12 Da were also observed (Fraction 2), which are
approximately 54 Da and 72 Da greater than the theoretical molecular weight. Again, the additional ladder of peaks with lower intensity are likely due to the same modification occurring at more than one site.

It is worth noting that these mass changes are not among the reported protein modifications in recombinant monoclonal antibodies. Additionally, searching ABRF Delta Mass database (www.abrf.org/index.cfm/dm.home) of common protein modifications did not yield plausible explanation for the observed mass increases of 54 Da or 72 Da either. Furthermore, it was not initially clear whether these two modifications were related. To elucidate the chemical nature and site of modifications, peptide mapping was carried out first to identify modified peptides.
Figure 2-1. The top panel is a typical WCX chromatogram of the recombinant monoclonal antibody after protein A purification (top and bottom traces were from cell culture M (modified) and N (normal), respectively, at day 9). The peaks labeled as Lys-0, Lys-1 and Lys-2 are antibody isoforms without the C-terminal Lys, with one C-terminal Lys and with two C-terminal Lys on the heavy chains, respectively. Peaks at 2.2 and 2.8 min were observed in antibody expressed in cell culture M and are denoted by Fractions 1 and 2, respectively, which are the focus of this study. The bottom panel shows the time dependence of the formation of these two species.
Figure 2-2. Deconvoluted mass spectra of the light chain and heavy chains in fraction 1 (A and B) and fraction 2 (C and D). The theoretical molecular weight of the light chain is 23408.13 Da (observed 23408.24); 23462.44 and 23480.66 Da in Pane A represent increase of mass of 54 and 72 Da, respectively. The theoretical molecular weight of the heavy chain is 50636.78 Da (observed 50637.95); 50691.04 and 50710 Da represent increase of mass of 54 and 72 Da, respectively. In antibody treated by authentic MGO (2.7 mM, 5 h), a series of peaks with
incremental mass increase of 54 and/or 72 were observed (see E and F). These additional peaks are absent from the control (G and H).
2.4.2 Localization of the modification sites

Peptide mapping was performed on fractions 1 and 2 in the weak cation exchange chromatogram. Initially, all theoretical tryptic peptides with mass increase of either 54 Da or 72 Da were manually searched but none were found. We surmised that the modifications might perturb proteolysis; hence, the manual search was expanded to tryptic peptides with one single mis-cleavage at either lysine or arginine, and several such peptides were observed. Two representative MS/MS spectra corresponding to the same peptide with +54 Da and +72 Da mass increase are shown in Figure 2-3. The internal arginine residue should be cleaved by trypsin but was not, suggesting it was likely modified. MS/MS was performed using an alternating fragmentation activation of CID performed in the ion trap and HCD performed in the collision cell to produce complimentary fragmentation spectra to provide better coverage. The major fragment ions in the spectra are from the b-ion series. A mass increase of 54 Da associated with \(b_6\) and \(b_{15}\)-ions localized the modification to the first six amino acids that include the only arginine residue in this peptide. In addition to \(b\)-ions, several \(y\)-ions were also observed. Most important was that while the \(y_{12}\) ion showed the predicted mass of the unmodified peptide fragment, the \(y_{13}\) ion (refer to Figure 2-3, Pane A) displayed a mass increase of 54 Da thus confirming that the modification was on the arginine. Similarly, for light chain peptide T3 (a doubly charged precursor ion of m/z of 1090.5), a mass increase of 72 Da was associated with both \(b_8\) and \(b_{15}\) ions that localized the modification to the first eight amino acids. Again, the observation of \(y_{12}\) ion with the predicted mass and \(y_{13}\) ion with a mass increase of 72 Da confirmed that the modification was on the Arg residue (refer to Figure 2-3, Pane B).
Figure 2-3. Representative MS/MS mass spectra ([1081.5]^{2+} at 31 min and [1090.5]^{2+} at 32 min, respectively) of peptides containing Arg residues modified by MGO forming a hydroimidazolone in cell culture (A and C) or from incubation with authentic methylglyoxal forming a dihydroxyimidazolidine (B and D). Modifications that resulted in the molecular weight increases of both 54 Da and 72 Da were localized to Arg based on the MS/MS fragmentation pattern. R* and R** denote the modified arginine by mass increases of 54 Da and 72 Da, respectively.
2.4.3 Deduction of the Structure of the Modification

Chemically reactive, arginine can be modified by a host of biological molecules and chemical reagents, the most common being carbonyls (aldehydes and ketones). The carbonyl-guanidyl adducts are formed via an initial addition mechanism (the mass of the adduct equals the combined mass of the carbonyl and arginyl peptide) and possibly a subsequent condensation (elimination of a water molecule of the initial addition adduct; thus, a further mass decrease of 18 Da). Based on these mechanisms, the molecules that modified arginine in our antibody should have an intact molecular weight of either 54 or 72 (54 +18) Da (for the +54 Da adduct), 72 or 90 (72+18) Da (for the +72 Da adduct). Furthermore, since both adducts were induced by changes in cell culture conditions, we surmised that one or more metabolites were the culprits. The following carbonyl molecules match the mass values: for 54 Da, 2-propynal; for 72 Da, methylglyoxal, malonaldehyde, glycidaldehyde, butanal and butanones; and for 90 Da, glyceraldehyde and dihydroxyacetone (glycerone). Methylglyoxal was our top candidates for the following considerations: (1) being a dicarbonyl, it forms fairly stable adducts with arginine; (2) production of methylglyoxal is known to be dependent on cell culture conditions, reminiscing autoinducer-2 (AI-2, another dicarbonyl metabolite) and (3) the two adducts shown in Scheme 1 can account for both observed mass increase. A similar approach has been used to identify unknown protein crosslinking in an antibody.

2.4.4 Incubation with Authentic MGO to generate reference.

In order to confirm that the modification of the monoclonal antibody in cell culture was indeed by MGO, Lys-0 antibody shown in Figure 2-1 was isolated using weak cation exchange fractionation and was subsequently incubated with authentic MGO at 35 ºC; then, the reaction
products were analyzed by weak cation exchange chromatography. As shown in Figure 2-4, peaks corresponding to fractions 1 and 2 in the weak cation exchange chromatogram (labeled as Peaks A and B, respectively) increased with the prolonged incubation. In comparison, incubation of the Lys-0 fraction without MGO under the same conditions for the entire 5 hours did not result in any observable changes in peak profile (data not shown). Peaks A and B (Figure 2-4) were collected from the sample incubated for 2 hours using weak cation exchange chromatography and the samples were analyzed by LC-MS in the same manner as for the samples from cell culture. As shown in Figure 2-2 (panes E and F), peaks with molecular weights of 23462 Da and 23480 Da correspond to molecular weight increases of 54 Da and 72 Da, respectively, compared to theoretical molecular weight of 23408 Da for the light chain. Similarly, peaks with molecular weights of 50691 Da and 50709 Da with molecular weight increases of 54 Da and 72 Da, respectively, as compared to theoretical molecular weight of 50637 Da for the unmodified heavy chain were also observed. Additional ladders of peaks with increments of 54 and/or 72 Da were also observed, as for the samples from cell culture. Similar to other PTM’s, formation of the initial MGO-adducts may alter protein conformation and dynamics, thus altering the kinetics of modifications at other sites. As a result, the formation of different adducts are interdependent, as indicated by the adduct distribution.
**Figure 2-4.** WCX chromatogram of a purified Lys-0 antibody incubated with and without authentic MGO (2.8 mM at 35 °C) for various durations as labeled. The bottom trace shows the chromatogram of the antibody generated from cell culture. Acidic peaks derived from incubation with authentic MGO are labeled as Peak A and Peak B, respectively. The retention times of these peaks are in good agreement with Fraction 1 and Fraction 2 displayed in the bottom trace which are the subject of this study.
2.4.5 **Comparison of the in vitro references and the cell culture samples.**

The peptide maps of the fractionated acidic species from the MGO spike in phosphate buffer and the corresponding cell culture acidic fractions were compared by LC/MS/MS. Modified tryptic peptides from both samples exhibit comparable retention times within the normal range of variation from run to run, e.g., 31.5 minutes and 32.3 minutes for 1081.5 \([M+2H]^{2+}\) corresponding to the peptide with a mass increase of 54 Da and 1090.5 \([M+2H]^{2+}\) corresponding to the peptide with a mass increase of 72 Da, respectively. In addition, MS/MS analyses indicated that the modification for both samples can be localized to the same arginine residues in both the cell culture derived antibody and the MGO stressed 0-Lys samples.

The MS/MS fragmentation patterns of the spectra from the antibody spiked with MGO in aqueous buffer and antibody modified during cell culture are shown in Figure 2-3 and are nearly identical; e.g., both spectra exhibit many b ions as well as some key y ions that allow conclusive localization of the modification as MGO-modified arginine.

2.4.6 **Global Analysis of Modifications by MGO**

After MGO was confirmed to be responsible for the modifications at the site described above, the entire primary structure of the antibody was assessed for both the dihydroxyimidazolidine (+72 Da) and the hydroimidazolone (+54 Da) adducts. Several sites in both variable and conserved domains were identified. All modified peptides exhibited the modification at arginine residues and subsequently resulted in mis-cleavages. The distribution was quantitated based on endoprotease Lys-C digest analyzed by LC/MS/MS peptide mapping.
Lys-C was chosen for it only cleaves at lysine but not at arginine or MGO-modified arginines, so peptide counterparts with and without MGO-modifications can be directly compared. The distribution and peak intensity of the modified arginine-containing peptides (both the +54 and +72 Da adduct) are shown in Figure 2-5. The differences are evident and likely to affect the elution behavior seen in the WCX chromatography.
**Figure 2-5.** Peak intensity of all methylglyoxal-modified peptides generated by Lys-C digestion of fraction 1 (black) and fraction 2 (red), which are normalized to the peak intensity of the corresponding native (unmodified) peptides. The checkered bars represent the +54 Da adduct and the solid bars represent the +72 Da adduct.
MGO may also react with lysine resulting in $N^\epsilon$-(carboxyethyl)lysine with a mass increase of 72 Da$^{39}$, but the reaction is considerably slower and the product much less stable than with arginine. A thorough examination for MGO modifications on lysine residues did not result in any matches, indicating the MGO modification is selective toward arginine under our conditions.

The majority of the modifications occur at the CDR region, which is more flexible than the other parts of the antibody. Arg30 of the light chain is particularly solvent accessible. These factors may contribute to its propensity for modification. Certainly, specific interactions with other residues, local environment and other factors may be involved as well $^{40-41}$.

### 2.4.7 Effects of MGO Modification on Charge

When an ionizable amino acid is chemically modified, its pKa value may be significantly perturbed. Using the Advanced Chemistry Development pKa prediction program accessed through SciFinder, the following values were obtained: guanidinium (12.55, experimental value), dihydroxyimidazolidine (7.13 +/- 0.6, calculated) and hydroimidazolone (6.93 +/- 0.4, calculated)$^{42}$ as shown in Figure 2-6. The lower pKa values imparted by MGO modifications$^{43}$ translate to more acidic species, consistent with an earlier elution by weak cation exchange chromatography. Additionally, the steric bulkiness conferred by MGO modifications may also interfere with the stationary phase interaction and reduce the binding strength. Conformational changes are another possible contributor to changes in chromatographic behavior. To this point, we compared the charge envelopes of both modified and unmodified heavy and light chains
obtained by mass spectrometry with respect to charge distribution$^{44-45}$. No apparent differences were seen (data not shown), suggesting no major alteration in high-order structures.
Figure 2-6: Calculated pKa of the core group of arginine and the two products of arginine modification by methylglyoxal. The pKa of the guanidinium core group is significantly depressed to 7.13 and 6.93 for the dihydroxyimidazolidine and hydroimidazolone core groups, respectively.

1. Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2013 ACD/Labs)
2. Gauthier et al., Biomacromolecules, 12:482-493, 2011
2.4.8 Relevance to other Contributing Factors to Acidic Species

The observation of acidic species formation over time and at elevated pH and temperature is fairly well understood. Several studies have shown that asparagine deamidation to aspartate and isoaspartate introduces an additional negative charge on proteins. The observation of an increase in acidic species occurring as the cell culture progresses appears to be due to something other than an increase in deamidation as all the samples underwent equivalent storage and the altered chromatograms differ from those generated in forced deamidation. Previously, some studies have shown that the introduction of a glycated lysine residue in a protein can result in an acidic shift seen in weak cation exchange chromatography by perturbing the charge on the protonated primary amine. In many cases, however, these known modifications cannot fully explain all the acidic species. Our findings suggest that arginine modification by MGO should be considered.

2.4.9 Formation of MGO as a New Critical Attribute for Cell Culture

Complicated metabolic pathways for methylglyoxal exist in CHO cells. MGO can be formed enzymatically or non-enzymatically from glucose, e.g., the ene-diol intermediate of dihydroxyacetone phosphate (DHAP). Not surprisingly, many reports of protein modifications by methylglyoxal come from research related to diabetes in which blood glucose levels are elevated; and MGO may be involved in the formation of advanced glycation end products (AGEs). In addition, because of the high reactivity and toxicity of MGO, dedicated catabolic mechanisms also exist. Previous studies highlighted that cells have biochemical pathways to effectively remove methylglyoxal. The glyoxalase pathway is a glutathione-mediated process which converts methylglyoxal to D-lactate by the glyoxylase I and glyoxylase II enzymes. In addition, MGO can be reduced to propanediol through the NADPH-dependant
aldose reductase pathway. Indeed, Chaplen and coworkers have observed that methylglyoxal can accumulate in CHO cell cultures due to perturbed regulation in MGO metabolism\textsuperscript{30-34, 40}.

Under our N (normal) and M (modified) conditions, no detectable differences in glucose levels were observed; but presumably, other difference did result in the accumulation of methylglyoxal. Changes in the flux of the glyoxalase pathway may be the cause of this unexpected appearance of methylglyoxal in the modified conditions. To the best of our knowledge, this is the first report of such a modification taking place in a CHO cell expression of a recombinant monoclonal antibody, affecting the quality of the protein products.

It is also important to consider that MGO not only affects the protein product being expressed, but also broadly affects many proteins in the host cells and possibly their biological functions. At the cellular level, vigor, viability, cell density and protein production yield may also be negatively affected. Conversely, elevated MGO formation also reflects a change in metabolic flex of competing pathways\textsuperscript{30, 32-33, 48-49, 51-53}. In summary, MGO can be considered a biomarker of a less than optimal cell culture.
2.5 Conclusions

Triggered by changes in cell culture media, a new modification of a recombinant monoclonal antibody by methylglyoxal (MGO) was identified by a combination of weak cation exchange chromatography, peptide mapping and mass spectrometry. Modification by MGO, e.g., the corresponding mass increase of 54 Da and 72 Da for the arginine derivatives, should be considered when assessing heterogeneity in recombinant proteins. In addition, our finding serves as a reminder that other unreported modifications are likely to exist in protein pharmaceuticals. Furthermore, the results demonstrate the critical role of cell culture conditions and downstream processes in controlling product quality.
2.6 References


SUPPORTING INFORMATION

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Figure S-1.: Comparison of peptide MS data between acidic peaks from cell culture and acidic fractions from methylglyoxal incubation. The data shows the corresponding mass spectra of a 3+ ion representing a mis-cleaved peptide with a mass increase of 54 daltons.
Figure S-2: The figure shows the total ion current (top), the native peptide (second pane), the peptide modified by one MGO (+54 da) and lastly, a trace showing no evidence of the second arginine within this peptide being modified. The site was identified by previous tryptic digest and the degree of modification was determined by the percent of the respective peaks in the XIC’s of the native and MGO modified peptides.
Figure S-3.: The figure displays a mass spectrum of a heavy chain Lys-C peptide showing the isotopic distributions of the +5 charge state (Arg93 internalized). In this manner, the native and the two MGO products of a single modified arginine can be seen within the same spectrum.
Figure S-4.: Mass spectra of the light chain for a pure 0 Lys are shown over the five hour period. The additional peaks of +54da and +72da have formed and have greatly increased the observed mass heterogeneity. The formation of the additional peaks is in agreement with the observed peaks from the acidic fractions isolated from cell culture. The control was incubated for the entire five hour period but without the addition of methylglyoxal and subsequently showed no added complexity or formation of additional peaks.
Figure S-5.: Mass spectra of the heavy chain for a pure 0 Lys are shown over the five hour period. The additional peaks of +54da and +72da have formed and have greatly increased the observed mass heterogeneity.
Table S-1.: Results of manual search of methylglyoxal modified peptides found in the recombinant antibody

**MGO Modified Tryptic Peptides (mis-cleavages)**

ASQGIR*NYLAWYQQKPGK
YNR*APYTFGQGTK
R*TVAAPSVMFPSDEQLK
EVQLVESGGGLVQPSR*SLR
DTLMISR*TPEVTCVVDVSHEDPEVK
EPQVYTLPSR*DELTK
SR*WQQGNVFSCVMHEALHNHYTQK
Table S-2: Raw data values of Lys-C peptide peak intensity from Cell Culture WCX fractions 1 and 2

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Table S-3: Relative Intensity of Lys-C peptides from Cell Culture WCX fractions 1 and 2

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A. Reduced Light Chain

Figure S-6: Detection limit of reduced LC/MS to detect MGO (+54 Da). The deconvoluted mass spectrum of reduced light chain spiked with MGO modified light chain (top pane is full view and bottom pane focuses on the +54 Da species). Modified light chain was spiked into native light chain to deduce the level at which the +54 Da peak could be detected without an enrichment step. The data shows overlaid traces at 10%, 7.5%, 5%, 2.5%, 2%, 1%, 0.5%, 0.1% and 0% modified antibody spikes. The +54 Da peak was detected at 2% modification and was unambiguously identified at 5% modification on our instrument.
Chapter 3  Discovery of a Chemical Modification by Citric Acid in a Recombinant Monoclonal Antibody

This chapter is based on a published paper with the same title

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3.1  Abstract

Recombinant therapeutic monoclonal antibodies exhibit a high degree of heterogeneity that can arise from various post-translational modifications. The formulation for a protein product is to maintain a specific pH and to minimize further modifications. Generally Recognized as Safe (GRAS), citric acid is commonly used for formulation to maintain a pH at a range between 3 and 6, and is generally considered chemically inert. However, as we reported herein, citric acid covalently modified a recombinant monoclonal antibody (IgG1) in a phosphate/citrate-buffered formulation at pH 5.2, and led to the formation of so-called “acidic species” that showed mass increases of 174 and 156 Da, respectively. Peptide mapping revealed that the modification occurred at the N-terminus of the light chain. Three additional antibodies also showed the same modification but displayed different susceptibilities of the N-termini of the light chain, heavy chain or both. Thus, ostensibly unreactive excipients under certain conditions may increase heterogeneity and acidic species in formulated recombinant monoclonal antibodies. By analogy, other molecules (e.g., succinic acid) with two or more carboxylic acid groups and capable of forming an anhydride may exhibit similar reactivities. Altogether, our findings again remind us that it is prudent to consider formulations as a potential source for chemical modifications and product heterogeneity.
3.2 Introduction

As most protein pharmaceuticals, recombinant monoclonal antibodies have a higher degree of inherent complexity as compared to traditional small molecule drugs. Various protein post-translational modifications (PTM’s) have been well documented as major contributors to heterogeneity observed in recombinant monoclonal antibodies1-6. Some of these processes occur during cell culture, such as modifications by reactive metabolites (e.g. methylglyoxal and homocysteine thiolactone)7-8, glycosylation and sialic acid incorporation9-17; while others can occur through production, purification and storage, such as oxidation18-21, deamidation22-27, crosslinking28-29, protein-protein interactions30 and fragmentation31-34.

An important part of drug development is to optimize formulation for a given biotherapeutic35-36. The formulation should minimize unwanted modifications or degradation during storage3,37. For example, polysorbate80 may be added to mitigate aggregation38-41. Free methionine may reduce the formation of methionine sulfoxide in proteins42-45. A critical aspect of formulation is the control of pH. One major reason is to minimize the deamidation of asparagine, a spontaneous non-enzymatic process that occurs in all monoclonal antibodies and the vast majority of protein pharmaceuticals16,22,24-26,46-47. Specifically, mildly acidic pH has been shown to reduce deamidation of asparagine22-27.

While almost all excipients added to the biotherapeutic formulation are Generally Recognized as Safe (GRAS) and considered chemically inert (i.e., free from reactions with the protein products), they may nonetheless display unexpected reactivities. For example, autoxidation of polysorbate 80 generated radicals that in turn increased the oxidative liabilities of the formulation, e.g., increases in methionine sulfoxide48. Photo-oxidation also induces cleavage, crosslinking and aggregation13,29,34,49-50. Glycation has been reported when glucose (a
reducing sugar with a hemiacetal or aldehyde group) was added to a lyophilized protein drug\textsuperscript{51}. As a result of this finding, sucrose (devoid of hemiacetal or aldehyde group) was used instead to reduce aggregation\textsuperscript{52}. Yet, in other studies, the glycosidic bond of non-reducing sucrose was shown to hydrolyze into glucose and fructose, resulting in glycation during storage\textsuperscript{53-54}. Pertinent to this work, photochemical degradation of citric acid led to acetonation of therapeutic proteins\textsuperscript{55}. Therefore, it is important to thoroughly evaluate the protein drug integrity following storage in the defined formulation and to screen for unexpected reactivity and modifications.

As reported herein, we observed an early eluting peak (i.e., acidic species) in the weak cation exchange (WCX) chromatogram for an antibody in citric acid formulation. Peptide mapping and mass spectrometric analysis revealed that covalent modifications by citric acid led to the formation of amides (mass increase of 174 daltons) and/or imides (mass increase of 156 daltons) at the N-terminus of the light chain\textsuperscript{56}. Furthermore, three additional recombinant monoclonal antibodies displayed a similar susceptibility of the N-termini of both the light and heavy chains. To the best of our knowledge, this is the first report of a citric acid modification of recombinant monoclonal antibodies. By analogy, other molecules (e.g., succinic acid) with two or more carboxylic acid groups and capable of forming an anhydride may exhibit similar reactivities\textsuperscript{57-58}. Altogether, our findings again remind us that it is prudent to carefully consider formulation excipients as a potential source for chemical modifications.
3.3 Materials and Methods:

3.3.1 Materials

The recombinant monoclonal IgG1 antibody was produced by stably transfected Chinese hamster ovary (CHO) cells cultured in a bioreactor and purified at AbbVie Bioresearch Center (Worcester, MA). Dithiothreitol (DTT) was from Sigma (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were from J.T. Baker (Phillipsburg, NJ). Formic acid (FA) was from EMD (Gibbstown, NJ). Trypsin was from Worthington (Lakewood, NJ). Endoproteinase Lys-C was from Roche (Indianapolis, IN). Guanidine-HCl was from Thermo Scientific (Rockford, IL). Citric acid (monohydrate, cat.# 0110-01) and sodium citrate (dehydrate, cat. # 3647-05) were from JT Baker (Phillipsburg, NJ). The citrate formulation consisted of a 1 mM sodium citrate, 6.5 mM citric acid combined with Na₂HPO₄, polysorbate 80 and mannitol with the pH adjusted to 5.2 with sodium hydroxide. The 20X citrate buffer consisted of 20 mM sodium citrate, 130 mM citric acid combined with Na₂HPO₄ with the pH adjusted to 5.2 with sodium hydroxide. Antibody A, Antibody A-S, Antibody B and Antibody C were all recombinant monoclonal antibodies (IgG1’s) produced at AbbVie Bioresearch Center, Worcester, MA.

3.3.2 Weak cation exchange (WCX) chromatography

The antibody in low salt buffer was loaded onto a ProPac 4 x 250 mm WCX-10 column (ThermoFisher, Sunnyvale, CA) at 94% mobile phase A (10 mM sodium phosphate, pH 7.5) and 6% mobile phase B (10 mM sodium phosphate and 500 mM sodium chloride, pH 5.5) at a flow-rate of 1 mL/min. The percentage of mobile phase B was increased from 6% to 16% over 20 min to elute the antibody monitored by UV absorbance at 280 nm. The column was then washed.
using 100% mobile phase B and then equilibrated using 6% mobile phase B for 9 minutes between injections.

3.3.3 LC-MS analysis of reduced antibody

Light chain and heavy chain of the antibody from different fractions were analyzed using an HPLC (Agilent 1260, Santa Clara, CA) with a reversed phase column (Vydac, C4, 1 x 150 mm, 5µ particle size) coupled to a Q-TOF mass spectrometer (Agilent, 6510). Antibody was reduced using DTT (10 mM final concentration) at 37°C for 30 minutes. Ten microliters of each sample was loaded at 95% mobile phase A (0.08% FA and 0.02% TFA in Milli-Q water) and 5% mobile phase B (0.08% FA and 0.02% TFA in acetonitrile) and then eluted using a gradient from 5% mobile phase B to 35% mobile phase B in 20 min. The column was washed using 90% mobile phase B and equilibrated using 5% mobile phase B for 10 min. The flow rate was 50 µL/min and column oven temperature was set at 60 °C. The mass spectrometer was operated in positive ion mode with a scan range from m/z 600 to 3200. Ion spray voltage was 4500 volts and the source temperature was 350 °C.

3.3.4 Tryptic digestion

Proteins were denatured using 6 M guanidine hydrochloride in 100 mM Tris, pH 8.0 and reduced using 10 mM DTT at 37 °C for 30 min. Alkylation was performed using 25 mM iodoacetic acid in 1M Tris pH 8.0 at 37 °C for 30 min. The samples were buffer exchanged to 10 mM Tris pH 8.0 using NAP-5 columns (GE Healthcare, Piscataway, NJ). The samples were digested with trypsin at 1:20 (w:w, enzyme:antibody) at 37 °C for 4 hours.
3.3.5 LC-MS analysis of peptides

An UPLC (Acquity, Waters, Milford, MA) equipped with a UPLC C18 column (Waters, 1 x 150 mm i.d., 1.7µ particle size) and a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, West Palm Beach, FL) were used to analyze peptide samples. Forty µL of each sample was loaded at 98% mobile phase A (0.08% formic acid and 0.02% TFA in Milli-Q water) and 2% mobile phase B (0.08% formic acid and 0.02% TFA in acetonitrile) and then eluted using a gradient from 2% mobile phase B to 35% mobile phase B in 80 min. The column was washed using 90% mobile phase B and equilibrated using 2% mobile phase B for 10 min. The flow rate was 50µL/min and column oven temperature was set at 60 ºC. The mass spectrometer was operated in positive ion mode with a scan range from m/z 400 to 2000 with alternating low energy collision induced dissociation (CID) and high energy collision induced dissociation (HCD) of the three most abundant parent masses. Ion spray voltage was set at 4500 volts and the source temperature was set at 350 ºC. The data were analyzed by searching extracted mass chromatograms (XIC) of tryptic peptides with mass increases of 156 and 174 daltons. The data were also searched against the theoretical primary structure using the Sequest algorithm and selecting the observed variant increases (Thermo Scientific, West Palm Beach, FL).

3.3.6 Reaction of citric acid buffer with the monoclonal antibodies

A 20X citric acid buffer (10 mM Na₂HPO₄, 20 mM Na citrate/130 mM citric Acid pH 5.2), in which the citric acid concentration was 20-fold of that of the formulation solution, was prepared. Antibody A was diluted to 1 mg/mL in this 20X buffer and stored at 40 ºC for up to 30 days. Antibody A incubated under the same conditions in actual 1X formulation buffer was used
as a control. In addition, Antibody A was stored in the 20X citrate buffer with the pH adjusted up to 7.0. Sample aliquots were removed at days 5, 10, 15, 20 and 30 and stored at -80 °C until analysis. The samples were analyzed by WCX-10 as described in the previous section.

### 3.3.7 N-Terminal Variants and Additional IgG’s

An analog of Antibody A was produced in which the N-terminal residues of the light chain and heavy chain were swapped (see Table 1). The resulting antibody (Antibody A-S) had a glutamate at the N-terminus of the light chain and an aspartate at the N-terminus of the heavy chain. Two other recombinant IgG1’s, Antibody B and Antibody C were also used for the study. The N-terminal sequences (first ten residues) of all the antibodies are shown in Table 1. Antibody A-S and the recombinant IgG1’s were formulated into the 20X citrate buffer at pH 5.2 as described in the previous section. The samples were incubated at 40 °C for 30 days. Following the incubation, the samples (including Antibody A in 20X citrate) were analyzed by reduced LC/MS and tryptic mapping with MS/MS detection for the presence and abundance of the citric acid modifications.
3.4 Results and Discussion

As detailed below, we found that citric acid covalently modified the N-termini of either or both the light and heavy chains in four different antibodies. Our analysis and results are consistent with the mechanism depicted in Scheme 1 with the anhydrides of citric acid as key intermediates.
Scheme 3-1: (I) Formation of a citric acid anhydride intermediate from citric acid and the subsequent reaction of the N-terminal amine with the anhydride; (II) The four possible products of the reaction; +174A and +174B represent adducts formed between the N-terminal amine and the citric acid anhydride. The +156A and +156B represent the subsequent imide products (5 and 6-membered rings, respectively) resulting from the cyclization of the newly formed amide and another carboxylic acid in citric acid. There are three carboxylic acids in citric acid: two are equivalent as denoted by the red dots and the other by the blue dot.
3.4.1 Unexpected covalent modifications by citric acid

A recombinant monoclonal antibody (Antibody A) was stored at 40 °C for 6 months in two different formulations to determine if there were any major differences in the protein stability. One formulation had 1 mM sodium citrate, 6.5 mM citric acid and the other formulation was without sodium citrate/citric acid; both had mannitol and polysorbate 80 and were at pH 5.2. Analysis of the samples by weak cation exchange (WCX) chromatography revealed that significant degradation and accumulation of multiple acidic species in both samples (see Figure 3-1). Most noticeably, the citrate formulation induced a very early eluting and well-defined peak (Peak A in Figure 3-1) that was absent in the other sample. This finding prompted us to perform subsequent analysis in order to determine the nature of these species.
**Figure 3-1:** The weak cation exchange chromatogram of the recombinant monoclonal antibody formulated with and without citrate. The top trace shows an early eluting acidic peak (Peak A) which is significantly smaller in the formulation without citrate. The control represents Antibody A in the citrate formulation stored at 4 °C.
3.4.2 Reduced LC/MS Analysis

Peak A fractions were examined by reduced LC/MS (see Figure 3-2). The major peak (observed mass 23408 Da) corresponded to the native light chain (theoretical mass 23408 Da). Two other masses of 23564 and 23582 were observed: increases of 156 and 174 Da, respectively. Pertinent to the mechanism of formation discussed later, these two masses differ by 18 Da and are likely due to the loss of a water molecule. In addition, a mass of 23570 Da was determined to be a glycation product (+162 Da).
Figure 3-2: Mass spectra of the light chain from reduced LC/MS analysis. Full scale spectrum is shown at the left and expanded view is shown at the right. All samples were stored in citric acid buffer at 40 °C except the control which was stored at 4 °C. (A) 1 mM sodium citrate, 6.5 mM citric acid at pH 5.2 for 6 months; (B) 1 mM sodium citrate, 6.5 mM citric acid at pH 5.2 for 1 months; (C) 20 mM sodium citrate, 130 mM citric acid at pH 5.2 for 1 month (D) 20 mM sodium
citrate, 130 mM citric acid at pH 7.0 for 1 month. (E) Light chain control (same as A except stored at 4 °C for 1 month).
3.4.3 Peptide Mapping and Determination of Sites of Modifications in antibody A

Peptide mapping with mass spectrometric detection revealed three tryptic peptides present in the formulation with citrate but were absent in the formulation without citrate. These peaks corresponded to doubly charged ions of peptides with masses of 2051.90 Da (Peptide B) and 2033.88 Da (Peptide C and D), respectively (Figure 3-3). Peptides C and D were isobaric yet chromatographically resolved on the C18 RP-HPLC column and both exhibited greater retention and thus greater hydrophobicity than Peptide B. The analysis of the MS/MS spectra of Peptides B, C and D were in good agreement to each other with the exception of the 18 Da mass shift between some of the b ions but clearly all three spectra were from the same fragmentation series. Manual de novo sequencing (Figure 3-4) performed on these peptides revealed high homology to the predicted y ion series of the N-terminal peptide of the light chain (Peak A). A comparison of these MS/MS spectra against the experimental MS/MS spectrum of the N-terminus of the light chain peptide showed high similarity between the fragmentation patterns as shown in Figure 3-5. The y ion series between Peak A (Native), Peak B (+174 Da), Peak C and Peak D (+156 Da) covered all residues in the peptide with the exception of the N-terminal aspartate. The b ion series, although limited, showed strong signal with coverage amongst the first three residues. Consequently, we were able to assign the observed mass increases to the N-terminus of the light chain. Thus, the data confirmed that the mass increases of 156 or 174 Da were from modifications on the light chain N-terminal amine (i.e., Asp1). Subsequent analysis of the heavy chain N-terminal peptide of Antibody A did not show any modification.
Figure 3-3: HPLC-MS analysis of a tryptic digest of the recombinant monoclonal antibody A following storage at 40 °C for 6 months in citrate buffer pH 5.2. The extracted ion chromatograms corresponding the +2 charge ions for the native light chain peptide (A, m/z = 1877.89), the same peptide with +174A and +174B (B) and mass increases of +156A and +156B (C), respectively.
Figure 3-4. De novo sequencing of doubly charged peptides corresponding to unique M/z’s of 2052.91 (Middle pane) and 2034.90 (Bottom pane). The data are compared to the light chain N-terminal peptide of M/z 1878.89 (Top pane). Major fragmentation neutral losses corresponding to a y ion series were in agreement and the deduced sequence is shown at the top of the figure.
Figure 3-5: MS/MS spectra of Antibody A light chain N-terminal peptide for the native, +174 Da, +156A Da, and +156B Da citrate modifications, respectively. Both y- and b-ion series support the modification was at the N-termini. The top spectrum corresponds to the native peptide while the second spectrum corresponds to the same peptide with the +174 Da modification on the light chain N-terminus. The bottom two spectra correspond to the two products for the +156 Da modification on the light chain N-terminus.
3.4.4 Elucidation of the chemical nature of the modifications

No protein modifications listed in either the ABRF Delta Mass database (www.abrf.org/index.cfm/dm.home) or the Unimod database (www.unimod.org) could give rise to the three observed species. As previously stated, citric acid was only present in the formulation of the sample where these modifications were found. The molecular weight of citric acid is 192 Da therefore the difference between the observed variants of +174 Da and +156 Da suggests two successive losses of water from citric acid. As illustrated in Scheme 3-1, we propose a mechanism that involves the initial formation of citric anhydride, the subsequent formation of an amide with an amino group in the protein (e.g., the N-terminus) that results in a molecular weight increase of 174 Da. Further condensation of the resulting amide and another carboxylic group in covalently attached citric acid leads to the formation of imides (either five- or six-membered), which confers a molecular weight increase of 156 Da. In addition, it is reasonable to expect that these two products would form at different rates favoring the 5-membered product and further supported by the two +156 isobaric peptides we observed (156A and 156B shown in Figure 3-3). This mechanism is consistent with the results reported on citrate modification of peptides and the propensity of citric acid to form an anhydride under acidic conditions.66-68, 60-61.

3.4.5 Reactions in citrate buffers (as compared to formulation)

To isolate and narrow down the factors involved in the modification, antibody A was incubated in citric acid buffer at the same pH (5.2) but without other formulation excipients (e.g.,
without mannitol and polysorbate 80) at 40 ºC for 1 month. Similar to the sample from citrate formulation, the weak cation exchange chromatogram (Figure 3-6) shows a clear time-dependent increase in the amount of acidic species. In addition, the formation of the distinct early eluting peak has a comparable retention time to peak A from the sample formulated in citrate. Similarly, the reduced LC/MS analysis of the light chain showed a major peak in good agreement with the theoretical mass and also showed two higher molecular weight masses with increases of +156 Da and +174 Da but at a higher abundance than the citrate formulation (Figure 3-2). And again, tryptic mapping confirmed on the same adducts localized to the N-terminus of the light chain (data not shown). Thus, these experiments supported our hypotheses that the citric acid was indeed the modifying agent causing the heterogeneity on the N-terminus of the light chain. In addition, we searched for the same modifications on the heavy chain N-terminal peptide and found trace levels of the +174 Da adduct and no detection of the +156 Da adduct thus we saw similar susceptibility as our stability sample (see Table 3-1).
Figure 3-6: The weak cation exchange chromatogram of Antibody A incubated in with citric acid (formulation and buffer alone). A: Analysis of Antibody A stored in 20X citrate buffer for 0, 5, 10, 15, 20 and 30 days and Antibody A in the citrate formulation for 6 months, all at 40 ºC. The data show the formation of a prominent early eluting acidic peak. In addition, the peak
aligns well with the Peak A from the sample stored in the citrate formulation for 6M/40C. **B:**

Time-dependent accumulation of peak A.
3.4.6 Prevalence of the citrate modification

To better understand the scope of this modification, several additional antibodies were examined (see Table 3-1). One was a variant of Antibody A (Antibody A-S) in which the N-terminus of the light chain had an aspartate substituted with a glutamate and the N-terminus of the heavy chain had a glutamate substituted with an aspartate; in essence, the two termini were swapped. LC/MS analysis of the light and heavy chains of Antibody A-S showed the same site of modification and similar susceptibility as Antibody A (see Table 3-1), suggesting protein structures (such as solvent accessibility) perhaps play a more dominant role than specific amino acid residues. Additionally, as shown in Table 3-1, Antibody B and Antibody C were also modified by citric acid at the N-termini of both the light chain and heavy chain. The modification was also observed on a heavy chain N-terminal alanine residue (data not shown), suggesting that this modification may occur on other residue at the N-terminus and the N-terminal acidic side chains (Asp or Glu) are not obligatory. Thus, the modification of the N-terminal primary amine by citrate appears to be common amongst recombinant IgG1 monoclonal antibodies but may be influenced by other factors such as the antibody structure and microenvironment. Furthermore, in all cases, the +174 Da species were more prominent than the +156 Da species, indicating the former are likely the initial products as proposed in Scheme 3-1.
Table 3-1: The percentage of citric acid modification found in the N-terminus of each chain in different antibodies: Antibody A in citrate formulation for 6 months at 40 °C; and Antibodies A, A-S, B and C in 20X citrate buffer for 30 days at 40 °C. The $+156 \text{ Da}^a$ and $+156 \text{ Da}^b$ refer to the two products formed after the second anhydride formation. The first ten residues of the N-terminal framework of the heavy chains and light chains of antibodies are also listed (n.d. denotes not detected).

<table>
<thead>
<tr>
<th>Recombinant IgG1</th>
<th>LC N-terminus</th>
<th>+174 Da</th>
<th>+156 Da$^a$</th>
<th>+156 Da$^b$</th>
<th>HC N-terminus</th>
<th>+174 Da</th>
<th>+156 Da$^a$</th>
<th>+156 Da$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody A (1X, 6M)</td>
<td>DIQMTQSPSS</td>
<td>1.3</td>
<td>1.2</td>
<td>0.4</td>
<td>EVQLVESGGG</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Antibody A (20X, 1M)</td>
<td>DIQMTQSPSS</td>
<td>7.0</td>
<td>2.0</td>
<td>1.4</td>
<td>EVQLVESGGG</td>
<td>0.02</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Antibody A-S (20X, 1M)</td>
<td>DIQMTQSPSS</td>
<td>8.7</td>
<td>1.3</td>
<td>0.6</td>
<td>DVQLVESGGG</td>
<td>0.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Antibody B (20X, 1M)</td>
<td>EVLVTQSPDF</td>
<td>4.8</td>
<td>0.1</td>
<td>0.1</td>
<td>EVQLVESGGAE</td>
<td>6.4</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Antibody C (20X, 1M)</td>
<td>DVLVTQSPLES</td>
<td>1.8</td>
<td>0.2</td>
<td>0.2</td>
<td>EVKLVESGGG</td>
<td>3.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
3.4.7 Influence of pH

As shown in Scheme 1, a key intermediate for the modification is citric acid anhydride\textsuperscript{57-58}. Citric acid has three pKa’s of 3.14, 4.75 and 6.39, so at pH 5.2, one of the carboxylic acids will be predominantly protonated, a first step for anhydride formation. As reported, formation of citric acid anhydride occurs between pH 3.0 to 6.0, with the maximum at pH 4.0 to 4.5\textsuperscript{56}. At pH 5.2 for our formulation, citric acid anhydride can still accumulate to a significant degree and modify the antibodies. Increasing the pH to neutral conditions, however, would markedly diminish the formation of the anhydride, thus little modification of the antibodies (see Figure 3-2, pH 7 data).

3.4.8 Selectivity of amines

We investigated whether there were any citrate modifications to the primary amines of lysine residues following the accelerated storage conditions. We searched the peptide mapping data using the Sequest algorithm (ThermoFisher Scientific) and did not find any modification to lysine residues. In general, N-terminal amines have a lower pKa (around 8) than those on the side chains of lysine (around 10). Under mildly acidic conditions (e.g., pH 5.2), though the vast majority of the N-terminal and lysyl amines are protonated, significant higher percentage of the N-terminal amines are deprotonated, thereby nucleophilic and can react with anhydride. Therefore, the observed selectivity of amines are consistent with the generally observed reactivities of N-terminal amines\textsuperscript{65-66}. 
3.5 Conclusions

Our results suggest the general susceptibility of the N-terminal amines to modifications by citric acid. The reactivity is likely influenced by multiple factors, including pH, pKa at the N-terminal amines and structural features, therefore the sites and extent of modification cannot be precisely predicted and thus should be investigated experimentally. In addition, formulations with elevated concentrations of citric acid would likely cause a greater extent of the modification therefore it would be prudent to consider other excipients which may be better suited for the desired pH range. In particular, other molecules containing two or more juxtaposed carboxylic acid groups may exhibit analogous reactivities (via the formation of anhydrides). Examples from the Generally Recognized as Safe (GRAS) list include adipic acid, malic acid, succinic acid and tartaric acid. Altogether, our findings are yet a reminder that the unexpected reactivity of excipients and formulation, though generally considered chemically inert and safe, should be carefully scrutinized.
3-6 References


Chapter 4  When Good Intentions Go Awry: Modification of a Recombinant Monoclonal Antibody in Chemically Defined Cell Culture by Xylosone, an Oxidative Product of Ascorbate

This chapter is based on a submitted paper with the same title

4.1 Abstract

With the advent of new initiatives to develop chemically defined media, cell culture scientists screen many additives to improve cell growth and productivity. However, the introduction or increase of supplements—typically considered beneficial or protective on their own—to the basal media or feed stream may cause unexpected detrimental consequences to product quality. For instance, because cultured cells are constantly under oxidative stress, ascorbic acid (vitamin C, a potent natural reducing agent) is a common additive to cell culture media. However, as reported herein, a recombinant monoclonal antibody (adalimumab) in cell culture was covalently modified by xylosone (molecular weight 148), an oxidative product of ascorbate. Containing reactive carbonyl groups, xylosone modifies various amines (e.g., the N-termini of the heavy and light chains and susceptible lysines), forming either hemiaminal (+148 Da) or Schiff base (imine, +130 Da) products. Our findings show, for the first time, that ascorbate-derived xylosone can contribute to an increase in molecular heterogeneity, such as acidic species. Our work serves as a reminder that additives to cell culture and their metabolites may become reactive and negatively impact the overall product quality, and should be carefully monitored with any changes in cell culture conditions.
4.2 Introduction

Until recently, cell culture scientists mostly focused on cell growth and protein expression level; but as demonstrated herein, the quality of the final products has recently emerged as another critical attribute to be considered. To this point, variations in product quality, or microheterogeneity, are mostly attributed to a myriad of post-translational modifications (PTMs)\textsuperscript{1-6}. In order to reduce variability in product quality, cell growth and expression levels, the current trend in recombinant monoclonal antibody production has been to move from complex undefined hydrolysate media to the utilization of chemically defined media\textsuperscript{7-8}. Furthermore, it has been shown that modulation of the supplemental feed can impact the product quality of the protein drug\textsuperscript{9-14}. For example, the addition of manganese and galactose to the medium can increase the amount of terminal galactosylation on the biantennary oligosaccharide in the CH2 domain of a recombinant monoclonal antibody\textsuperscript{10}.

A common additive is the much-storied vitamin C (ascorbic acid). In addition to its function as a cofactor for collagen synthesis\textsuperscript{15}, it has been implicated as an antioxidant (biological reductant) and potent scavenger of reactive oxygen species (ROS)\textsuperscript{16-19}. Large scale production of recombinant monoclonal antibodies require higher levels of oxygen to maintain higher cell densities\textsuperscript{20}. Such conditions may generate reactive oxygen species\textsuperscript{21-22}. Therefore, including antioxidants such as ascorbic acid is generally considered protective and desirable. Such a view is also held for human nutrition and health, perhaps epitomized by the mega-dose championed by Linus Pauling\textsuperscript{23}.

Pertinent to this study, media components or additives have been shown to affect product quality and protein modifications\textsuperscript{24-25}. The best known example is arguably glycation of proteins by glucose\textsuperscript{26-28}, an essential nutrient as the main energy source of cultured cells. While
unavoidable, glycation nevertheless is predictable. On the other hand, it is challenging to predict and detect modifications by secondary metabolites or by-products. For example, we previously reported that a recombinant monoclonal antibody was unexpectedly modified by an accumulation of methylglyoxal (MGO) during cell culture due to a change in media that was perceived as beneficial\textsuperscript{29}. Methylglyoxal is a dicarbonyl compound that is generated as a by-product of glycolysis\textsuperscript{29,30}. As a reactive metabolite, it modifies the side chains of susceptible arginine residues forming adducts with mass increases of 72 Da and 54 Da, respectively. Under optimal conditions, methylglyoxal is effectively removed by the glutathione dependent glyoxylase I/II pathway\textsuperscript{30}. However, changes in the cell culture conditions, specifically the redox state, can affect this important balance, ultimately leading to increased amounts of this modification\textsuperscript{31}. Modifications from other reactive species include cysteinylation\textsuperscript{32}, glutathionylation\textsuperscript{33,34}, and N-homocysteintylation from homocysteine thiolactone\textsuperscript{35}. Therefore, changes to the cell culture medium may result in unexpected changes in product quality\textsuperscript{36,37}.

As reported herein, we observed an increase in acidic species in a recombinant monoclonal antibody supplemented with ascorbic acid during cell culture. Additionally, we observed two unidentified masses in the reduced LC/MS analysis of acidic fractions which exhibited molecular weight increases of 130 and 148, respectively. Detailed analyses revealed that these modifications occurred on the primary amines of the N-termini of heavy and light chains and also susceptible lysine residues. This was confirmed by \textit{in vitro} incubation of native antibody with increasing concentrations of ascorbic acid. Given that the molecular weight of ascorbate is 176, it was hypothesized that metabolites or degradation products of this nutrient were the culprits. This was confirmed in more detailed mechanistic investigations using \textsuperscript{13}C labeled ascorbate. As illustrated in Scheme 1, ascorbate is first oxidized to dehydroascorbic acid.
Subsequent decarboxylation generates xylosone$^{38-39}$. Xylosone is highly reactive and is capable of modifying susceptible primary amines, resulting in mass increases of 148 Da and 130 Da for the hemiaminal and Schiff base, respectively (Scheme 1). To the best of our knowledge, this is the first report of an ascorbate-originated xylosone modification of a recombinant monoclonal antibody in vitro and in cell culture.
4.3 Materials and Methods

4.3.1 Materials

The recombinant monoclonal antibody (adalimumab) was produced by stably transfected Chinese hamster ovary (CHO) cells cultured in a bioreactor and purified at AbbVie Bioresearch Center (Worcester, MA). Dithiothreitol (DTT) was from Sigma (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were from J.T.Baker (Phillipsburg, NJ). Formic acid (FA) was from EMD (Gibbstown, NJ). Trypsin was from Worthington (Lakewood, NJ). Ascorbic acid was from Sigma (St. Louis, MO). $^{13}\text{C}$ labeled ascorbate reagents labeled at either the carbon 1, carbon 2 or carbon 3 position were from Sigma (St. Louis, MO). Guanidine-HCl was from Thermo Scientific (Rockford, IL).

4.3.2 Weak cation exchange (WCX) chromatography

The antibody in low salt buffer was loaded onto a ProPac 4 x 250 mm WCX-10 column (Thermo Scientific, Rockford, IL) at 94% mobile phase A (10 mM sodium phosphate, pH 7.5) and 6% mobile phase B (10 mM sodium phosphate and 500 mM sodium chloride, pH 5.5) at a flow-rate of 1 mL/min. The percentage of mobile phase B was increased from 6% to 16% over 20 min to elute the antibody monitored by UV absorbance at 280 nm. The column was then washed using 100% mobile phase B and then equilibrated using 6% mobile phase B for 9 minutes between injections.

4.3.3 LC-MS analysis of reduced antibody
Light chain and heavy chain of the antibody from different fractions were analyzed using an HPLC (Agilent 1260, Santa Clara, CA) with a reversed phase column (WR Grace, C4, 1 x 150 mm i.d., 5µ particle size) coupled to a Q-TOF mass spectrometer (Agilent, 6510). Antibody was reduced using DTT (10 mM final concentration). Two microliters of each sample were loaded at 95% mobile phase A (0.08% FA and 0.02% TFA in Milli-Q water) and 5% mobile phase B (0.08% FA and 0.02% TFA in acetonitrile) and then eluted using a gradient from 5% mobile phase B to 35% mobile phase B in 20 min. The column was washed using 90% mobile phase B and equilibrated using 5% mobile phase B for 10 min. The flow rate was 50 µL/min and column oven temperature was 60 ºC. The mass spectrometer was operated in electrospray positive ion mode with a scan range from m/z 600 to 3200. Ion spray voltage was 4500 volts and the source temperature was 350 ºC.

4.3.4 Fractionation of Acidic Species

In order to understand the chemical nature of the new acidic peaks, ascorbate supplemented samples were analyzed using weak cation exchange (WCX) chromatography, and the distinct acidic peaks were fractionated. The pooled fractions were concentrated using an Amicon Ultra 10 KDa MWCO (Millipore) and subjected to analysis by reduced LC/MS analysis as described previously.

4.3.5 Tryptic digestion

Protein fractions from WCX chromatography were denatured using 6 M guanidine hydrochloride in 100 mM Tris, pH 8.0 and reduced using 10 mM DTT at 37 ºC for 30 min. Alkylation was performed using 25 mM iodoacetic acid at 37 ºC for 30 min. The samples were
buffer exchanged to 10 mM Tris pH 8.0 using NAP-5 columns (GE Healthcare, Piscataway, NJ). The samples were digested with trypsin at 1:10 (w:w, enzyme:antibody) and incubated at 37 °C for 4 hours.

### 4.3.6 LC-MS analysis of peptides

An UPLC (Acquity, Waters, Milford, MA) equipped with a UPLC C18 reversed phase column (Waters, 1 x 150 mm i.d., 1.7µ particle size) and a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, Waltham, MA) were used to analyze peptide samples. Forty µL of each sample was loaded at 98% mobile phase A (0.08% formic acid and 0.02% TFA in Milli-Q water) and 2% mobile phase B (0.08% formic acid and 0.02% TFA in acetonitrile) and then eluted using a gradient from 2% mobile phase B to 35% mobile phase B in 80 min. The column was washed using 98% mobile phase B and equilibrated using 2% mobile phase B for 10 min. The flow rate was 50µL/min and column oven temperature was 60 °C. The mass spectrometer was operated in positive ion mode with a scan range from m/z 300 to 2000 with alternating CID and HCD of the three most intense parent masses. Ion spray voltage was set at 4500 volts and the source temperature was set at 350 °C. The data were analyzed by searching extracted mass traces of tryptic peptides with mass increases of 130 and 148 daltons. The data were also searched against the theoretical primary structure using the Sequest algorithm and selecting the observed variant increases (Thermo Scientific, West Palm Beach, FL).

### 4.3.7 in vitro incubation of Monoclonal antibody with ascorbic acid

Ascorbic acid (Sigma, St Louis, MO) was dissolved in phosphate–buffered saline (20 mM Na₂HPO₄, 150 mM NaCl, pH 7.2) at a concentration of 3 mg/mL (17 mM) in order to simulate the high ascorbate conditions from the cell culture experiments. The isoform of the
recombinant monoclonal antibody without any C-terminal Lys (denoted as Lys-0) was diluted into the ascorbate solution and incubated in at 37 °C for 14 days, the typical duration of the cell culture experiments. The antibody was buffer exchanged to 10 mM Tris pH 7.0 following the incubation. Lys-0 antibody incubated under the same conditions in PBS alone was used as a control. The samples were analyzed by weak cation exchange chromatography and reduced LC/MS as described in the previous sections. Specific peaks that appeared in the acidic region of the chromatogram in the sample incubated with ascorbate were fractionated for further analysis.

4.3.8 Regio-labeled ascorbate to elucidate the mechanism of the modification

Ascorbic acid that was specifically regio-labelled with $^{13}$C (at C1, C2 or C3 carbon, respectively) was obtained from Sigma-Aldrich (see Figure 4-14). These ascorbic acid isomers were used to elucidate and confirm the mechanism for the formation of the reactive degradant (xylosone) and the final modifications to the antibody. The antibody was incubated with each of the labeled ascorbate reagents for 14 days at 37 °C. The samples were analyzed by reduced LC/MS and by tryptic mapping with LC/MS/MS for analysis of the resulting adducts.
4.4 **Results and Discussion**

As detailed below, we have discovered that a recombinant monoclonal antibody (adalimumab) was modified by ascorbic acid both in vitro and in cell culture. Furthermore, mass spectrometric and mechanistic investigation has attributed the modifications to xylosone, a degradation product of ascorbate. The modifications occurred on primary amines, including the N-termini of both light and heavy chains and also susceptible lysine residues.

4.4.1 **Ascorbate supplement in cell culture induced new acidic variants**

A recombinant monoclonal antibody was expressed in shake flasks using various supplementation and additives. Of specific interest were cell culture conditions under which the concentration of ascorbate was increased (0, 0.1, 1 and 3 mg/mL, respectively). When antibodies were analyzed by weak cation exchange chromatography, the formation of new acidic species directly correlated with ascorbate concentration (Figure 4-1). This observation led us to initiate a detailed structural analysis of the recombinant monoclonal antibody in order to determine the chemical nature and cause of the increase in charge heterogeneity.
Figure 4-1: WCX-10 chromatograms of the recombinant monoclonal antibody control (top, no ascorbic acid) and supplemented with 0.1, 1 and 3 mg/mL of ascorbate in cell culture, respectively. The increase in ascorbate concentration corresponds to an increase in the early eluting species and a well defined peak (Peak A).
4.4.2 Reduced LC/MS analysis of antibody stored in ascorbate buffer

Without separating antibody variants, reduced LC/MS analysis of the recombinant monoclonal antibody heavy chain and light chain from the ascorbate supplemented cultures did not show obvious differences in the mass spectra (Figure 4-2). Therefore, fractionation of the earlier eluting peaks and the Lys-0 peak from weak cation exchange (Figure 4-1) was employed. The analysis of the Lys-0 fraction by reduced LC/MS revealed a deconvoluted light chain mass spectra which was in good agreement with the expected light chain mass (Figure 4-3, A).

Analysis of the mass spectra resulting from Peak A, however, exhibited discernible differences as shown in Figure 4-3 (B). The deconvoluted light chain mass spectra showed the expected mass as well as several lower abundance peaks with higher molecular-weight. Two of the masses corresponded to mass increases of 148 Da and 130 Da.

In order to elucidate the nature of the additional species, a pure fraction of the Lys-0 species was treated with ascorbic acid in vitro. The deconvoluted spectrum (Figure 4-3, C) of the in vitro sample was highly similar to that from cell culture (Figure 4-3, B). First, the observed molecular weight of 23408.5 Da represented the unmodified light chain from Lys-0 incubated with ascorbate and was in good agreement with the theoretical value of 23408.1. Secondly, two other lower intensity peaks were also observed with masses of 23538.3 Da and 23556.6 Da which corresponded to mass increases of 130 Da and 148 Da, respectively (Figure 4-3, C). Furthermore, these two masses are in good agreement with the two masses representing +130 Da and +148 Da from cell culture suggesting that they were generated due to the ascorbic acid supplementation. Analysis of the heavy chain from the ascorbate supplementation experiments was complicated by the presence of inherent glycosylation. Treatment with
PNGaseF and carboxypeptidase B simplified the heavy chain mass spectra (Figure 3-4), which suggests the same ascorbate-related modifications are present although not as conclusive as shown in the light chain data due to other low intensity peaks in the spectra.
Figure 4-2: Light Chain spectra from reduced LC/MS analysis of unfractionated Antibody A.

Two masses of +130 and +148 Da increase within the mass spectra as more ascorbate is added to the cell culture.
Figure 4-3: LC/MS analysis of the recombinant monoclonal antibody light chain after reduction of antibody. A: Deconvoluted mass spectrum of the 0 Lys peak (theoretical MW = 23408.13 Da; Observed MW = 23408.34 Da). B: Deconvoluted spectrum of Peak A from cell culture. The main peak (23408.44) agrees with the theoretical molecular weight of the light chain (23408.13). Several lower intensity peaks are also observed including those with mass increases of 130 Da and 148 Da, respectively. C: Deconvoluted mass spectrum of light chain from Antibody A incubated in buffer containing 3 mg/mL ascorbate for 14 days at 35 °C, showing the same peaks as from cell culture.
Figure 4-4: Deglycosylated heavy chain spectra from reduced LC/MS analysis of fractionated Peak A. Although two masses of +130 and +148 Da are observed, the heavy chain mass spectra was less conclusive than the light chain mass spectra.
4.4.3 Tryptic mapping and LC/MS/MS detection

Tryptic mapping was performed on the recombinant monoclonal antibody produced in cell culture supplemented with up to 3 mg/mL of ascorbate and on the 0 Lys isoform incubated with ascorbate \textit{in vitro}. It is important to note that the mass difference between these two species is 18 daltons therefore they may be related and involve the loss of a water molecule, reminiscing modifications of lysine or arginine residues by methylglyoxal (MGO) or other carbonyl-containing molecules. Therefore, we searched for mis-cleaved tryptic peptides and found none with an internal arginine. Then, we examined potential modifications of primary amines, specifically the N-termini and mis-cleaved lysine containing peptides, which did indeed produce several possible sites of modification as shown in Table 4-1.

Specifically, the light chain N-terminal peptide had two chromatographically resolved +148 Da peaks with close elution to the native peptide and a +130 peak that eluted later as shown in Figure 4-5. The MS/MS spectra were analyzed for the native and modified +148 Da and +130 Da peptides that all had y ion series which were in good agreement with the predicted amino acid sequence and covered the entire peptide except the first two N-terminal residues as shown in Figure 4-6. The b ion series was limited but had strong signal for the first three residues at the N-termini. In addition, a strong signal for the native and modified $a_1$ and $a_2$ ions was also present. All together, our data conclusively localized both modifications (+148 Da and +130 Da) to the N-terminal amine of the light chain (Asp1).
Table 4-1. Peptides identified with modifications by xylosone (sites are denoted by asterick * and NH$_2$- denotes N-terminal amine).

<table>
<thead>
<tr>
<th>Chain</th>
<th>Peptide Sequence</th>
<th>Increase in Mass (Da)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>*NH$_2$-DIQMTQSPSSLSASVGDR</td>
<td>148, 130</td>
<td>N-terminus</td>
</tr>
<tr>
<td>LC</td>
<td>NYLAWYQQK*PGK</td>
<td>148</td>
<td>Variable Region</td>
</tr>
<tr>
<td>LC</td>
<td>APK*LLIYAASTLQSGVPSR</td>
<td>148</td>
<td>Variable Region</td>
</tr>
<tr>
<td>LC</td>
<td>APYTFGQGTK*VEIK</td>
<td>148</td>
<td>Variable Region</td>
</tr>
<tr>
<td>LC</td>
<td>VEIK*R</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>LC</td>
<td>EAK*VQWK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>LC</td>
<td>VQWK*VDNALQSGNSQESVTEQDSK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>LC</td>
<td>DSTYSLSSTLTLSK*ADYEK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>LC</td>
<td>HK*VYACEVTHQGLSSPVTK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>LC</td>
<td>VYACEVTHQGLSSPVTK*SFNR</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>*NH$_2$-EVQLVESGGGLVQPGR</td>
<td>148, 130</td>
<td>N-terminus</td>
</tr>
<tr>
<td>HC</td>
<td>DNAK*NSLYLQMNSLR</td>
<td>148</td>
<td>Variable Region</td>
</tr>
<tr>
<td>HC</td>
<td>GPSVFPLAPSK*SGSGGTAALGCLVK</td>
<td>148</td>
<td>Variable Region</td>
</tr>
<tr>
<td>HC</td>
<td>DYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYIC</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>NVNHK*PSNTK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>VSNK*ALPAPIEK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>ALPAPIEK*TI9K</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>DELTK*NQVSLTCLVK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>TTPPVLDSDGSFFLYSK*LTVDK</td>
<td>148</td>
<td>Constant Region</td>
</tr>
<tr>
<td>HC</td>
<td>LTVDK*SR</td>
<td>148</td>
<td>Constant Region</td>
</tr>
</tbody>
</table>
Figure 4-5: Extracted ion chromatograms (XIC) from the recombinant monoclonal antibody tryptic map corresponding to the doubly charged light chain N-terminal peptide and N-terminal peptides with +148 Da and +130 Da adducts. The +148 Da adduction resulted in two chromatographically resolved species in the XIC.
Figure 4-6: The MS/MS spectra of the light chain N-terminal tryptic peptides from Peak A fractionated from the recombinant monoclonal antibody supplemented with 3 mg/mL ascorbate. The top spectra is for the unmodified peptide; the two middle spectra show the two +148 Da peptides, respectively; and the bottom spectra is for the +130 Da peptide. In all cases, the $a_1$ ions definitively localize the modifications to the N-termini of the peptides.
The N-terminal peptides of the heavy chain also exhibited a parent peak in the native extracted mass chromatogram, two isobaric peaks in the extracted mass chromatogram corresponding to a mass increase of +148 Da and two isobaric peaks in the extracted mass chromatograms corresponding to a mass increases of +130 Da (Figure 4-7). Similar to those of the light chain N-termini, the MS/MS spectra for all five of the peaks again showed a very strong y ion series with good agreement with the expected amino acid sequence as shown in Figure 4-8. Once again, the b ion series was used to definitively assign the +148 Da and +130 Da modification to the N-termini of the two sets of resolved modified peptides.

The analysis of other peptides from the recombinant monoclonal antibody showed the modification of mis-cleaved lysine residues (see Figures 4-9 and 4-10). However, lysines residues exhibited lower susceptibility to modification as compared to the N-terminal primary amines as shown in the analysis of the in vitro ascorbate incubation over time and at increasing concentrations of ascorbate (Figure 4-11). The observation is in good agreement with the lower pKa of the N-terminal amines (~ 8) as compared to the pKa of the lysyl amine on the side chain (~ 10)\(^{40-41}\); and of course, these modifications are also likely to be influenced by other factors such as the antibody structure and microenvironment\(^{24, 29, 35, 42-44}\). In addition, it is important to note that only a +148 Da species was seen for all modified lysine residues in the antibody, again suggesting local environment is likely to affect the nature and distribution of various chemical forms, as discussed in greater details later.
Figure 4-7: Extracted ion chromatograms from the heavy chain N-terminal peptide. The XIC for the +148 mass produced two peptides which elutes slightly later than the unmodified XIC. The ratio is approximately 2:1 suggesting one of the isomers is favored over the other. The XIC for the +130 mass also produced two peaks which also had a 2:1 ratio which suggests both species resulted from a Schiff base formation as shown in Scheme 1.
Figure 4-8: MS/MS spectra from the heavy chain N-terminal peptide. The data conclusively localizes the +148 Da and +130 Da modifications from the peaks shown in S-2 to the N-terminus of the heavy chain.
Figure 4-9: Extracted ion chromatograms of an unmodified (top) peptide with an internal lysine and the same peptide modified by a xylosone adduction. Only the +148 Da modification was observed for all modified lysine containing peptides.
Figure 4-10: MS/MS spectra of the unmodified (top) and modified (bottom) lysine containing peptides shown in S-5. The data localizes the +148 xylosone adduction to the internal lysine residue.
Figure 4-11. Relative susceptibilities of representative peptides modified by xylosone
The same exercise was performed for the tryptic maps of the pure 0 Lys isoform treated with ascorbate \textit{in vitro} (0.1, 1.0 and 3.0 mg/mL) over time (3, 5, 11 and 14 days). The same modifications of the heavy chain and light chain N-terminal peptides were observed by the presence of mass increases of +130 Da and +148 Da on these peptides, respectively (Figure 4-12 and Figure 4-13). Additionally, the MS/MS data once again localized the increase to the respective N-terminal residues (data not shown). Furthermore, miscleaved lysine containing peptides were also observed to a similarly lesser degree. The relative susceptibilities and rate of the modification to these representative peptides are shown in Figure 4-11. Reduced LC/MS analysis of the light chain also showed a correlation between the duration of the \textit{in vitro} incubation and the extent of these modifications (Figure 4-14). Thus, the modifications observed in the cell culture samples supplemented with ascorbate were in good agreement with the modifications seen from \textit{in vitro} treatment, narrowing down the modification species to ascorbic acid or its derivatives.
Figure 4-12: Comparison of XIC’s of modified light chain N-terminal peptides generated in cell culture (Peak A) or during in vitro incubation with ascorbate. The overall chromatographic profiles between the two samples are in good agreement suggesting the same chemical modifications are generated in both conditions.
Figure 4-13: Comparison of XIC’s of modified heavy chain N-terminal peptides generated in cell culture (Peak A) or during in vitro incubation with ascorbate. Once again, the overall chromatographic profiles between the two samples are in good agreement suggesting the same chemical modifications are generated in both conditions.
Figure 4-14: Reduced LC/MS of light chain from recombinant mAb incubated with xylosone over time. The data show that the levels of the +130 and +148 Da modifications increase over time.
4.4.4 Elucidation of the modification agent as xylosone

The mass of ascorbate is 176 Da which is 28 Da greater than the +148 modification observed, so it was unlikely that ascorbate itself modified the antibody, but rather its degradation product(s) was the likely culprit(s). Another hint is the 18 Da difference (130 vs 148 Da) between the two observed modifications, suggesting a dehydration (elimination of a water molecule) following the initial reaction; this is reminiscent of modifications of lysine or arginine residues by methylglyoxal (MGO) or other carbonyl-containing molecules. All together, we postulated that the bona fide reactive species should be degraded from ascorbate and also contain reactive carbonyl group(s). Xylosone thus emerged as a likely candidate as it has been reported as a degradation product of ascorbate (see Scheme 4-1 for its formation pathway)\textsuperscript{38}.

Furthermore, xylosone has a mass of 148 Da and two carbonyl groups. The 130 Da adduct may be due to the loss of a water molecule following the initial addition reaction between the carbonyl group of xylosone and the amines in the protein (hemiaminal to Schiff base as shown in Scheme 4-1).
Scheme 4-1:

```
[O]  

H OH OH OH O
OH OH OH OH O  dehydroascorbic acid
ascorbic acid (176 Da)  (174 Da)

-O CO2

H OH OH O
OH OH O

xyllosone (148 Da)

H N
Protein

H OH H N
O
O
O
Schiff base (+130 Da)

H OH OH H N
O
O

hemiaminal (+148 Da)
```
4.4.5 Incubation of antibody with $^{13}$C Regio-labeled ascorbate

Isotopic labeling and tracing often provides detailed mechanistic insights$^{45-47}$. In order to confirm that ascorbate was degraded to xylosone (losing the carbon atom at 1 position), which in turn modified the antibody, we used ascorbate regio-specifically labeled with $^{13}$C (at C1, C2 or C3 carbon, respectively, as shown in Figure 4-15, A). Following incubation with unlabeled ascorbate or one of the three regio-labeled ascorbate molecules, the antibody was analyzed by tryptic peptide mapping with mass spectrometry detection. The results (Figure 4-15, B) lend conclusive evidence supporting the mechanism in Scheme 4-1: labeling at the C1 position ($^{13}$C or $^{12}$C) produced the same mass spectra; in contrast, ascorbate with $^{13}$C labeling at C2 or C3 shifted the peaks to 1 m/z higher compared to $^{12}$C ascorbate (+149 vs +148 and 131 vs 130, respectively). In addition, MS/MS localized the modification to the light chain N-terminal residue or heavy chain N-terminal residue including the heavy label for the C2 and C3 regio-labeled ascorbates as shown in Figure 4-16 and Figure 4-17, respectively. Thus, the data have verified that the C1 carbon in ascorbate is lost but neither C2 nor C3. Therefore, our data confirmed that ascorbate was converted to xylosone with the concomitant loss of C1 carbon atom as illustrated in Scheme 4-1.
Figure 4-15: **A**: Structures of the four different isotopic isoforms of ascorbate used to probe the structure of the +130 Da and +148 Da adducts. The red dot denotes carbon-13 labeling at a given position. **B**: The mass spectra of the doubly charged modified light chain N-terminal tryptic peptide of Antibody A incubated with 3 mg/mL of ascorbate. The pattern of mass shift, indicates that the carbon atom at 1 position in ascorbate is cleaved off in the final adducts, consistent with the proposed mechanism of xylosone being the reactive intermediate (see Scheme 4-1).
Figure 4-16: MS/MS spectra of light chain modified by xylosone derived from ascorbic acid or ascorbic acid with 13C at C1, C2 or C3, respectively. The heavy label is not retained in the ascorbate labeling at C1 but is retained when labeled at C2 and C3 thus conforming that ascorbate is degrading to xylosone which subsequently modifies susceptible primary amines.
Figure 4-17: MS/MS spectra of heavy chain modified by xylosone derived from ascorbic acid or ascorbic acid with 13C at C1, C2 or C3, respectively. The data supports the conversion of ascorbate to xylosone and subsequent modification to the heavy chain N-terminal primary amine.
4.4.6 Chemical Nature of the Modifications

When a carbonyl reacts with an amine, two products may form: the initial addition reaction leads to a hemiaminal (see Scheme 4-1) with a mass equals to the total of the masses of the two reactants (amine and carbonyl; for xylosone, 148 Da); a subsequent elimination of a water molecule (18 Da) leads to a Schiff base (imine, see Scheme 4-1) with a mass that is 18 Da less than the hemiaminal (148-18=130 Da). Hence the masses of the two adducts match with the observed masses.

The underlying chemistry, including stereo-, regio- and positional isomers of the adducts, also explains the multiple isobaric peaks observed (see Figures 4-5 and 4-7). For instance, the formation of the hemiaminal can result in two stereoisomers (the chiral center is denoted with an * in Scheme 4-1). Two other factors further complicate the situation: first, two carbonyls exist in xylosone; and second, xylosone may exist in both cyclic and acyclic forms (see Schemes 4-2 and 4-3), and each may lead to the hemiaminal and Schiff base forms described above. Furthermore, the Schiff bases can undergo further cyclization as well (Scheme 4-3).

For this antibody, no modification of arginine was observed; at first, it was somewhat surprising to us as both xylosone and methylglyoxal contain two adjacent carbonyl groups. However, upon closer inspection, as shown in Scheme 4-2, several hydroxyl groups in xylosone can readily form stable cyclic hemiacetal or hemiketal with one of the carbonyl group, thereby leaving only one reactive carbonyl group for further reactions with amines that is similar to that of glycation of amines. Of course, protein structures and local environments can certainly affect the stability of the products described above\textsuperscript{24}. For example, for some other proteins, modification of arginine by xylosone was reported\textsuperscript{48}.
Ascorbate oxidative degradants have been reported as a source of chemical modifications in human eye lens and were shown to modify lysine and arginine residues in model systems\textsuperscript{38-39}. However, neither a +148 Da or +130 Da mass deviation nor are listed as a xylosone modification in the ABRF Delta Mass database (\url{www.abrf.org/index.cfm/dm.home}) or the Unimod database (\url{www.unimod.org}). To the best of our knowledge, this is the first report of a xylosone modification of a recombinant monoclonal antibody.
Scheme 4-2. Reaction scheme of cyclic xylosone with a protein primary amine.
Scheme 4-3: Reaction scheme of acyclic xylosone with a protein primary amine.
4.4.7 Cell Culture Media Additives

Cell culture scientists are under constant pressure to increase titers, enhance cell culture performance and improve product quality. The practice of modifying the cell culture medium is the predominant tool used to address these goals\textsuperscript{10,49-54}. However, it is difficult to predict whether a specific additive will address a specific biochemical need of the host cell with the desired outcome or whether these good intentions will go awry (see Table 4-2). Occasionally, a cell culture additive may have negative implications on product quality\textsuperscript{55-58}. In our study, a recombinant monoclonal antibody (adalimumab) exhibited a difference in product quality following a change to the cell culture conditions. Our initial observations were confounded by the fact that ascorbate unexpectedly degraded quite rapidly to xylosone that in turn exhibited reactivity with susceptible primary amines. It has been well established that increasing glucose levels in a cell culture will increase the potential for glycation to occur through well understood chemistry\textsuperscript{26-27}. However, the discovery that ascorbate supplementation to the cell culture of a recombinant monoclonal antibody induced a novel glycation-like modification by xylosone was quite surprising and is a reminder that product quality is another parameter that must be considered when making changes to the cell culture feeding strategies. Furthermore, it is worth noting that xylosone almost certainly modifies a myriad of proteins of the host cells, thereby directly affecting a broad range of biological activities ultimately impacting the culture viability.
Table 4-2: List of cell culture additives shown to affect the product quality

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type</th>
<th>Product Quality Impact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Amino Acid</td>
<td>Low glutamine decreased sialylation and increased hybrid and high mannose N-glycans</td>
<td>[1]</td>
</tr>
<tr>
<td>Cystine</td>
<td>Amino Acid</td>
<td>Reduced protein aggregation</td>
<td>[2]</td>
</tr>
<tr>
<td>Cys, Ile, Leu, Trp, Val, Asn, Asp, Glu</td>
<td>Amino Acid</td>
<td>Increased sialylation</td>
<td>[3]</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sugar</td>
<td>Increased glycation</td>
<td>[4, 5]</td>
</tr>
<tr>
<td>Galactose</td>
<td>Sugar</td>
<td>Increased G1/G2 N-glycans</td>
<td>[6]</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sugar</td>
<td>Low glucose reduced full glycosylation</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low glucose reduced glycation</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Changes in antigen binding due to changes in glycosylation</td>
<td>[9]</td>
</tr>
<tr>
<td>ManNAc</td>
<td>Sugar</td>
<td>Increased sialylation</td>
<td>[10]</td>
</tr>
<tr>
<td>GlcNAc, uridine, ManNAc</td>
<td>Sugar</td>
<td>Increased glycan antennarity</td>
<td>[11]</td>
</tr>
<tr>
<td>Mn</td>
<td>Trace Metal</td>
<td>Increased G1/G2 N-glycans</td>
<td>[12]</td>
</tr>
<tr>
<td>Cu</td>
<td>Trace Metal</td>
<td>Promoted disulfide bond reformation</td>
<td>[13]</td>
</tr>
<tr>
<td>Fe</td>
<td>Trace Metal</td>
<td>Increased protein oxidation</td>
<td>[14]</td>
</tr>
<tr>
<td>Co</td>
<td>Trace Metal</td>
<td>Increased G1/G2 N-glycans</td>
<td>[15]</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>Vitamin</td>
<td>Increased peptide bond breakage and deamination</td>
<td>[16]</td>
</tr>
<tr>
<td>DMSO</td>
<td>Non-nutrient</td>
<td>Reduced sialylation</td>
<td>[17]</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Non-nutrient</td>
<td>Increased sialylation, decreased aggregation</td>
<td>[18]</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Non-nutrient</td>
<td>Decreased aggregation</td>
<td>[19]</td>
</tr>
</tbody>
</table>


4.4.8 Acidic Species

The modification of a recombinant monoclonal antibody by xylosone led to an increase in acidic species. Acidic species has been widely studied with regards to recombinant biotherapeutics. Common contributors include asparagine deamidation, glycation of primary amines, incorporation of sialic acid, etc. and our recent studies showing methylglyoxal (MGO) modification of arginine residues and covalent adduction of the N-terminal primary amines by citrate. In all these cases, there is a change in formal charge either due to the introduction of a carboxylate into the molecule or the perturbation of a basic residue protonation due to a depression in its ionizable group’s pKa. The adduction of the N-terminus of heavy and light chains and lysine primary amines of the recombinant monoclonal antibody by xylosone appears to follow this second case. Thus, in depth studies of the underlying cause of acidic species helps analytical scientists establish general chemical susceptibilities that provide insight and may ultimately allow the assignment of all molecular variants which exist in this highly heterogeneous region.

4.5 Conclusions

Cell culture supplementation with ascorbic acid caused an unexpected change to the product quality of a recombinant monoclonal antibody, therefore the use of ascorbic acid as a supplement should be taken with caution. The recombinant monoclonal antibody was modified by xylosone, a highly reactive species generated as an oxidative degradation product of ascorbate, but not directly introduced into the culture. In addition, xylosone almost certainly modifies a myriad of proteins of the host cells, thereby directly affecting a broad range of
biological activities which may impact the culture viability. With the advancement in protein mass spectrometry and the increasing awareness of issues highlighted in this paper, similar modifications and mechanisms will likely be revealed in other systems and proteins. Altogether, better understanding and critical consideration of the latent reactivities of any addictives — and particularly, deleterious consequences — would be prudent to ensure the quality of protein products.
4.6 References:


Chapter 5. Perspectives and Future Directions

5.1 Perspectives and Future Directions

From a quote previously mentioned “analytical chemists are very good at finding what they know”. Recombinant proteins are extremely complex molecules and in addition to changes driven due to primary structure (deamidation, oxidation, N-terminal pyroglutamate, etc.), they are also subject to modifications by enzymes, reactive metabolites, cell culture additives and formulation excipients. The intracellular environment is in constant flux which is exacerbated by the high demands of the protein drug expression, high cell densities and flux in redox. All of these can induce changes to the environment where reactive molecules may appear.

As discussed in this dissertation, both advanced glycation end products and xylosone have been implicated in protein crosslinking. More recently, it has been shown that protein crosslinks may be detected using $^{18}$O labeling with an algorithm which looks for a signature crosslink pattern based on the presence of two C-termini\(^1\). Such an approach should be applied to samples where the prevalence of AGEs or xylosone is apparent. In addition, such an approach may uncover other reactive species in cell culture.

The targeted search for events known to be associated with ROS may prove fruitful. For instance, racemization has been associated with protein aging and may be facilitated in environments when the levels of reactive oxygen species increase. Recent reports present analytical methodologies which may be applied to help detect and quantify the levels of racemization which may be occurring\(^2\). Hydrogen-deuterium exchange of the alpha carbon proton has proven a valuable approach in assessing which residues in the primary structure underwent the formation of the racemization intermediate thereby providing regions worth investigating further\(^3\). These occurrences along with changes in chromatographic behavior could
effectively probe the primary structure of the antibody for racemization as a result of a stressed cellular environment.

As discussed previously, sulfenylation has emerged as another posttranslational modification linked to the formation of ROS\(^4\). They should certainly be considered in recombinant monoclonal antibody production. The recently reported trapping strategies may prove quite useful. In addition, other variants associated with cysteine residues such as trisulfides should be further investigated\(^5\). Like sulfenylated products, the trisulfides are also labile therefore they may be more prevalent than currently thought.

The current work presented focused on the recombinant monoclonal antibody product. However, modifications of other endogenous CHO cell proteins by reactive species such as methylglyoxal could also have impactful consequences to the cell culture. Analytical studies to focus of CHO proteins should be performed. Proteomics analysis of cell culture lysates from varying degrees of stress could probe for proteins susceptible to this modification. The influence such a modification could have on the cell viability or the expression levels should be investigated.

The structure-function relationship of recombinant monoclonal antibodies exhibiting chemical modification would prove prudent. Chemical modifications to the CDRs may likely affect the ability of the antibody to bind its intended target. An investigation of antibody function using surface plasmon resonance may provide clues to underlying chemical modifications. If resolved regions of the ‘acidic species’ have reduced binding to the epitope, it would suggest that a chemical modification is a likely cause. Additionally, modifications to the FC region of the antibody could influence the Effector Functions. This could have further reaching implications beyond the protein drug. These conserved regions share homology with
endogenous human IgG1 therefore sites susceptible to reactive species in CHO cell culture will likely extrapolate to susceptible human IgG.

The mass spectrometry search algorithms are lacking. These tools are capable of reporting chemical modifications which are considered by the application. They are not capable of reporting mystery mass shifts however due to the algorithms de-coupling of MS and MS/MS data. The MS/MS spectral data used to identify a peptide should be linked with the observed parent mass. Should the fragmentation profile be in good agreement with neutral losses corresponding to a region of the fasta sequence but the parent mass deviate from the theoretical, it should flag it along with the mass shift. In this way, the target peptide, the mass change and potentially the target residue would be reported. Seldom, do modifications happen at only a single site therefore the presence of multiple flags for the same variant would increase the likelihood of its validity. Such information would be a major breakthrough for the identification of unknown chemical modification. Communicating with groups like Protein Metrics or Peaks on needs for future versions of their data analysis packages could make this approach a reality.

Mass spectrometry is constantly evolving to meet the demands of drug discovery. Accordingly, instruments are achieving greater sensitivity, faster scan rates and higher resolution. In addition, fragmentation technologies are also improving to where Top Down and Middle Down approaches are becoming more and more robust\(^6\)\(^-\)\(^7\). Such approaches may be able to detect lower abundance mass shifts enabling easier identification of variants. In addition ETD, electron transfer dissociation, has matured into a valuable tool for measuring more labile chemical modification. ETD has been used to successfully perform Top Down analysis on the heavy and light chains of a recombinant monoclonal antibody\(^8\). Using this approach to carefully
dissect and discern isolated regions of the weak cation exchange profile may provide a molecular mapping of the antibody elution profile and help to uncover what is influencing it.

Alternative approaches to evaluating charge heterogeneity should be investigated. The Agilent OffGel Fractionator may be applied to the separation of charge variants in a recombinant monoclonal antibody. The separation mechanism is equivalent to isoelectric focusing, however, discrete bands separated by differences in pI can be recovered to measurable amounts and subjected to analysis by mass spectrometry. This approach would differ from weak cation exchange chromatography in that charge differences not associated with surface residues could be elucidated.

Alternatively, chromatofocusing is becoming more widely used for the analysis of charge heterogeneity. It is a hybrid technique which utilizes ampholytes to produce a pH gradient thus influencing the charge as the pH approaches the isoelectric point. Additionally, the interaction between the protein and the weak cation exchange column still relies on surface charge. The intriguing aspect of chromatofocusing is the possibility of coupling this technique directly to a mass spectrometer. Salt based separations are not compatible with mass spectrometry but a separation which uses MS friendly ampholytes may be able to bridge the gap between isolation and analysis. This approach deserves further investigation.

In conclusion, the most critical aspects to discover an unknown variant in recombinant monoclonal antibody is staying well informed of the science. A continued pursuit in understanding chemical biology, biochemistry, advances in analytics and the current reports in the literature pertaining to antibody heterogeneity are essential. Being well informed of possible reactive species increases the likelihood that an unassuming peak in a mass spectrum is not missed. It is of course through my training over the past five years that I have come this point
where I have contributed to what is known about recombinant monoclonal antibody heterogeneity and as I look towards the future, it is the continued pursuit of the science which will lead to the yet unwritten chapters.
5.2 References


