Cysteine-Linked IgG2 Antibody-Drug Conjugates: How Antibody Structure Affects Conjugation Locations and Stability

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UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

CYSTEINE-LINKED IgG2 ANTIBODY-DRUG CONJUGATES: HOW ANTIBODY STRUCTURE AFFECTS CONJUGATION LOCATIONS AND STABILITY

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Antibody-drug conjugates (ADCs) are a newer class of targeted biologics that combine the specificity of an antibody with the cytotoxic potency of a small molecule. Only four ADCs are currently approved for market, and approximately 60 molecules are in active clinical trials in the US. Several modalities of ADCs are currently under development, one of which uses thiol-maleimide chemistry to conjugate drug-linkers at the antibody’s endogenous interchain cysteines. The most popular framework used for cysteine-linked ADCs is the IgG1 subclass, for which extensive physicochemical and structural information is available in literature.

Cysteine-linked antibody-drug conjugates (ADCs) produced from IgG2 monoclonal antibodies (mAbs) are more heterogeneous than ADCs generated from IgG1 mAbs, as IgG2 ADCs are comprised of a wider distribution of molecules,
typically containing 0 – 12 drug-linkers (DL) per antibody. The three disulfide isoforms (A, A/B, and B) of IgG2 antibodies confer differences in solvent accessibilities of the interchain disulfides and contribute to the structural heterogeneity of cysteine-linked ADCs. Furthermore, IgG2s are more susceptible to disulfide bond scrambling events that are favored in neutral or high pH conditions, such as those typically employed for thiol-maleimide reactions.

Variably conjugated ADCs produced under either acidic or alkaline conditions using IgG2-A and IgG2-B mAbs were compared to better understand the influence of disulfide isoforms on the conjugation profiles of cysteine-linked ADCs. Higher drug-to-antibody ratios (DARs) were observed in the IgG2-A ADCs processed under acidic conditions, indicating that bond connectivity influences the rate of interchain disulfide reduction. On the other hand, both IgG2-A and IgG2-B ADCs generated using the alkaline process exhibited comparable average DARs and distributions of conjugated species, suggesting that conversion of the A to the B disulfide configuration occurs under alkaline conditions. Although the parent mAb disulfide configurations differed, the primary conjugation sites for both A and B isoforms were determined to be the hinge region cysteines for ADCs generated under both acidic and alkaline conditions.

Significant efforts went into pinpointing which specific hinge cysteines are conjugated, however, further work remains to be done on this area. Several approaches were explored for this purpose, including middle-down characterization methods and novel techniques for mapping scrambled IgG2
disulfides. Additional work described in this dissertation include work initiated to solve the high-resolution structure of a full-length IgG2 antibody and its ADCs.
DEDICATION

To my husband Mike whose unending love and support kept me focused on the finish line, and to my daughter Sabine whose adorable baby antics made me laugh every time.

To all the girls and women around the world, may you always know in your heart that you are smart, talented, worthy, and never too old to pursue your passion.
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To Dr. Gayathri Ratnaswamy for her continuous encouragement, words of wisdom, for inspiring me to become a better scientist, and for putting up with my endless requests for meetings.

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To my family and friends for always being there for me, no matter where in the world they may be.

To my dear husband Mike, whose love, patience, mindfulness, and adaptability allowed us to overcome all the challenges our family faced as I pursued this path.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADC</td>
<td>Antibody-drug conjugate</td>
</tr>
<tr>
<td>CEX-HPLC</td>
<td>Cation exchange high pressure liquid chromatography</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>DAR</td>
<td>Drug-to-antibody ratio</td>
</tr>
<tr>
<td>DL</td>
<td>Drug-linker</td>
</tr>
<tr>
<td>DSB</td>
<td>Disulfide bond or disulfide-bonded</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron-capture dissociation</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron-transfer dissociation</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>HH</td>
<td>Heavy-heavy</td>
</tr>
<tr>
<td>HHL</td>
<td>Heavy-heavy-light</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>ISD</td>
<td>In-source decay</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
</tbody>
</table>
m/z  Mass-to-charge ratio
mAb  Monoclonal antibody
MALDI  Matrix-assisted laser desorption ionization
mcMMAF  Maleimidocaproyl monomethyl auristatin F
MPA  Mobile phase A
MPB  Mobile phase B
MS  Mass spectrometry
RP-UHPLC  Reversed-phase ultra-high pressure liquid chromatography
SE-HPLC  Size exclusion high pressure liquid chromatography
TCEP-HCl  Tris(2-carboxyethyl)phosphine hydrochloride
TFA  Trifluoroacetic acid
TOF  Time-of-flight
vcMMAE  Maleimidocaproyl valine-citrulline para-aminobenzoic acid monomethyl auristatin E
Chapters 1, 3, 4, 5, and 6:

Versions of material in these chapters have been published or submitted for publication in:


Chapter 1 - Introduction

1.1 Therapeutic Antibodies

Antibodies, or immunoglobulins (Ig), are specialized recognition biomolecules that are part of the innate humoral immune response, which are able to elicit different types of immune responses to eliminate foreign pathogens. Coating of a pathogen with antibodies inhibits infection by blocking the pathogen’s ability to reach cell-surface targets (neutralization), and marks the coated pathogen for degradation via phagocytosis (opsonization) or by activating the complement cascade. Endogenous antibodies against specific protein antigens are produced by B cells that become activated after recognition by the B-cell surface immunoglobulin and subsequent signaling from a helper T-cell.¹

Activated B-cells can produce five different antibody isotypes (classes) that perform effector functions in different areas of the body. IgM antibodies are found mostly in the blood as pentameric complexes, and are the first isotype to be produced in the humoral response.² Class switch recombination (CSR) involving rearrangement of the heavy chain gene leads to isotype switching in B-cells, promoting the production of IgA, IgG, IgE, or IgD classes.³ IgA molecules are the primary isotype in epithelial secretions and can exist in either monomeric or dimeric forms. The IgG and IgE isotypes are always in monomeric form; IgGs are the most common antibodies and primarily function in body tissues, and IgEs are found on mast cells in mucosa and connective tissues and mainly respond to allergens. IgGs are the most common antibody class researched and engineered as
biotherapeutics. The function of IgDs are still unclear, but may be involved in mucosal immunity and pro-inflammatory responses.\textsuperscript{2, 4}

Since the approval of the first recombinant antibody in 1986 (muromonab-CD3, an anti-CD3 murine IgG2a), there has been increasing interest in the clinical development of monoclonal antibodies (mAbs) and antibody-derived biomolecules for a growing repertoire of both cancer and non-cancer indications.\textsuperscript{5} Currently, more than 50 mAbs and mAb-derivatives have been approved for market in the US and EU, and over 400 mAbs were being evaluated in Phase 1 – 3 clinical trials as of 2016.\textsuperscript{6-7} The majority of therapeutic mAbs are developed for treatment of cancer or inflammatory diseases.

1.1.1 Immunoglobulin Structure

Antibodies are Y-shaped heterodimeric glycoproteins comprised of two light chains (LC) and two heavy chains (HC) covalently linked via disulfide bonds. Each antibody chain contains both constant and variable regions. The constant regions determine the Ig isotype and subclass, and the variable regions contain the antigen complementarity regions (CDRs) which directly bind to the antigen.\textsuperscript{8}

Specifically referring to IgGs, the approximate molecular weight (MW) of the total antibody is 150 kDa. The size of each HC is approximately 50 kDa and contain a conserved glycosylation site at N297. The constant region of the HC is classified as three subdomains (C\textsubscript{H1}, C\textsubscript{H2}, and C\textsubscript{H3}), with the C\textsubscript{H1} and C\textsubscript{H2} connected via a flexible hinge loop. Light chains can be either \textlambda or \kappa types, and also contain a constant and a variable region of a total approximate size of 25 kDa each. Each subdomain in the IgG consists of two \beta sheets connected via a single
intrachain disulfide bond, and this structure is known as the immunoglobulin fold. All IgGs contain 12 intrachain disulfide bonds, with one located on each antibody subdomain. Interchain disulfide bonds connect the LC to the HC at conserved residues that depend on the IgG subclass, and the two HCs are connected at the hinge region. The total number of interchain disulfide bonds depends on the IgG subclass: IgG1 and IgG4 subclasses have four, IgG2s have six, and IgG3s have thirteen interchain disulfides.

Early research using IgG molecules led to the definition of three major antibody fragments that result from enzymatic degradation: two Fab (fragment antigen-binding) and one Fc (fragment crystallizable). The $V_H + C_{H1}$ regions alone are known as the Fd fragment. The structure schematic of an IgG1 is shown in Figure 1.1

![Figure 1.1. Structure of a typical antibody](image)

An IgG1 molecule with glycosylation at the conserved N297 site is depicted, with HCs shaded blue and LCs shaded gray. The subdomains are indicated, where “C” denotes constant and “V” denotes variable, and the subscript H or L represent HC or LC, respectively. CDR loops on the $V_H$ and $V_L$ are represented as white lines. The hinge loop connecting the Fab and Fc regions is shown in teal and the interchain disulfide bonds in red. Intrachain disulfide bonds within each constant and variable domain are not depicted.
The biological activity of an IgG relies on the Fab region for antigen recognition and on the Fc region for inducing the immune response. The function of glycosylation variants is still poorly understood, but it has been related to clearance and stability in serum. The effector functions that therapeutic IgGs typically elicit are antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), although neutralizing therapeutic IgGs have also been described to a lesser extent.

1.1.2 IgG Subclasses

IgGs are further classified into four subclasses (IgG1, IgG2, IgG3, or IgG4) that differ based on the sequence of the conserved regions and the number and configuration of interchain disulfide bonds (Figure 1.2). In addition to structural variability, each subclass also exhibits differences in the abilities to elicit effector functions, to cross the placenta, in serum half-life, and stability in the drug product form (Table 1.1). Monoclonal antibodies (mAbs) of the IgG1, IgG2, and IgG4 subclass have been developed as biotherapeutics. IgG1s are the most commonly researched subclass for therapeutic use, possibly due to its structural simplicity, stronger ability to elicit ADCC and CDC, higher half-life, and lower propensity for aggregation in solution. IgG4 antibodies are structurally similar to IgG1s, but these molecules have a propensity for conversion of the hinge interchain to intrachain disulfides. The formation of intrachain disulfides can lead to the dissociation of the IgG4 half-antibody and lead to undesirable attributes such as Fab exchange with serum albumin. IgG3s are highly effective at inducing
effector functions, but its short half-life and hinge region complexity complicate their development for clinical use.

**Figure 1.2. Structures of IgG1, IgG2, IgG3, and IgG4.** Representation of the four IgG subclasses with configurations of both intrachain and interchain disulfide bonds. Figure taken from Liu and May (2012).[^9]

<table>
<thead>
<tr>
<th>Attribute</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (kDa)</td>
<td>150</td>
<td>150</td>
<td>170</td>
<td>150</td>
</tr>
<tr>
<td>Amino acids in hinge region</td>
<td>15</td>
<td>12</td>
<td>62 (!)</td>
<td>12</td>
</tr>
<tr>
<td>Inter-heavy chain disulfide bonds</td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Half-life in serum (days)</td>
<td>21</td>
<td>21</td>
<td>7-21</td>
<td>21</td>
</tr>
<tr>
<td>C1q binding (CDC)</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Fc receptor affinity (ADCC, Effector)</td>
<td>+++</td>
<td>-</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Table 1.1. Summary of structural and functional attributes of each IgG subclass.**

IgG2 antibodies are more structurally complex compared to IgG1s. The hinge region of IgG2s contain four cysteines that can be bonded in at least three different
configurations, namely A, A/B, and B disulfide isoforms. The IgG2 disulfide isoforms are distinguished by the configuration of the LC-HC interchain disulfide bonds, as shown in Figure 1.3. The IgG2-A structure is the canonical IgG interchain disulfide connectivity, where the LC and HC are disulfide-bonded in the Fab region. In the IgG2-B configuration, the Fab is connected to the hinge region in a “crossed” form, where the CH1 interchain cysteine is disulfide bonded to the second hinge cysteine of the opposite HC, and the LC interchain cysteine forms a disulfide bond with the N-terminus hinge cysteine. The A/B configuration is a hybrid structure, containing one Fab arm in the A configuration and the other Fab arm in the B configuration. Previous studies have shown that IgG2 disulfide isoforms exhibit differences in solvent accessibilities, hydrophobicity, isoelectric points, and antigen binding.

Figure 1.3. Cartoon representations of the IgG2 A, A/B, and B disulfide isoforms.

The IgG2 disulfide isoforms differ on their configurations of the interchain disulfide bonds (orange). The A configuration (left) is the canonical IgG disulfide bonding pattern, the B configuration (middle) connects the C_{H1} interchain cysteine to a hinge cysteine of the opposite HC, and the A/B configuration (right) has one Fab arm connected in the A configuration and the other Fab arm in the B configuration.
1.1.3 Manufacturing Therapeutic IgGs

The design of therapeutic IgGs commonly involve engineering of the CDR and framework sequences. Desirable outcomes of the engineered antibody include enhanced antigen affinity (binding and specificity), improved pharmacokinetics (increased potency and reduced clearance and immunogenicity), and better pharmaceutical properties (concentration, stability, purity).24-27 The next challenge in the generation of therapeutic antibodies is boosting its manufacturability, which is remediated by engineering cell lines, cell culture and purification conditions, and formulation. In addition to manufacturability, achieving scalability, batch consistency, and the target product quality profile are also vital aspects of drug development.

The flow of a typical mAb manufacturing process is shown in Figure 1.4. Manufacturing processes are commonly divided into two units: the upstream cell culture and the downstream purification units.28-29 The upstream process involves expansion of the cells until the full production scale, and monitoring the cell culture fluid (CCF) variables until predefined endpoints are met (such as days, titer, or cell viability).30 Separation of the CCF from the cell mass can be done by centrifugation and/or depth filtration. The IgG is then purified from the CCF via a sequence of chromatographic processes that usually start with a capture step (Protein A), followed by polishing steps (cation and anion exchange, size exclusion, mixed-mode purification).31 A final filtration sequence removes viral particles and formulates the purified IgG pool into the desired buffer and concentration.28
In the case of IgG2 mAb production using Chinese hamster ovary (CHO) cells, the distribution of disulfide isoforms depend on the length of cell culture. The newly assembled mAbs in the endoplasmic reticulum are largely in the IgG2-A configuration.\textsuperscript{20} The secreted IgG2 antibodies convert from the A to the A/B and, finally, to the B configuration based on the age of the cell culture fluid. The cysteine and glutathione ingredients in the cell culture media are believed to facilitate the rearrangement of disulfide bonds.\textsuperscript{32-33} The disulfide isoforms co-purify in the downstream process and therefore the final, formulated antibody bulk inevitably contains a heterogeneous mixture of isoforms.

\section*{Figure 1.4. Standard IgG manufacturing process}
The diagram of a generic mAb manufacturing process is shown. The upstream process is shown on the top row and the downstream process on the bottom row. Figure reproduced from Gronemeyer, et.al. (2014).\textsuperscript{28}
1.2 Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) are a rapidly growing class of targeted therapeutics, mostly for oncology indications, that utilize a recombinant antibody to deliver a highly potent cytotoxic small molecule to the target cells. The mechanism of action of an ADC relies on the cellular internalization of the molecule, followed by intracellular degradation which releases the cytotoxic drug and disrupts essential cell processes to cause cell death (Figure 1.5).

Figure 1.5. Mechanism of action of antibody-drug conjugates.
The mechanism of action of ADCs rely on the cellular internalization after the antibody binds the target antigen. Lysosomal degradation of the ADC leads to the intracellular release of the cytotoxic drug. The solid black arrows illustrate the intended effects of the ADCs, and the dashed arrows are the unintended effects. Figure reproduced from Senter and Sievers (2012).37

The majority of ADCs in clinical development utilize the IgG1 antibody subclass as the framework, and the popularity of this framework might be due in part to the simplicity of the IgG1 interchain disulfide structure and the historical prevalence of IgG1s in the field of antibody therapeutics. However, IgG1 antibodies may elicit strong effector responses that would result in incompatible effects with the mechanism of action (MOA) of an ADC, such as extracellular drug release and off-target cytotoxicity. IgG2 antibodies, on the other hand, are weak inducers
of effector responses and could therefore be more compatible with the desired MOA, where internalization of the ADC complex and intracellular drug release are the advantageous effects. Moreover, a previous study comparing cysteine-linked ADCs derived from IgG1, IgG2, and IgG4 antibodies showed similar tolerability and toxicity profiles between the IgG1 and IgG2 ADCs.41 At least 9 clinical-stage ADCs use an IgG2 or IgG4 mAb and knowledge gained from IgG1 ADCs may not necessarily apply to these molecules.34, 42

1.2.1 Survey of ADC Modalities in Clinical Investigation

In theory, the modular nature of ADC design allows for limitless choices of conjugation chemistry, linker type, and payload structures. Endogenous cysteine and lysine residues are currently the most popular conjugation targets for ADCs in clinical development, but other locations such as N-linked glycans and engineered natural and unnatural amino acid substitutions are also under investigation.34, 43-46 Two general categories of stable linkers are currently used, cleavable or non-cleavable, and the chemical structure depends on the antibody conjugation target, the cytotoxic component (payload), and desired enhancements of other properties such as stability in serum.47-48 The cleavable linkers release the cytotoxic component after chemical or lysosomal degradation. Non-cleavable linkers rely solely on the lysosomal degradation of the ADC for payload release into the intracellular compartment. With respect to the payload variety, most ADCs in advanced stages of clinical development disrupt DNA or microtubule processes that cause cell cycle arrest or cell death, and are too potent to be administered alone as a first-line chemotherapeutic agent.34, 43 However, some ADCs are
conjugated with commonly used chemotherapeutic agents to treat cancers that are difficult to target medically. Examples of these chemical agents include microtubule polymerization inhibitors (auristatins, maytansinoids, tubulysins, rhizoxins, cryptophycins), DNA-alkylating agents (calicheamicins, duocarmycins, benzodiazepines, anthracyclines), and transcription/translation inhibitors (camptothecin analogues, α-amanitin, spliceostatins, thailanstatins). Illustrations and structures of some common forms of ADCs in clinical development are shown in Figure 1.6.

Currently, four ADCs have gained FDA approvals for market (Adcetris®, Kadcyla®, Mylotarg®, and Besponsa®) and over 50 ADCs were in active clinical trials as of 2016. Cysteine-linked ADCs that target conjugation to endogenous cysteines involved in interchain disulfide bonding constitutes approximately half of all active clinical ADCs. The most popular drug-linker molecules coupled to cysteine-linked ADCs are auristatins that contain cleavable or non-cleavable maleimide linkers (vcMMAE or mcMMAF, respectively).

Monomethyl auristatins E (MMAE) and monomethyl auristatins F (MMAF) are synthetic analogues of the marine dolastatin 10, a microtubule polymerization inhibitor. The structures of mcMMAF and vcMMAE are shown in Figure 1.7. The “vc” linker refers to the maleimidocaproyl moiety with a valine-citrulline dipeptide, the latter of which is a target for degradation by cathepsin B. The “mc” linker stands for maleimidocaproyl and this linker is not enzyme-cleavable, and therefore drug release relies on full degradation of the ADC complex. Several drug-linker compounds also contain a self-immolative spacer, such as p-aminobenzoyl
alcohol, $p$-aminobenzoic acid (both abbreviated as PABA) or $p$-aminobenzoyl oxycarbonyl (PABC).\textsuperscript{52-53}

Figure 1.6. Examples of common ADC modalities in clinical development. Illustrations of ADCs represent various forms of conjugation, such as lysine conjugation (A, B), endogenous cysteine conjugation (C), and site-specific conjugations (D, E, F). Also shown are structures of linker and payload of the approved ADCs (A, B, C), and drug-linkers of other ADCs undergoing clinical investigation (D, E, F). Figure reproduced from Beck, et.al. (2017).\textsuperscript{34}
1.2.2 Structural Attributes of Cysteine-Linked Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) with drug-linkers conjugated at interchain cysteine residues are produced by partial reduction of the interchain disulfides, followed by a covalent attachment of the drug-linker to the reduced cysteines. The unreacted drug-linker is quenched with a free cysteine compound, such as N-acetyl L-cysteine. The last step is the ultrafiltration/diafiltration (UF/DF) of the solution to remove the impurities and formulate the ADC in the desired buffer and concentration. A generic cysteine-linked ADC production scheme is shown in Figure 1.8.

Since the release of cysteines is carried out via chemical reduction using a low concentration of reducing reagent, typically DTT or TCEP, this type of reaction generates a heterogeneous mixture of ADC molecules, or drug-loaded species, conjugated with 0 – 8 drugs per IgG1 mAb or 0 – 12 drugs per IgG2 mAb. The possible distribution of drug-loaded species reflects the number of interchain
disulfide bonds in the IgG1 or IgG2 mAb, and intrachain disulfide bonds are undisturbed by this process since the most labile disulfide bonds are the interchain disulfides.\textsuperscript{55} Depending on whether the conjugation occurs on the hinge or the Fab interchain cysteines, diversity of conjugation positional isoforms is also possible. Figure 1.9 shows a simplified collection of possible drug-loaded species and conjugation positional isomers for IgG1 and IgG2 ADCs. The possible combinations of hinge conjugation sites are not shown in this figure.

The primary conjugation isoforms differ for IgG1 and IgG2 ADCs, and correlate with the rank order of reduction susceptibility of the interchain disulfides.\textsuperscript{55, 57} The first conjugation sites to fill up in IgG1s are the Fab cysteines, whereas hinge
cysteines are the first targets for IgG2s comprised of a distribution of A, A/B, and B isoforms. Studies performed using IgG1 ADCs have shown that the distribution of conjugated species and their conjugation positional isomers have an impact on the drug product’s bioactivity and in vivo stability. Similar experiments using IgG2 ADCs have not been reported, but it is reasonable to assume that conjugation isomers could also impact the biological function of IgG2 ADCs.

In-depth characterization of the drug substance and drug product is an important aspect during clinical development of therapeutic candidates, as deeper understanding of molecular attributes enhance the quality and batch consistency of the drug product. Recent analytical improvements present new approaches for the detailed structural analysis of ADCs, underscoring the importance of using modern techniques to better characterize the heterogeneity of cysteine-linked ADCs. However, most of the physicochemical characterization of cysteine-linked ADCs pertain to the IgG1 framework and may not necessarily apply to IgG2 ADCS. The increased structural variability of IgG2 ADCs influenced by drug-loading, conjugation positional isomers, and disulfide bond isoforms multiplies the complexity for the in-depth characterization of these molecules compared to the IgG1 counterparts. Additional contributors to the complexity of IgG2 ADC characterization are the presence of hinge trisulfide bonds in some IgG2 mAbs and the higher concentration of endogenous unpaired cysteines.
<table>
<thead>
<tr>
<th>DAR</th>
<th>IgG1 and IgG2 positional isomers</th>
<th>Expected fragments (intact condition)</th>
<th>Expected fragments (reduced condition)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td><img src="image1.png" alt="Diagram" /></td>
<td>mAb</td>
<td>L0 H0</td>
</tr>
<tr>
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<td><img src="image2.png" alt="Diagram" /></td>
<td>mAb(hinge) HHL L</td>
<td>L0 L1 H0 H1</td>
</tr>
<tr>
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<td>HHL HH HL L</td>
<td>L0 L1 H1 H2</td>
</tr>
<tr>
<td>6</td>
<td><img src="image4.png" alt="Diagram" /></td>
<td>HH HL H L</td>
<td>L0 L1 H2 H3</td>
</tr>
<tr>
<td>8</td>
<td><img src="image5.png" alt="Diagram" /></td>
<td>HHL H L</td>
<td>L0 (IgG2) L1 H3 H4 (IgG2)</td>
</tr>
<tr>
<td>10</td>
<td><img src="image6.png" alt="Diagram" /></td>
<td>HHL H L</td>
<td>L0 L1 H4 H5</td>
</tr>
<tr>
<td>12</td>
<td><img src="image7.png" alt="Diagram" /></td>
<td>H L</td>
<td>L1 H5</td>
</tr>
</tbody>
</table>

**Figure 1.9. Possible drug loading and conjugation positional isomers for IgG1 and IgG2 cysteine-linked ADCs.**

Structures of possible IgG1 and IgG2 cysteine-linked ADCs are shown, where the blue stars represent the conjugated drug-linkers at the interchain cysteines, and circles in between the HCs represent unconjugated hinge cysteines. For simplicity, the possible conjugation positional isomers at the hinge cysteines are not shown. Figure reproduced from Wiggins, et.al. (2015).\(^{42}\)
The impacts of each IgG2 disulfide isoform on the ADC conjugation profile remains unclear. The physicochemical attributes imparted by the higher-order structural differences as a result of disulfide connectivity could influence the conjugation positions in cysteine-linked ADCs. Additionally, the biological activity, safety, and off-target cytotoxicity of IgG2 ADCs could be affected by the locations of drug attachment as a function of the disulfide configuration of the parent mAb.\textsuperscript{9, 18, 58-60, 68} Since the sites of conjugation are considered critical quality attributes (CQAs) of ADCs, a precise understanding of the distribution and positions of conjugated drug-linkers are important for the development of effective and stable ADCs. This dissertation research focuses on the impact of disulfide isoforms on the conjugation profile and stability of IgG2 ADCs, and specific aims are outlined in the next chapter.
Chapter 2 - Research Aims

The aim of this dissertation research is to understand how the IgG2 antibody disulfide isoforms influence the conjugation profiles of IgG2 cysteine-linked ADCs. The distribution of the disulfide isoforms in the starting material (mAb intermediate) is influenced by the upstream cell culture conditions and copurify during downstream purification. In the case of cysteine-linked ADCs, the diversity of interchain disulfide configurations may influence the distributions of both the drug-loaded species and their conjugation positional isomers. The impact of each disulfide isoform on the final ADC drug product conjugation profile remains unclear. The model antibody for this thesis research is an anti-CD37 human IgG2κ mAb produced from Chinese hamster ovary (CHO) cells. Thus, the specific aims of this research are:

1. Investigate the influence of the IgG2 antibody interchain disulfide configuration on the drug loading and primary conjugation positions of cysteine-linked ADCs
2. Determine the effect of conjugation reaction pH on the formation of IgG2 ADCs
3. Evaluate the thermodynamic stability of the ADCs as a function of drug loading and mAb disulfide isoform

In addition, work was carried out on localizing the specific conjugated hinge cysteines, and towards understanding the structure of a full-length IgG2 mAb and IgG2-vcMMAE ADC. These additional goals and are discussed in Chapter 6.
Chapter 3 - Materials and Methods

Sections 3.1 – 3.9 of this chapter were published in mAbs, as a Report titled “Influence of disulfide bond isoforms on drug conjugation sites in cysteine-linked IgG2 antibody-drug conjugates,” with Lily Liu-Shin, Adam Fung, Arun Malhotra, and Gayathri Ratnaswamy as co-authors. DOI: 10.1080/19420862.2018.1440165

Section 3.10 of this chapter was submitted for publication in mAbs, as a Report titled “Evidence of disulfide bond scrambling during production of an antibody-drug conjugate,” with Lily Liu-Shin, Adam Fung, Arun Malhotra, and Gayathri Ratnaswamy as co-authors.

Section 3.12 of this chapter was submitted for publication in mAbs, as a Report titled “Rapid characterization of complex IgG2 disulfide-bonded peptides via MALDI-TOF/TOF,” with Anja Resemann, Lily Liu-Shin, Guillaume Tremintin, Arun Malhotra, Adam Fung, Fang Wang, Gayathri Ratnaswamy, and Detlev Suckau as co-authors.

3.1 Materials

Fully human IgG2κ monoclonal antibody was obtained from the Process Sciences and Manufacturing group at Agensys, Inc., an affiliate of Astellas. The IgG2 mAb starting material containing a mixture of A, A/B, and B disulfide isoforms was produced using upstream and downstream protocols established in-house. The antibody was stored at -80 °C as a 25 mg/mL formulated bulk in acetate/sucrose buffer at pH 5.0. High-purity maleimidocaproyl valine-citrulline para-aminobenzoic acid monomethyl auristatin E (mc-vc-PABA-MMAE, or
vcMMAE) was supplied by the Agensys ADC Chemistry group as a 10 mM solution in DMSO. L-cysteine, N-acetyl-L-cysteine, and DMSO were purchased from Sigma Aldrich (Cat #s 168149, A7250, and D2650). Cystamine dihydrochloride (AAB2287314), TRIS base (T395-500), 5 M NaCl solution (BMA51202), glacial acetic acid (A38-212), TCEP-HCl (PI20491), sucrose (S6-12), LC/MS-grade trifluoroacetic acid (TFA, A116-10X1AMP), 0.5 M sodium phosphate monobasic solution (50-843-058), 0.5 M sodium phosphate dibasic solution (50-843-060), ammonium sulfate (A702-500), No-Weigh® dithiothreitol (DTT, PI20291), and LC/MS-grade formic acid (A11710X1-AMP) were all purchased from Fisher Scientific. Guanidine HCl (IC048205-40), disodium EDTA salt (BDH4616-500G), HPLC-grade isopropanol (IPA, BDH20880.400) and LC-MS grade acetonitrile (CH$_3$CN, BDH83640.400) were purchased from VWR. EMD Millipore Amicon Ultra centrifugal devices with 10 kDa MWCO Ultracel membranes were used for buffer exchange, unless otherwise noted. All chromatographic separations were carried out on a Thermo Scientific Ultimate 3000RS UHPLC equipped with a 100 µL split-loop injector, pre-column heater, post-column cooler, and a diode-array detector (DAD). Chromatography data analyses were performed using Thermo Scientific Chromeleon version 6.8 software.

### 3.2 Enrichment of A and B Isoforms from Starting Material via Reduction-Oxidation of Interchain Disulfide Bonds

Enrichment for either the IgG2-A or IgG2-B disulfide isoform was done by modifying conditions previously reported$^{18}$ using the IgG2 starting material containing a mixture of A, A/B, and B disulfide isoforms. For enrichment of the A
and B isoforms, the starting material was diluted to 5 mg/mL in enrichment buffer and transferred into Thermo Scientific Slide-A-Lyzer™ 10K MWCO dialysis cassettes (PI66453). The mAb was incubated in redox enrichment buffer for 5 days at 4 °C, protected from light. The enrichment buffer to promote conversion to the B isoform was comprised of 0.3 M TRIS-HCl, 1.5 mM cystamine dihydrochloride, 9 mM L-cysteine, pH 8.0. The same buffer composition with an additional 1.5 M guanidine-HCl was used as the enrichment buffer for the conversion to the A isoform. The enriched samples were buffer exchanged using Amicon Ultra-15 (UFC901024) into 20 mM sodium acetate, pH 5.0, in preparation for loading onto the CEX column.

### 3.3 Fractionation of A- and B-Enriched Antibody by Cation Exchange High-Pressure Liquid Chromatography (CEX-HPLC)

The enriched IgG2-A and IgG2-B antibodies in acetate buffer were fractionated using cation exchange high-pressure liquid chromatography (CEX-HPLC) on an Ultimate 3000RS UHPLC equipped with an AFC-3000 fraction collector. The redox-enriched samples were loaded onto a Sepax Technologies Antibodix WCX-NP5 column (602NP5-4625), 10 mm ID x 250 mm L, that had been equilibrated with the mobile phase A (MPA) comprised of 20 mM sodium acetate, pH 5.0. Column was set to 40 °C and gradient elution occurred at a flow rate of 0.5 mL/min, from 35% B to 65% B over 14 minutes. The mobile phase B (MPB) was comprised of 20 mM sodium acetate, 450 mM sodium chloride, pH 5.0 + 5% isopropanol. Collected fractions were concentrated and buffer exchanged into the acetate/sucrose formulation buffer at pH 5.0 using Amicon Ultra-15 centrifugal
devices. Fractionated samples were stored frozen at -80°C until conjugation or analysis. The final concentrations were measured using a Thermo Scientific Nanodrop 2000c, determined as 48 mg/mL for the IgG2-A and 23 mg/mL for the IgG2-B antibodies.

### 3.4 Generation of Cysteine-Linked IgG2-vcMMAE Conjugates

The IgG2-A, IgG2-B, and starting IgG2 material were diluted to 5 mg/mL in the reduction/conjugation buffer comprised of 20 mM sodium acetate, 150 mM sodium chloride, 1 mM EDTA, pH 5.3. Partial reduction of the antibody was carried out in an Eppendorf Thermomixer set at 37 °C and 300 RPM via incubation in nanomolar concentrations of TCEP-HCl prepared in the reduction/conjugation buffer. After the 3-hour partial reduction time period, TCEP-HCl was removed using Amicon Ultra-0.5 (UFC5010BK). The partially-reduced samples were adjusted to a minimum concentration of 8% DMSO (v/v) prior to addition of the drug-linker. vcMMAE was added at a theoretical 5-fold molar excess to the amount of reduced cysteines, and allowed to react for 1 hour at 25 °C and 300 RPM. The unreacted vcMMAE was quenched using excess N-acetyl-L-cysteine, and incubated for 30 minutes at room temperature. The antibody-drug conjugate solution was purified of excess reagents via buffer exchange into the acetate/sucrose buffer at pH 5.0.

### 3.5 Reversed-Phase Ultra-High Pressure Liquid Chromatography (RP-UHPLC) for Separation of mAb Disulfide Isoforms

The disulfide isoform distributions of the purified fractions were analyzed under non-reducing conditions using a mildly-denaturing RP-UHPLC method as follows. Five micrograms of sample diluted in formulation buffer to a target between 2.5
and 5.0 mg/mL were injected onto an Agilent Technologies Zorbax RRHD 300SB-C8 (857750-906), 2.1 mm ID x 50 mm L column with a 1.8 µm particle size. The column had been previously equilibrated at 82 °C in 75% MPA (0.1% TFA, 2% IPA, 97.9% water) and 25% MPB (0.1% TFA, 24.9% CH₃CN, 75% IPA). Separation of the antibody disulfide isoforms was achieved at 0.4 mL/min using a multi-step gradient ramping from 25.0% B to 27.5% B over 6 minutes, 27.5% B to 28.0% B over 1 minute, and 28.0% B to 30.0% B over 1 minute. A post-column cooler set to 30 °C was used to cool the solution prior to UV detection at λ = 280 nm.

3.6 Size-Exclusion High-Pressure Liquid Chromatography (SE-HPLC)

Size exclusion analysis utilized a Waters (Milford, MA) XBridge Protein BEH SEC column (7.8 mm ID x 300 mm L), packed with 3.5 µm particles with pore size of 200 Å. Forty-five micrograms of sample in formulation buffer were separated isocratically on the 25 °C column, utilizing a mobile phase comprised of 25 mM sodium phosphate, 175 mM NaCl, pH 6.8 + 15% isopropanol flowing at 0.8 mL/min. Component species were detected using the UV signals at λ₁ = 230 nm, λ₂ = 280 nm, and λ₃ = 248 nm.

3.7 Hydrophobic Interaction Chromatography (HIC)

HIC analysis utilized a Tosoh Bioscience TSKgel Butyl-NPR column (4.6 mm ID x 35 mm L, cat # 14947). The column was equilibrated with 100% MPA, comprised of 25 mM sodium phosphate, 1.1 M ammonium sulfate, pH 6.7. Fifty micrograms of sample containing 2.5 M NaCl were injected onto a 35 °C column,
and eluted using gradient from 0 – 100% MPB (25 mM sodium phosphate, pH 6.7 + 25% IPA) at 0.8 mL/min over 18 minutes. Absorbance was detected at $\lambda_1 = 214$ nm and $\lambda_2 = 280$ nm.

### 3.8 RP-UHPLC Analyses of Non-Reduced and Reduced mAbs and ADCs

Analysis of the partially-reduced mAb intermediate and the ADC in both reduced and non-reduced forms utilized the same Agilent Zorbax RRHD 300SB-C8 column described previously. Gradient elution was carried out with MPA comprised of 0.1% TFA in water and MPB comprised of 0.08% TFA in 90% CH$_3$CN, 10% water. UV absorbances at $\lambda_1 = 280$ nm and $\lambda_2 = 248$ nm were used for peak integration.

The partially-reduced mAb intermediate and the non-reduced ADCs (nrRP-UHPLC) were diluted with equal volumes of 2% formic acid, 50% CH$_3$CN, 48% water. Five micrograms of protein were loaded onto a 75°C column. Separation of the component species was carried out at a flow rate of 0.5 mL/min by ramping up from 30% to 60% B over 14 minutes. Peak identification by matching theoretical to observed masses via LC-MS is described in the next section (3.9 - LC/MS Analysis of Non-Reduced ADCs).

Analyses of the reduced ADC (rRP-UHPLC) were carried out by reducing the remaining interchain disulfide bonds via incubation in 20 mM DTT for 15 minutes at 37°C to release variably conjugated light and heavy chain fragments. The ADC samples were adjusted to pH ~8 using 1 M TRIS-HCl, pH 9 prior to reduction with DTT. Excess reducing reagent was quenched by addition of equal volume of 2%
FA, 50% CH₃CN, 48% water. Seven and a half micrograms of protein were loaded to the column set at 70 °C, and elution used a multi-step gradient at 0.8 mL/min. Initially, 34.5% B was ramped up to 38.0% B over 3 minutes, then to 38.5% B over 2.5 minutes, and finally to 55.0% B over 20 minutes.

Average DAR was calculated by weighting the relative peak areas of each light and heavy chain based on the number of drug linkers attached, as described in the following equation:

\[
DAR = 2 \times \left( \sum_{0}^{1} \frac{\text{Light chain peak area} \times n_{\text{drug}}}{\text{Total light chain peak area}} + \sum_{0}^{5} \frac{\text{Heavy chain peak area} \times n_{\text{drug}}}{\text{Total heavy chain peak area}} \right)
\]

### 3.9 LC/MS Analysis of Non-Reduced ADCs

All reagents used for the LC/MS analysis of the non-reduced ADCs were purchased from Fisher Scientific or VWR at HPLC or LC/MS grade as described in Section 3.1. Waters Acquity I-class UPLC inline with a Waters LCT Premier/XE ToF mass spectrometer were used for the peak identification of the ADC components detected in the nrRP-UHPLC analyses. The gradient and column temperature of the LC method were slightly modified from the nrRP-UHPLC method described in the preceding section (3.8 - RP-UHPLC Analyses of Non-Reduced and Reduced mAbs and ADCs) to adjust for instrument-related peak resolution variability. Fifteen micrograms of fully glycosylated sample were loaded onto the Agilent Technologies Zorbax RRHD 300SB-C8, 2.1 mm ID x 50 mm L column with 1.8 µm particle size. The column was equilibrated at 65 °C with 0.1% TFA in water (mobile phase A) and 0.08% TFA, 90% acetonitrile, 10% water
(mobile phase B) prior to analysis. Gradient elution occurred at 0.5 mL/min, increasing from 28% B to 65% B over 28.5 minutes. The capillary and cone voltages were set to 3500 V and 35 V, respectively. The source and desolvation temperatures were set at 150 °C and 450 °C, respectively. Deconvolution of the full scan ESI mass spectra collected in ES+ mode used the MaxEnt1 algorithm in the Waters MassLynx software.

3.10 Differential Scanning Calorimetry (DSC) of mAbs and ADCs

Samples were buffer exchanged into 20 mM sodium acetate, 5% (w/v) sucrose, pH 5.0 and diluted to 1 mg/mL. A Malvern MicroCal VP-Capillary DSC was used for analysis with a scan rate of 60 °C/h, from 20 °C to 100 °C, and no re-scans were performed. Origin 7 software was used to process the buffer-subtracted thermograms, using the non-2 state model for peak fitting and determination of melting transition temperatures.

3.11 Preparation of Fd, LC, and Fc Fragments from mAbs and ADCs

SpeB and IdeS (FabULOUSTM and FabRICATORTM, Genovis, Lund, Sweden) were used to generate (Fab’)2, Fd, LC, and Fc fragments. Deglycosylation was achieved after overnight incubation of 50 mU peptide-N-glycosidase F (PNGaseF, QA Bio, San Mateo, CA) with the mAb or ADC at 5 mg/mL in formulation buffer adjusted to pH 7.5 with 1 M TRIS, pH 9 (final TRIS concentration ~25 mM). SpeB or IdeS digestion followed via overnight incubation at 37 °C with 1 U of enzyme / 1 µg of protein. Protein digestion with SpeB required co-incubation with 5 mM of TCEP-HCl.
Separation of Fab and Fc fragments utilized the Amicon® Pro Affinity Concentrator kit (EMD Millipore, Bellica, MA) using protein A resin and a 50 kDa MWCO spin concentrator per manufacturer’s instructions. Briefly, 5 mL of sample were loaded onto 200 µL of resin previously equilibrated with D-PBS (Gibco, location) + 150 mM NaCl. The sample was allowed to bind to the resin for 1.5 hours on a shaking platform set to 80 RPM, and the unbound species (Fd, (Fab')2, and LC fragments) were collected after 2 x 500 µL washes with D-PBS + 150 mM NaCl. The bound species (Fc fragment and intact IgG) were subsequently eluted with 500 µL of the elution buffer provided with the kit, neutralized using the buffer provided, and concentrated per the product insert. The sample concentrations were determined via Nanodrop 2000c using D-PBS as the blank solution.

3.12 LC-MALDI-TOF/TOF Analysis of IgG2 Disulfide Bond Isoforms

3.12.1 Collection of IgG2-A, -A/B, and –B Fractions via RP-UHPLC

Fractionation of IgG2 disulfide isoforms utilized a Thermo Scientific Ultimate 3000RS UHPLC equipped with an AFC-3000 fraction collector. The starting material comprised of a mixture of 22% A, 33% A/B, and 45% B isoforms was fractionated using an Agilent Technologies Zorbax RRHD 300SB-C8, 2.1 mm ID x 50 mm L column with a 1.8 µm particle size and 300 Å pore size. The LC conditions were as follows: mobile phase A (MPA) comprised of 0.1% TFA, 2% IPA, 97.9% water and mobile phase B (MPB) comprised of 0.1% TFA, 25% CH₃CN, 74.9% IPA were flowed through the 82 °C column at 0.4 mL/min. The gradient delivered 25.0% - 27.5% MPB over 6 minutes, followed by 27.5% - 28.0% MPB over 1 minute, and finally 28.0 % - 30.0% MPB over 1 minute. Fraction
collection was programmed by time based on the elution time and peak width of each disulfide isoform.

3.12.2 Lys-C/Trypsin Digestion under Non Reducing Conditions

The IgG2 mAb fractions were denatured and free thiol groups alkylated via incubation for 3 h at 37 °C in 5.8 M guanidine-HCl and 7 mM NEM. The samples were purified and buffer exchanged using Amicon Ultra-0.5 centrifugal filters with 10 kDa MWCO. Denatured and alkylated samples were digested overnight at 37 °C with Mass Spec Grade trypsin/Lys-C mix (Promega), at an enzyme-to-substrate ratio of 1:10 in the presence of 0.175 mM NEM in a sodium phosphate dibasic, pH 5.8 buffer (Sigma).

3.12.3 LC Separation and Spotting of Digested Peptides

The peptides derived from trypsin/Lys-C digestion were separated by RP-nanoLC (Ultimate 3000 RSLCnano LC system, ThermoFisher Scientific). A Thermo Scientific Acclaim PepMap RSLC C18 column (15 cm x 300 μm, 2 μm particle size, 100 Å pore size) was equilibrated with 100% MPA (water with 0.1% TFA) flowing at 4 μL/min. A gradient from 0% to 45 % B over 30 min was applied using MPB comprised of 0.1% TFA, 90% CH₃CN, 9.9% water.

Fifteen second fractions were collected by the PROTEINEER fc II LC-MALDI fraction collector (Bruker) on MTP AnchorChip sample plates (Bruker) with hydrophilic anchors as follows. In an initial screen for the detection of all DSB-peptides containing one SS-bond (therefore, not the hinge peptides), fractions were spotted on 800 μm anchors with a sheath flow of 0.5 μL per spot of a prepared HCCA solution consisting of 36 μL saturated HCCA (in 90% CH₃CN/10 % water),
mixed with 748 µL of 85 % acetonitrile/15 % water, 8 µL of 100 mM ammonium phosphate, and 8 µL of 10 % TFA in water. Calibrant spots were prepared with a mixture of synthetic peptides (peptide calibration standard II, Bruker) plus bovine Insulin, (Sigma) and matrix to cover a calibration range from 700 to 7000 Da allowing for external calibration with <10 ppm mass accuracy.

For the detection of hinge peptides greater than 7 kDa, the fractions were spotted manually on the 2 mm anchors of a MTP BigAnchor 384 target (Bruker), in 0.5 µL of sDHB matrix solution consisting of 50 mg sDHB (Bruker) dissolved in 0.1 % TFA in 50% water/50% CH$_3$CN. Proper crystallization of the matrix was verified visually.

3.12.4 Mass Spectrometry

Data acquisition and processing were generated and processed using rapifleX-TOF/TOF using compass for flex series 2.0 with standard methods. Spectra were acquired at positive reflector ion mode with acceleration voltage of 20 kV. Eight thousand laser shots were accumulated per fraction in MS mode at a laser shot rate of 5 kHz, and 5000 shots were accumulated for every MS/MS spectrum with same laser frequency. LC-MALDI datasets were imported in BioPharma Compass 2.0 (BPC 2.0) and peptides identified using in silico digestion of the sequences of the IgG2 LC and HC. The collection of identified peptides was then further processed in DisulfideDetect 1.2.2 (Bruker) to automatic identification and quantification of the DSB peptides in the LC-MALDI dataset.

Multiply disulfide-bonded peptides required manually matching the MS/MS fragments to the theoretical MWs of hinge peptides with up to 2 missed cleavages.
Fractions containing mass spectral peaks matching the theoretical hinge peptide masses of the B form were interrogated again for mis-cleaved proteolytic digest products or possible reduced forms of the hinge peptide. MS/MS spectra from all possible precursors were matched with their theoretical structures by annotating typical MALDI-CID fragment patterns of disulfide-bonded peptides. This approach allowed the identification of large DSB-peptides that did not achieve full sequence coverage by CID.

3.13 IgG2 mAb Crystallization Screening

IgG2-B antibody was prepared at 9.2 mg/mL in 20 m sodium acetate, 5% (w/v) sucrose, pH 5.0, or at 24 mg/mL in 20 mM sodium acetate, 150 mM NaCl, 5 mM Na2-EDTA, pH 5.3. Crystallization buffer screening was performed using hanging drop vapor diffusion on greased 24-well plates. The reservoir was filled with 600 µL of buffer and a coverslide with a 2 µL drop (1 µL sample + 1 µL reagent) was sealed above the reservoir. Hampton Research (Aliso Viejo, CA) crystallization screening kits used were Index (HR2-144), Grid Screen™ Ammonium Sulfate (HR2-211), Grid Screen™ PEG 6000 (HR2-213), Crystal Screen (HR2-110), and Crystal Screen 2 (HR2-112).

3.14 Homology Modeling of an IgG2 Antibody

Homology modeling used the Antibody Homology Modeling function in Schrödinger Bioluminate 2016 as follows. Two models were generated using a full-length human IgG1 structure (PDB ID: 1HZH) and full-length human IgG4 structure (PDB ID: 5DK3) as templates. The heavy and light chains were
independently modelled and energy-minimized by applying the OPLS-2005 force field. The full IgG2 model was assembled in the A configuration, by first automatically creating the disulfide bonds using the Protein Preparation Wizard, followed by loop refinement of the hinge loops. The interchain disulfide bonds required manual assembly, and local loop refinement and full-molecule energy minimization was applied after each bond was created.

As the sequence identity was lower when using 1HZH as template (77% for heavy chain, ~80% for light chain), the (Fab’)2 region was first modelled using PDB ID 4HAF as template (>90% sequence identity), as previously described. The Fab was assembled to the IgG2 Fc region modelled using 1HZH as template. Energy minimization was then applied to the full molecule.
4.1 Introductory Remarks

The contents of this chapter were published in mAbs, as a Report titled "Influence of disulfide bond isoforms on drug conjugation sites in cysteine-linked IgG2 antibody-drug conjugates," with Lily Liu-Shin, Adam Fung, Arun Malhotra, and Gayathri Ratnaswamy as co-authors. DOI: 10.1080/19420862.2018.1440165

In-depth characterization of the drug substance and drug product is an important aspect during clinical development of therapeutic candidates, as deeper understanding of molecular attributes enhance the quality and batch consistency of the drug product. Recent analytical improvements have allowed the optimization of tandem and novel mass spectrometry methods developed specifically for detailed structural analysis of ADCs. Nevertheless, limited structural and biophysical information is available regarding IgG2 ADCs because these molecules have not been a popular choice for clinical development. This gap in the knowledge of the characteristics of an IgG2 ADC poses a challenge to the design of better ADC therapeutics with enhanced drug product homogeneity, stability, efficacy, and improved therapeutic window. The aim of this investigation is to understand how the disulfide isoforms of IgG2 mAbs influence the site(s) of drug attachment in cysteine-linked ADCs, and determine whether the disulfide isoform distribution should be controlled as a critical attribute of the mAb intermediate.
Characterization of IgG2 mAbs and ADCs is more challenging when compared to their IgG1 counterparts. The complexity of IgG2 ADCs results from the multiple configurations of the mAb interchain disulfide bonds (Figure 1.3). In addition to a higher possible number of drug-linkers conjugated per mAb compared to an IgG1, the increased number of interchain disulfide bonds in an IgG2 mAb also equates to a larger number of conjugation positional isoforms. Moreover, some IgG2 mAbs potentially contain more trisulfides in the hinge region than IgG1s, which can affect the quality attributes of cysteine-linked ADCs. To our knowledge, the effect of the mAb disulfide isoforms on the conjugation profile has not been previously evaluated.

For this study, we generated and evaluated the ADCs derived from IgG2-A and IgG2-B mAbs produced under the same reaction conditions. The first reaction step involved the release of cysteines from interchain disulfide bonds via partial reduction using low concentrations of tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), followed by conjugation of the cysteines with molar excess of maleimidocaproyl valine-citrulline para-aminobenzoic acid monomethyl auristatin E (vcMMAE). The partially reduced mAbs and the corresponding ADCs were characterized with respect to distribution and locations of conjugated drug-linkers.

### 4.2 Enrichment and Purification of IgG2-A and IgG2-B Antibodies

The starting material used for this study was a CHO-derived fully-human IgG2 mAb, containing 22% IgG2-A, 33% IgG2-A/B, and 45% IgG2-B (Table 4.1). Enrichment for either the IgG2-A or IgG2-B isoform was achieved by first shuffling the disulfide bonds under redox conditions in a buffer containing L-cysteine and
cystamine dihydrochloride at pH 8.0, modified from previously published procedures.\textsuperscript{18-19} Conditions that promote rearrangement of disulfide bonds into the A isoform required the addition of 1.5 M guanidine-HCl to the redox buffer, consistent with previous reports.\textsuperscript{18-19}

<table>
<thead>
<tr>
<th>Disulfide Isoform\textsuperscript{a}</th>
<th>Starting Material\textsuperscript{b}</th>
<th>Enriched for IgG2-A</th>
<th>Enriched for IgG2-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + A'</td>
<td>22%</td>
<td>Redox only\textsuperscript{c}</td>
<td>Redox + CEX\textsuperscript{d}</td>
</tr>
<tr>
<td>A/B</td>
<td>33%</td>
<td>65%</td>
<td>100%</td>
</tr>
<tr>
<td>B</td>
<td>45%</td>
<td>26%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 4.1. Disulfide isoform distribution of the IgG2 mAb preparations
\textsuperscript{a}Distribution of disulfide isoforms were determined via nrRP-UHPLC. \textsuperscript{b}Initial IgG2 mAb sample (“starting material”) is comprised of a mixture of A, A/B, and B disulfide isoforms. \textsuperscript{c}Distribution of disulfide isoforms after dialysis in redox buffer for 5 days at 4 °C, protected from light. \textsuperscript{d}Distribution of disulfide isoforms of redox-enriched A and B mAb further purified via CEX-HPLC fractionation.

Levels of the desired mAb isoforms after incubation in redox buffer increased to 65 – 70%, and cation exchange high-pressure liquid chromatography (CEX-HPLC) was used to fractionate each isoform.\textsuperscript{19, 70} The disulfide isoforms were resolved on a weak cation exchange (WCX) analytical column using a sodium acetate/NaCl mobile phase system optimized for this IgG. Both IgG2-A and IgG2-B mAbs were 100% pure after CEX fractionation as determined by a non-reduced reversed-phase ultra-high pressure liquid chromatography (nrRP-UHPLC) method that resolves disulfide isoforms (Figure 4.1). The nrRP-UHPLC method utilizes a TFA/IPA/CH\textsubscript{3}CN gradient and provides better resolution than CEX-HPLC. However, the severe conditions of the nrRP-UHPLC assay irreversibly denatures the mAb, which precluded its use for fractionation purposes. The IgG2-B elutes
first by nrRP-UHPLC, followed by the A/B and A isoforms. The IgG2-A isoform is further resolved as two species using this method, and were assigned as the A and A' species. The A' species has the same disulfide configuration as the A-form, but contains a hinge disulfide that is protected from reduction.

Figure 4.1. Disulfide isoform distributions of starting IgG2 mAb and A- or B-isoform enriched material.
Disulfide isoform distribution was analyzed via nrRP-UHPLC and shown are chromatograms of the starting material (A), redox-enriched A and B isoforms (B), and CEX-fractionated IgG2-A and IgG2-B mAbs (C).

4.3 Susceptibility of Interchain Disulfide Bond Reduction Based on Antibody Isoform

Partial reduction of the mAb is the first step in the preparation of ADCs conjugated at the cysteines involved in interchain disulfide bonding. The starting material, purified IgG2-A, and purified IgG2-B mAbs were partially reduced at pH 5.3 with TCEP-HCl:mAb molar ratios ranging from 4:1 – 46:1. Typical partial reduction and drug-linker conjugation reactions involved in the production of cysteine-linked ADCs use buffers with pH ≥ 7, and normally generate two moles of reactive thiol per mole of reducing reagent. Because IgG2 disulfide bonds can rearrange in alkaline or reducing environments, the partial reduction conditions were optimized at pH 5.3 to control for disulfide scrambling. However, preliminary experiments determined that the reduction kinetics of the interchain
disulfide bonds are slower at pH 5.3 (data not shown), thereby requiring higher molar equivalents of TCEP-HCl:mAb to tune the generation of ADCs with higher average DAR values. Control experiments were also performed using typical ADC manufacturing parameters at a higher pH, and the results are discussed in the next chapter.

We monitored the extent of interchain disulfide reduction using via RP-UHPLC analyses of the partially-reduced mAbs. The chromatographic conditions for this method were different than those used for the separation of disulfide isoforms. The TFA/CH₃CN gradient used in this analysis provides baseline resolution of the peaks corresponding to the intact mAb and light chain, but does not resolve the mAb disulfide isoforms (Figure 4.2). The HC and other covalently-associated antibody fragments partially co-elute with the intact mAb peak, and the increasing amplitude of these peaks correspond to higher amount of interchain disulfide reduction.

Increased levels of antibody fragments and free LC with a concomitant decrease of the intact mAb peak were observed for the IgG2-A when compared to the IgG2-B and the starting material treated with the same amount of reducing reagent. Focusing specifically on the extent of LC-HC bond reduction, higher percent of free LC was generated after partial reduction of the IgG2-A antibody. The amount of free LC increased linearly with the concentration of TCEP-HCl, topping at 16% for the IgG2-A mAb and 4% for the IgG2-B mAb. With respect to the B isoform, our analysis of the partially-reduced IgG2-B mAb showed a higher
amount of intact mAb and other covalently associated species in the samples treated with higher TCEP-HCl:mAb ratios.

![Diagram](image)

**Figure 4.2. Overlay of the nrRP-UHPLC chromatograms of the partially-reduced and quantified % free LC in IgG2-A, IgG2-B, and IgG2 starting material.**

Top panels show the nrRP-UHPLC chromatograms of IgG2-A (top), IgG2-B (middle), and IgG2 starting material comprised of a mixture of A, A/B, and B isoforms (bottom) partially-reduced using TCEP-HCl:mAb ratios of 4 (A), 24 (B), and 46 (C). Bottom plot shows the percent of LC quantified by nrRP-UHPLC analysis of the partially-reduced mAb as a function of TCEP-HCl:mAb ratio (D). Closed circles (●) are the IgG2-A, open circles (○) are the IgG2-B, and filled triangles (▲) are the non-enriched IgG2 starting material.

The control for this experiment was the starting material containing a mixture of A, B, and A/B isoforms. The chromatographic profiles and percent free LC of the starting material align in between the results observed for the purified A and B isoforms. The higher similarity of the starting material to the B isoform reflects the
distribution of disulfide isoforms of the starting material, where 39% of Fab arms are in the A configuration and 61% in the B configuration.

Size variant distributions of the partially-reduced mAbs were evaluated under non-denaturing conditions and found to be mostly in monomeric form. The combined amounts of high and low molecular weight species (HMWS and LMWS, respectively) were less than 2% across the range of TCEP-HCl:mAb ratios (Figure 4.3).

![Figure 4.3. Percent LMWS and % HMWS detected by SE-HPLC.](image)

% LMWS and % HMWS as a function of TCEP-HCl:mAb are plotted for mAb (A, B) and ADC (C, D). The IgG2-A isoforms are depicted in closed circles (●), and IgG2-B isoforms in open circles (○).

4.4 Characterization of Conjugate Distribution of IgG2-A and IgG2-B ADCs

The ADCs were produced by conjugating vcMMAE to the partially-reduced mAbs using the same reagent stoichiometries and reaction conditions for both A and B isoforms. To highlight the influences conferred by the mAb disulfide configuration on the ADCs, we selected TCEP-HCl:mAb molar equivalents of 4, 24, and 46 to represent conditions necessary to target relatively low, intermediate,
and high average DARs. We characterized the distribution of conjugated species in the resulting ADC samples under intact, reduced, and denaturing conditions. The ADCs were determined to be >97% monomeric for the TCEP-HCl range evaluated (Figure 4.3). While an increase in LMWS was observed for the IgG2-A ADC in comparison to its mAb counterparts, the highest relative percent of LMWS detected was only 1.6% for the ADC.

Figure 4.4. SE-HPLC chromatograms of IgG2-A and IgG2-B ADCs. ADCs generated from partial reduction using TCEP-HCl:mAb ratios of 4 (A, D), 24 (B, E), and 46 (C, F). The largest peak in the chromatogram is the monomer, and peaks eluting before and after the monomer are the HMWS and LMWS, respectively. The insets show the SE-HPLC profiles zoomed in on the x- and y-axes to show the peak detail of the HMWS and LMWS.
A characteristic that was unique to the IgG2-A ADCs was the broadening of the monomer peak at the higher TCEP-HCl:mAb ratios (Figure 4.4). Broad peaks in the SE-HPLC analysis could be due to the presence of multiple species with similar hydrodynamic radii or due to secondary interactions between the column packing material and the analyte, but was not characterized further to support this study.

Conjugate distributions and average DARs are CQAs for the ADC, and can be characterized by a combination of chromatographic analyses of both the intact and reduced molecules. The distributions of conjugated species were evaluated by hydrophobic interaction chromatography (HIC), where the ADCs elute in order of the increasing number of conjugated drug-linkers per mAb in a gradient of decreasing ammonium sulfate and increasing isopropanol. The relative abundance of ADCs conjugated with two (D2) and four drug-linkers (D4) per antibody at the low TCEP-HCl:mAb level is similar between the two isoforms, although the IgG2-B sample contained a higher amount of unconjugated mAb (Figure 4.5). The differences between ADCs produced from A and B isoforms become more apparent at the intermediate TCEP-HCl:mAb condition, and grow further accentuated at the high conjugation level. The multiply conjugated D4, D6, and D8 molecules are the predominant species in the IgG2-A ADC derived from 46 molar equivalents of TCEP-HCl, and contrast with D2 and D4 being the major conjugated species for the B isoform produced under the same conditions. D10 and D12 species were not detected by HIC, and is attributed to a limitation of the sample preparation for the HIC analysis where those species irreversibly precipitate when diluted with sample diluent containing NaCl. Although
particulates were not observed upon visual inspection, low protein recovery was a problem during the analyses of the highly conjugated ADCs, particularly for the purified IgG2-A isoform. Nonetheless, the non-covalently associated fragments related to D8, D10, and D12 were detected under denaturing assay conditions of non-reduced ADCs (nrRP-UHPLC).

![Figure 4.5. Hydrophobic interaction chromatograms and distribution of the drug-loaded species for the IgG2-A and IgG2-B ADCs. Shown are ADCs generated with low, intermediate, and high conjugation levels. Unconjugated mAb (D0) is filled with diagonal lines and the various conjugated species are filled with solid colors.](image)

The relative peak areas integrated from the HIC profile are widely used for the calculation of average DAR in IgG1 cysteine-linked ADCs. In the case of the IgG2 ADCs, however, the poor resolution of the D8, D10, and D12 species precludes accurate integration and leads to errors in the average DAR calculation. Recent
advances in analytical technologies have provided alternate methods for quantifying drug loading based on mass, such as via coupling two-dimensional LC with mass spectrometry or by determination of the concentration of drug-linker after enzymatic deconjugation. Instead, we used the distributions of conjugated light and heavy chains derived from the RP-UHPLC analysis of the reduced ADCs (rRP-UHPLC) to calculate the average DAR values, where species elute according to differences in hydrophobicity between light chain and heavy chain and the number of drug-linkers conjugated. For an IgG2 ADC, the expected species are unconjugated light chain (L0), LC conjugated with one drug-linker (L1), unconjugated heavy chain (H0), and HC conjugated with one to five drug-linker molecules (H1 – H5).

Figure 4.6. Average DAR as a function of TCEP-HCl:mAb ratio.
IgG2-A ADCs are represented by closed circles (●) and IgG2-B ADCs by open circles (○).

Average DAR was plotted as a function of the TCEP-HCl:mAb ratio to compare the conjugation levels of the A and B isoforms (Figure 4.6). Both IgG2-A and IgG2-B ADCs had similar average DARs when reduced with low (4) TCEP-HCl equivalents, calculated to be 0.8 and 0.5 average DAR, respectively. The average DAR vs. TCEP-HCl:mAb curves of the A and B isoforms diverged as the concentration of TCEP-HCl increased, with the IgG2-A showing higher drug
loading than the IgG2-B ADCs. This corresponded to 1.9 drug-linkers per mAb for every 10 TCEP-HCl molar equivalents for the A isoform, and 1.2 drug-linkers per mAb for the B isoform. The average DARs of the IgG2-A ADCs were calculated as 4.0 and 6.3 at the intermediate and high conjugation conditions, in contrast to 2.5 and 4.2 for the IgG2-B ADCs produced under the same conditions.

Figure 4.7. Reduced RP-UHPLC chromatograms and distribution of the reduced species for the IgG2-A and IgG2-B ADCs. Shown are ADCs generated with low, intermediate, and high conjugation levels. Light chains are shaded orange and heavy chains are shaded blue. The unconjugated species (L0 and H0) are filled with diagonal lines and conjugated species (L1, H1, H2, H3, H4, and H5) are filled with solid colors.

The distributions of conjugated heavy chains were significantly influenced by the mAb disulfide isoform and correlated with the average DAR values (Figure 4.7). Higher levels of HC conjugated with three or more drug-linkers (H3, H4, H5) were
detected in the IgG2-A ADC at the intermediate and high TCEP-HCl:mAb levels. In the case of the IgG2-B ADCs, heavy chains conjugated with zero, one, or two drug-linkers (H0, H1, H2) were the predominant species when generated using the same reaction conditions. Notably, very low amount of conjugation to the light chain was detected at 0 – 5%, depending on the TCEP-HCl:mAb levels. An interesting observation from the highly-conjugated IgG2-A showed that this sample was comprised of 17% H0, compared to 12% H0 in the B isoform.

4.5 Conjugation Positional Isomer Distributions of IgG2-A and IgG2-B ADCs

The preceding experiments showed prevalent reduction/conjugation at the HC for both disulfide isoforms. We therefore elucidated the main conjugation positional isomers of the D2, D4, D6, and D8 species. The conjugation sites were assigned to cysteines in the Fab or the hinge regions by reconstructing the ADCs based on the species detected under denaturing conditions. Peaks detected via nrRP-UHPLC were identified by matching the observed masses obtained by LC/MS-ESI-TOF to the theoretical masses, as listed in Table 4.2.

The collection of possible component species associated with each conjugated variant are cataloged in Figure 4.8. For example, a D2 molecule conjugated at the LC-HC interchain cysteines on one Fab arm would result in the detection of a conjugated light chain (L1) and a heavy-heavy-light chain fragment with one drug ((HH)1L0). nrRP-UHPLC profiles of IgG2-A and IgG2-B ADCs with average DARs of ~2.5 and ~4.0 are shown in Figure 4.9 to represent the differences observed in distribution of conjugated species as a function of average DAR and mAb disulfide
configuration. The primary conjugation positional isomers reconstructed from the data are illustrated in Figure 4.10.

<table>
<thead>
<tr>
<th>Peak identification</th>
<th>Calculated mass$^a$</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>L0</td>
<td>23231.9</td>
<td>23234.3</td>
</tr>
<tr>
<td>D0</td>
<td>146034.9</td>
<td>146056.8</td>
</tr>
<tr>
<td>L1</td>
<td>24548.4</td>
<td>24550.6</td>
</tr>
<tr>
<td>(HH)0L0</td>
<td>122823.0</td>
<td>122821.0</td>
</tr>
<tr>
<td>D2</td>
<td>148667.9</td>
<td>148684.3</td>
</tr>
<tr>
<td>(HH)1L0</td>
<td>124139.5</td>
<td>124137.2</td>
</tr>
<tr>
<td>(HH)2L0</td>
<td>125456.0</td>
<td>125458.9</td>
</tr>
<tr>
<td>D4</td>
<td>151300.9</td>
<td>151318.7</td>
</tr>
<tr>
<td>(HH)5L0</td>
<td>129405.5</td>
<td>129404.9</td>
</tr>
<tr>
<td>H4L0</td>
<td>78287.5</td>
<td>78292.2</td>
</tr>
<tr>
<td>H3</td>
<td>53741.1</td>
<td>53743.2</td>
</tr>
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<td>H3L0</td>
<td>76971.0</td>
<td>76976.5</td>
</tr>
<tr>
<td>(HH)8</td>
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<td>110121.7</td>
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<tr>
<td>D8</td>
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<tr>
<td>H5</td>
<td>56374.1</td>
<td>56380.5</td>
</tr>
<tr>
<td>(HH)10</td>
<td>112730.1</td>
<td>112762.0</td>
</tr>
</tbody>
</table>

Table 4.2. List of observed masses in ADC peaks detected by nrRP-UHPLC

$^a$Calculated masses assume glycosylation with G0F/G0F, all intrachain and interchain disulfide bonds where applicable, heavy chain N-terminal pE formation, and heavy chain C-terminal K truncation.

Intact D2 and D4 were detected as the predominant conjugation isomers in both A and B isoforms. However, unique peak shapes and retention times were observed for the conjugates derived from each disulfide isoform. Referring to Figure 4.8, detection of intact D2 and D4 species reveal that the drug-linkers are attached at the hinge cysteines, although we were unable to pinpoint the exact conjugated hinge cysteines using this approach. A noteworthy difference between the two isoforms is that the D2 species in the IgG2-B ADCs was observed as a peak doublet, and LC/MS data showed a 2 Dalton (Da) mass difference between the peaks. Fab conjugations were detected at minor levels in component species...
derived from D2 and D4 of IgG2-A ADCs. The higher peak amplitudes of L1, (HH)0L0, (HH)1L0, and (HH)2L0 species in the IgG2-A ADCs provide further evidence of conjugation at the LC-HC cysteines. Also unique to the A isoform ADCs was the detection of a 23.8 kDa fragment co-eluting with the L1 peak. This fragment was not characterized, but it could be derived from in-source fragmentation of the drug-linker of the singly conjugated LC or from peptide bond hydrolysis of the HC.79-80

Figure 4.8. Possible component species for IgG2 ADC conjugation positional isomers analyzed under denaturing conditions.
For simplicity, only cartoon representations of the A isoform ADCs are shown. The hinge conjugations are shown for illustrative purposes and do not represent the specific hinge cysteine that was reduced and conjugated. The black lines represent interchain disulfide bonds and the blue starts represent the drug-linker conjugated at an interchain cysteine.
Figure 4.9. nrRP-UHPLC chromatograms comparing ADCs with average DAR ~2.5 and average DAR ~ 4.0 conjugated from IgG2-A and IgG2-B.

Peak codes listed in the figure were identified by matching observed molecular weights (MW) by LC-MS to the calculated MW of the expected component species as listed in Table 4.2. Cartoon representations of the component species are shown in Figure 4.8.

Component species that correspond to D6 in both A and B isoforms were detected primarily as (HH)5L0, which is the HHL fragment with 5 drug-linkers distributed on the heavy chains. This component species was observed as a peak doublet with equivalent masses, and potentially represents positional isomers of (HH)5L0. The A isoform ADCs showed higher abundance of the later eluting (HH)5L0 peak in the doublet pair, whereas the IgG2-B ADCs contained higher abundance of the earlier eluting peak. Reconstruction of the D6 molecule shows that the primary isomer contains four HC conjugations and two Fab conjugations. No intact D6 was detected, but baseline amounts of H3L0 were detected in both isoforms.

The main component species corresponding to D8 was H4L0, indicating that the hinge-conjugated D8 is the prevalent isomer. Constituents reflecting D8 with Fab conjugations (i.e., (HH)7L0 and (HH)6) were detected at baseline levels in the
highly conjugated ADCs derived from both isomers (data not shown). Components pertaining to D10 and D12 were identified as L1, H5, (HH)8, and (HH)10. As discussed earlier, D10 and D12 were not detected via HIC due to their higher propensity to salt-out during sample preparation. We were unable to determine the main positional isomer of D10 using the current approach due to the low abundance of its component species.

Curiously, intact D8 and (HH)10 peaks were only detected in the IgG2-B ADCs. These species were not expected under the denaturing assay conditions, as all interchain disulfides have been disrupted. It is possible that either species was formed via conjugation to cysteines involved in both interchain and intrachain disulfide bonding, but no orthogonal evidence supported reduction of intrachain disulfide bonds.

Figure 4.10. Main conjugation positional isomers detected IgG2 ADCs via nrRP-UHPLC analysis.
Two possible pathways for the formation of D6 to D10 species are shown. Only the IgG2-A isoform is depicted for clarity, where the black lines indicate disulfide bonds and the blue stars represent drug-linkers attached at cysteines. The “SH” labels indicate reduced interchain cysteines that are not conjugated.
4.6 Discussion

Most ADCs in clinical development utilize the IgG1 framework, and previous studies have shown that physicochemical attributes may differ between IgG1 and IgG2 cysteine-linked ADCs.\textsuperscript{34, 42-43} Therefore, characterization knowledge gathered using IgG1 ADCs as the model may not translate to the IgG2 ADCs. One critical difference between the IgG subclasses is the presence of two additional hinge disulfide bonds in the IgG2 antibodies, which allow for a distribution of 0 – 12 drug-linkers. Also unique to the IgG2s is the existence of antibody disulfide isoforms, and their impact on the generation of ADCs was previously unknown. In this study, we characterized the partially reduced mAbs and ADCs derived from pure IgG2-A and IgG2-B isoforms.

Consistent differences in retention times of the partially reduced antibodies and the intact ADCs from the purified isoforms suggest that no disulfide scrambling occurred during the reduction step under the conditions used in this study. Results obtained from the material containing a mixture of disulfide isoforms also supports this inference. Data acquired from the partially reduced mAbs also showed that the reduction kinetics is influenced by the disulfide configuration, with IgG2-A mAbs revealing a higher rate of reduction than the B isoform. This result indicates that IgG2-B hinge disulfides are more solvent-protected, which is in agreement with the closer Fab/C\textsubscript{H}2 interdomain interactions reported for this isoform as measured by hydrogen-deuterium exchange mass spectrometry (HDX-MS) and electron microscopy (EM).\textsuperscript{81-82} Additional evidence was observed as the hinge-conjugated D8 and (HH)10 species in the IgG2-B ADCs analyzed under denaturing
conditions. Though all hinge disulfides have been disrupted by conjugation, the HCs likely remained associated under denaturing conditions due to the strong non-covalent interactions between the Fab arms and the C\(_\text{H2}\) domains in the conformation imparted by the B configuration. In contrast, H4L0 and H5 fragments were detected in the IgG2-A samples, which indicates weaker Fab-C\(_\text{H2}\) interactions.\(^8^1\) Future improvements to mass spectrometric applications for disulfide mapping in heterogeneous samples should allow for a straightforward examination of disulfide configurations in ADCs.

The prevalence of the mAb peak and other covalently associated fragments (e.g., HHL and HH) point towards the hinge disulfides being the main targets for reduction in IgG2 antibodies. As disulfide isoforms differ in the bonding patterns to the hinge cysteines, each of the four hinge disulfides may exhibit differences in the rank order of vulnerability to reduction based on their connectivity. The influence of the disulfide bonding pattern on the sequence of hinge disulfide bond reduction is currently being investigated. We considered the possibility that the preferential reduction and conjugation sites for the A isoform could be the cysteines involved in the LC-HC interchain disulfide bond, given that the IgG2-A configuration is similar to an IgG1.\(^5^5,\,^5^7\) Comparison of the starting material and purified IgG2-A and IgG2-B mAbs showed that higher amounts of free LC were generated after partial reduction of the IgG2-A antibody. However, the absolute amount of free LC is relatively low in the A isoform, implying that the LC-HC disulfide is not the main target of reduction. One explanation could be attributed to the position of the C\(_\text{H1}\) cysteine involved in interchain disulfide bonding, which
resides in the middle of the Fab rather than proximal to the hinge region as is the case for the IgG1 isotype.⁹, ⁸³

Our characterization of the ADCs showed that a higher rate of disulfide bond reduction correlates with a higher average DAR. Analysis of the intact and reduced ADCs via HIC and RP-UHPLC shows that highly conjugated species were more prevalent in the A isoform. Hence, unconjugated mAb and lesser-conjugated species were the major components in the IgG2-B ADCs. However, an interesting result was observed in the highly conjugated ADCs, where the A isoform contained more unconjugated HC (H0) than the B isoform. While we did not further characterize the H0 sub-population in the IgG2-A, we speculated that perhaps the presence of the A’ isoform with a protected hinge disulfide could play a role in this instance.⁷¹, ⁸⁴

The preference for hinge conjugation is consistent with the observations from the partially reduced mAb analysis. We can therefore infer that the release of reduction of disulfide bonds determines the locations of drug-linker conjugation. Examination of the major conjugation isomers of each variably conjugated ADC allowed us to discern the regional progression of disulfide reduction. Our approach directly analyzed the heterogeneous DAR mixture under denaturing conditions and used the detected components to reconstruct the original conjugation isomers. Identical primary conjugation isomers were detected for the A and B isoforms, suggesting that the disulfide configuration does not play a significant role in directing the reduction/conjugation locations. Hinge-conjugated isomers were the primary species detected for the D2, D4, and D8 conjugates. The low abundance
of D10 in our samples precluded the identification of its primary conjugation isomer, but all isomers shown in Figure 9 were detected in both the A and B isoforms.

Formation of D6 deviated from the hinge conjugation trend, as its main isomer contains a pair of Fab conjugations. A low level of hinge-conjugated D6 was identified as the H3L0 species, indicating that all four hinge disulfides were reduced but only three cysteines were conjugated. Therefore, the reduction of three hinge disulfide bonds likely progresses promptly towards the reduction of the fourth disulfide. This hypothesis also rationalizes the predominance of the hinge-conjugated D8, as this molecule probably progressed from further hinge reduction of the mAb that generated D4. Under native conditions, the hinge-conjugated D6 likely remains associated in LHHL form via non-covalent interactions.

The hypothesis of rapid reduction of two hinge disulfides has been previously suggested for the IgG1 ADC, where the main D6 conjugation positional isomer is derived from a minor conjugation isomer of D4 that likely results from the consequent reduction of both hinge disulfides. The IgG2 antibody possibly undergoes global conformational changes after the reduction of two hinge disulfides, thereby increasing solvent accessibility at the hinge region. Other mechanisms such as thiol-disulfide exchange aided by a nearby reduced cysteine, or conversion of interchain to intrachain disulfides similar to the putative mechanism suggested for the IgG4 could also facilitate the rapid reduction of the remaining two hinge disulfides in the IgG2 mAb.
Peak profile differences observed during the denatured analysis of ADCs suggested that the disulfide isoforms potentially drive the reduction/conjugation to different hinge region cysteines. The most remarkable differences between isoforms are the D2 and (HH)5L0 species. The intact D2 peak appeared as a single peak in the A isoforms, whereas a peak doublet was detected in the B isoforms. The 2 Da mass difference between the two peaks suggests the possibility that one isomer contains a pair of reduced, but unconjugated, hinge cysteines. However, the possibility that the two peaks pertain to different hinge conjugation sites should also be considered. Similarly for the (HH)5L0 species, the distribution of the doublet pair is specific to each disulfide isoform.

In this study, we analyzed the effect of the IgG2 disulfide isoforms on the formation of cysteine-linked ADCs. The mAb disulfide configurations influence the solvent accessibilities of the interchain disulfide bonds, but do not affect the primary sites of reduction and conjugation. The impact of expected process variabilities on conjugation sites, potency, and cytotoxicity remains to be addressed. Future explorations will focus on characterizing the hinge conjugation positions, and the correlations between biological activity and the ADCs derived from different disulfide isoforms.

Greater availability of data derived from IgG2 ADCs will contribute to the development of ADCs with improved quality profiles and clinical benefit. While IgG2 ADCs may be more structurally heterogeneous compared to IgG1 or IgG4 ADCs, batch consistency with respect to disulfide isoform distribution, average DAR, and composition of drug-loaded species is presently attainable in large-scale
processes. Moreover, cysteine-linked IgG2 ADCs are composed of higher amounts of the proper D4 species where the intended DAR target is 4 drugs/mAb, compared to the binomial distribution typically reported for cysteine-linked IgG1 ADCs. Improved understanding of process parameters on the CQAs of IgG2 mAbs and ADCs will allow fine-tuning of the product quality. From a biological perspective, the reduced ability of IgG2s to stimulate effector functions could be a key benefit that can improve internalization into the target cells and minimize the incidence of off-target toxicity. Recent efforts for improving drug product consistency have popularized the development of site-specific ADCs with homogeneous DAR, which makes this an attractive modality with regard to the chemistry, manufacturing, and controls requirements. It remains unclear, however, whether DAR homogeneity correlates to clinical benefit for all molecules, as other factors such as off-target binding and drug-linker chemistry may also contribute to the reported dose-limiting toxicities. Continuing efforts evaluating the associations between product quality and clinical activity will help elevate the impact of ADCs as therapeutic options for oncology and other indications.
Chapter 5 - Interchain Disulfide Bonds Scramble During the Production of IgG2 Cysteine-Linked ADCs

5.1 Introductory Remarks

The contents of this chapter have been submitted for publication in mAbs, as a Report titled “Evidence of disulfide bond scrambling during production of an antibody-drug conjugate,” with Lily Liu-Shin, Adam Fung, Arun Malhotra, and Gayathri Ratnaswamy as co-authors.

Antibody-drug conjugates (ADCs) that are produced using thiol-maleimide chemistry are commonly generated by reactions that occur at or above neutral pH. One concern for the ADC manufacturing process is the potential for disulfide bond rearrangement, which can occur at the preferred pH range for the partial reduction and conjugation reactions. The rate of intramolecular thiol-disulfide exchange is accelerated when the conditions favor the degradation of disulfides and the formation of the thiolate anion, such as under the presence of a reducing reagent and/or in neutral or alkaline environments. IgG2 mAbs are particularly susceptible to disulfide bond scrambling, and have been reported to spontaneously rearrange from the A → A/B → B configurations under reducing conditions. Antibodies of the IgG4 subclass have also been reported to undergo disulfide bond scrambling that results in formation of intrachain disulfides and subsequent dissociation of the half-antibody.

The preceding chapter demonstrated that the IgG2-A isoform interchain disulfide bonds reduce at a faster rate than the IgG2-B isoform, therefore generating higher average DARs. This earlier set of experiments were carried
out at low pH to prevent disulfide bond scrambling during the reduction/conjugation process and therefore preserved the original antibody disulfide configuration. The aim of the investigation discussed in this chapter is to determine if the typical ADC manufacturing process conditions at higher pH are conducive to disulfide bond scrambling and how it may affect the conjugation profile of the ADC. An IgG2 was selected as the model for this study since disulfide scrambling events can be detected as interconversion between disulfide isoforms. IgG2-A and IgG2-B mAbs were conjugated with the DL under alkaline and acidic conditions, and the drug loading profiles were characterized with respect to average drug-to-antibody ratios (DARs) and distributions of intact, denatured, and reduced conjugated species. Identification of the reaction step that supports disulfide bond scrambling was also assessed by examining the hydrophobic and thermodynamic properties of the mAbs and ADCs.

5.2 Influence of Process pH on ADC Conjugation Profiles

IgG2-A and IgG2-B mAbs were selectively generated from a mixture of A, A/B, and B disulfide isoforms (see Section 4.2 - Enrichment and Purification of IgG2-A and IgG2-B Antibodies). Conjugation conditions typically used to generate an IgG2-vcMMAE ADC with an average DAR of 3.8 include partial reduction of interchain disulfide bonds with TCEP at pH 9 followed by reaction with excess drug-linker. Since TCEP is an effective reducing agent over a broad pH range, ADCs with a range of average DARs between 0.5 – 7.0 were generated from IgG2-A and -B isoforms at either pH 5.3 or 8.9. The IgG2 starting material containing a mixture of 22% A, 33% A/B, and 45% B isoforms was used as the control in
these experiments. Where possible, equivalent process parameters such as antibody concentration, incubation times and temperatures, and purification and formulation procedures were utilized for both alkaline and acidic reactions. Since disulfide bond reduction kinetics depend on the reaction pH, ratios of TCEP:mAb ranged from 2 – 12 molar equivalents for the alkaline reactions and 4 – 46 molar equivalents for the acidic reactions to generate similar amounts of free cysteines between the two conditions. The conjugation distributions of each ADC pool produced at each TCEP:mAb ratio were evaluated in intact, denatured, and reduced forms.

Figure 5.1 and Table 5.1 compares the HC and LC drug-loading from ADCs generated under alkaline or acidic conditions. ADCs generated under alkaline conditions were largely comprised of similar distributions of conjugated LC and HC regardless of the parent mAb disulfide configuration (Figure 1 and Table S1 of Supplementary Information). The largest differences were observed in ADCs generated using the lowest and highest TCEP:mAb concentrations (panels A and C in Figure 1), though differences between isoforms were not as pronounced compared to the ADCs generated under acidic conditions. The IgG2-A ADCs derived using acidic conditions were comprised of higher amounts of multiply conjugated heavy chains (H3 – H5) compared to the IgG2-Bs and the starting material. Higher DAR of approximately 6 was only achieved using the IgG2-A and the starting material under low pH conditions.

The average DARs of the A and B isoform ADCs produced alkaline conditions were more similar compared to the ADCs produced at pH 5.3 (Figure 5.2). The
average DARs calculated for the ADCs generated at pH 8.9 ranged from 1.6 – 7.3 for the A isoform and 1.8 – 6.8 for the B isoform, in contrast to 0.8 – 6.3 for the IgG2-A and 0.5 – 4.1 for the IgG2-B ADCs generated at low pH.

Figure 5.1. Distribution of reduced species detected in the ADCs derived from IgG2-A, IgG2-B, and starting material.

The top panels (white background) are ADCs produced under alkaline conditions, and the bottom panels (gray background) were from acidic conditions. The x-axis list the reduced unconjugated and conjugated species, where L and H are light and heavy chains, respectively, and 0, 1, 2, 3, 4, 5 refer to the number of attached DL. Black bars represent IgG2-A, gray bars represent IgG2-B, and white bars represent IgG2 starting material comprised of a distribution of A, A/B, and B isoforms. The concentration of TCEP used to achieve each average DAR is indicated. For the pH 8.9 process, the IgG2-A, IgG2-B, and starting material achieved similar average DAR using the same concentration of TCEP. For the pH 5.3 process, IgG2-A, IgG2-B, and starting material required different TCEP concentrations to achieve the same DAR.
Table 5.1. Relative peak areas integrated from rRP-UHPLC analysis of reduced ADCs generated under alkaline and acidic conditions.

Relative peak areas from IgG2-A (“A”), IgG2-B (“B”), and non-enriched IgG2 starting material (“NE”) generated via reduction using TCEP-HCl:mAb ratios from 2 – 12 (alkaline process) or 4 – 46 (acidic process). Average DARs calculated from the distribution of reduced species are also listed.

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Figure 5.2. Average DAR curves of ADCs generated under alkaline and acidic conditions as a function of TCEP-HCl:mAb ratios.

Gray circles indicate IgG2-A; black circles, IgG2-B; and white circles, the IgG2 starting material containing a mixture of A, A/B, and B isoforms.
Intact ADC analyses also showed similarities between IgG2-A and IgG2-B ADCs produced under alkaline conditions, and significant differences from ADCs generated under acidic conditions (Figure 5.3). ADCs with average DARs of ~4 derived from the high pH reaction showed similar peak shapes and retention times of the D2, D4, and D6 species. Although the average DARs between the A and B isoforms differed by only 0.2, some noteworthy differences were observed in the relative abundances of unconjugated mAb (D0). Higher amounts of D0 were detected in the IgG2-B ADCs produced at pH 8.9. Additionally, a small amount of D10 was detectable in ADC derived from the IgG2-A mAb, but accurate quantification of this species was not possible due to the poor peak resolution and sloping baseline. Nevertheless, the distribution of drug-loaded species in the IgG2-A and IgG2-B ADCs generated under alkaline conditions are largely more similar to each other than the A and B ADCs generated under acidic conditions (Table 5.2). Acidic process conditions resulted in dissimilar distributions of conjugated species for the ADCs generated from mAbs with A and B disulfide isoforms. IgG2-B ADC was comprised of 46% D4, whereas only 24% was detected in the ADC from the IgG2-A mAb. Disparate amounts of D0, D1, D3, and D8 were also found between the A and B isoforms.
Table 5.2. Relative peak areas integrated from HIC analysis of intact ADCs generated under alkaline and acidic conditions.

Distributions of intact drug-loaded species detected in ADCs generated from pure IgG2-A ("A"), and the pure IgG2-B ("B"), using TCEP:mAb equivalents of 2 – 12 (alkaline process) or 4 – 46 (acidic process).

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<td>A</td>
<td>B</td>
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The table presents the relative peak areas integrated from HIC analysis of intact ADCs generated under alkaline and acidic conditions. Distributions of intact drug-loaded species detected in ADCs generated from pure IgG2-A ("A"), and the pure IgG2-B ("B"), using TCEP:mAb equivalents of 2 – 12 (alkaline process) or 4 – 46 (acidic process).
Figure 5.3. Chromatograms of partially-reduced mAbs and the resulting ADCs analyzed under intact and reduced conditions. nrRP-UHPLC profiles of partially-reduced mAbs (left column), HIC profiles of intact ADCs (middle column), and distribution of reduced species obtained via rRP-UHPLC of ADCs (right column) are shown. The white backgrounds represent samples treated under alkaline conditions and gray backgrounds under acidic conditions. Gray lines are IgG2-A and black lines are IgG2-B.

The main conjugation isomers were reconstructed from the analysis of non-covalently associated species that dissociate under denaturing conditions. Reversed-phase chromatography of the non-reduced ADCs (nrRP-UHPLC) using a high temperature TFA/CH₃CN gradient was employed for this purpose. For example, the D4 isomer containing a pair of DLs at the Fab region and another
pair on the hinge cysteines would result in the dissociation of conjugated light chain (L1) from HHL with three DLs (denoted as (HH)2L), whereas the hinge-conjugated D4 isomer would be detected as the full LHHL antibody with four drug-linkers (Figure 5.4).

The dissociated components showed that the alkaline process yielded ADCs with similar conjugation isomers, which are mainly comprised of hinge-conjugated D4 species for an average DAR ~4. Moreover, specific chromatographic features such as the D2 doublet and the predominance of the early-eluting (HH)5L0 peak were common between the disulfide isomers. Contrasting the results described previously, IgG2-A and -B ADCs generated under acidic conditions were comprised of different conjugated species, however, both achieved average DARs of ~3.8. The L1, H4L0, and H5 species were detected in higher amounts in the IgG2-A, while the D4 species was predominant in the B isoform. The chromatographic profiles of the component species were also unique to each disulfide isoform. The profiles of the IgG2-B ADCs generated at low pH were similar to the chromatograms for the ADCs derived from reaction at pH 8.9.

The distributions of non-covalently associated species for high average DAR ADCs are shown in Panel C of Figure 5.4. Both A and B isoforms treated under alkaline conditions were able to achieve an approximate average DAR of 7 after partial reduction of the antibodies with 12 molar equivalents of TCEP-HCl, whereas only the A isoform processed under acidic conditions achieved an average DAR of 6.3 after reduction with 46 TCEP molar equivalents. The profiles of ADCs generated via the alkaline process resulted in similar conjugate distributions at high
DAR, particularly the highly conjugated HC (H5 and (HH)10 species). Increased amounts of L1 indicated an increase in conjugation in the Fab region. The IgG2-A ADC derived from the acidic reaction exhibited similar levels of L1 and H5 to the ADCs formed at pH 8.9, but the distributions and profiles of the peaks eluting between L1 and H5 were different compared to the ADCs generated at alkaline pH.

Figure 5.4. Comparisons of nrRP-UHPLC chromatograms obtained from IgG2-A and IgG2-B ADCs generated via low and high pH reactions. Top left panel (A) compares the IgG2-A (gray) and IgG2-B (black) ADCs at average DAR ~3.5 generated under alkaline conditions (white background). Top right panel (B) compares the ADCs generated at low pH to a DAR ~ 3.8 under acidic conditions (gray background). Panel C compares the highly conjugated ADCs with average DAR ~7.0 (solid lines are pH 8.9 reactions, and dotted line is the IgG2-A from the pH 5.3 reaction).
The cartoon illustrations of the components are shown in the lower right corner, and only the IgG2-A is depicted for simplicity purposes, where lines represent disulfide bonds and stars represent vcMMAE. The specific conjugated hinge cysteines have not been determined.

5.3 Characterization of Intact and Partially-Reduced mAbs Derived from Acidic and Alkaline Reactions

The rate-limiting step in the formation of cysteine-linked ADCs is the reduction of disulfide bonds, which also determines the conjugation sites. The preceding characterization of the ADCs demonstrated that alkaline conditions generates similar conjugation profiles for both A and B isoforms. This suggests that disulfide bond scrambling occurred during the alkaline process, and we therefore evaluated which reaction step could allow for scrambling. The mAbs incubated in each reaction buffer with and without added reducing reagent were analyzed for this purpose.

The distribution of disulfide isoforms was directly evaluated for the non-reduced mAbs via a mildly-denaturing RP-UHPLC method (Figure 5.5), whilst the partially-reduced mAbs were analyzed using a strongly denaturing RP-UHPLC method (Figure 5.6). Incubation of the intact mAbs in reaction buffer alone did not induce disulfide bond scrambling. Therefore, in agreement with previous observations in vitro and in vivo, the rearrangement of disulfides in IgG2 antibodies requires the presence of a reducing agent. The mAbs treated with three concentrations of reducing reagent are shown to represent the trend throughout the entire TCEP-HCl titration curve (Figure 5.6). The profiles of the IgG2-A and IgG2-B mAbs reduced at alkaline pH were comparable after partial reduction. Moderate differences attributable to the disulfide configuration became apparent only at the
higher TCEP-HCl concentrations. Increased levels of LC and HC with concomitant
decreased mAb peak were detected in the IgG2-A reduced with ≥8 molar
equivalents of TCEP-HCl. In contrast, different rates of reduction were observed
between the A and B isoforms treated under acidic conditions. A lower amount of
intact mAb was detected in the IgG2-A isoform compared to the IgG2-B isoform.
Moreover, a higher amount of LC was detected in the A isoform at all TCEP-HCl
concentrations.

Figure 5.5. Disulfide isoform distributions of IgG2-A and IgG2-B mAb
incubated in the reaction buffer for 3 hours at 37 °C.
The solid lines are the mAbs incubated in the 20 mM sodium acetate, 150 mM NaCl, 1
mM EDTA, pH 5.3 buffer, and dashed lines are the mAbs incubated in the 25 mM sodium
borate, 150 mM NaCl, 1 mM EDTA, pH 8.9 reaction buffer.
5.4 Thermal Stability of mAbs and ADCs Based on Disulfide Configuration

The similarities observed between isoforms with respect to the ADC conjugation profile and the extent of reduction of the mAbs suggested unidirectional disulfide bond rearrangement from the A to the B configuration under alkaline process conditions. Further support for the directionality from A $\rightarrow$ B...
conversion is revealed in the similarities of the HIC and RP-UHPLC chromatographic profiles of the ADCs generated at high pH compared to the IgG2-B ADC generated at low pH. The disulfide scrambling likely yields the thermodynamic product during the partial reduction step, as suggested in previous mAb investigations.$^{18, 32, 96}$

We analyzed the thermal stability of the IgG2 mAb disulfide isoforms and ADCs generated under acidic conditions using differential scanning calorimetry (DSC), and the melting curves are shown in Figure 5.7 and Figure 5.8. The mAb disulfide isoforms yielded unique melting curves, similar to previous reports.$^{18, 97}$ The B isoform showed separate transitions for the $\mathrm{C}_2$, $\mathrm{C}_3$, and Fab domains, in contrast to the overlapping transitions observed in the A isoform. The melting temperatures ($T_m$) of each domain depend on the disulfide configurations (Table 5.3), and DL conjugation primarily affected the stability of the $\mathrm{C}_2$ domain. Additionally, a second melting transition of the $\mathrm{C}_2$ was observed in both IgG2-A and IgG2-B ADCs. Increased drug loading resulted in destabilization of the lower-temperature $\mathrm{C}_2$ transition for both isoforms, and increased stabilization of the higher-temperature $\mathrm{C}_2$ transition for the A isoform only. The Fab and $\mathrm{C}_3$ domains were affected by conjugation in the IgG2-B only, with opposing effects for each domain. The IgG2 mAb and ADC containing a mixture of disulfide isoforms showed $T_m$ values in between the IgG2-A and IgG2-B results. It is important to note that the current data set was derived from ADCs comprised of a heterogeneous distribution of drug-loaded species, and therefore only reflect an average of the contributions from each structure. The thermal denaturation curves
of cysteine-linked ADCs are sensitive to variations in distributions of both conjugated species (D0 – D12) and conjugation positional isomers (Fab vs. hinge conjugations).

Figure 5.7. DSC thermograms of IgG2-A and IgG2-B mAb and ADCs
DSC thermograms obtained from IgG2-A (gray lines) and IgG2-B (dashed black lines) scanned from 20 – 100 °C at 60 °C/h are shown. Top panel (A) is the overlay of the mAb thermograms and bottom panel (B) is the thermogram overlay obtained from ADCs with an average DAR of ~3.3.

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Table 5.3. Melting temperatures of the IgG2-A, IgG2-B, and IgG2 starting material (mixture of A, A/B, and B isoforms) mAbs and ADCs up to average DAR of 3.5.
Figure 5.8. DSC curve fittings for thermograms derived from IgG2 mAbs and ADCs.

Curve fittings for thermograms derived from IgG2-A (A, B), IgG2-B (C, D), and starting material (E, F) mAbs (left panel) and ADCs (right panel) are shown. The average DAR of the ADCs ranged from 3.2 – 3.5. Black traces indicate the thermogram obtained via DSC, red traces are the curve fittings for the CH2 transitions, blue traces are the curve fittings of the Fab transitions, and green traces are the curve fittings for the Fab/CH3 transitions.
5.5 Discussion

The conjugate distribution, average DAR, and conjugation locations are critical quality attributes (CQAs) for cysteine-linked ADCs. Typical ADC production processes are optimized to achieve a target average DAR while maximizing reaction efficiency. The optimal pH for antibody partial reduction and thiol-maleimide coupling is also favorable for disulfide bond scrambling, particularly for IgG2 and IgG4 mAbs. Therefore, understanding whether disulfide scrambling occurs and how this impacts the ADC conjugation profile is important for the design of the ADC manufacturing process.

In this study, we have demonstrated that disulfide bond scrambling occurs with IgG2s processed under alkaline conditions. Our characterization of the IgG2-A and IgG2-B mAbs and ADCs found evidence of conversion of the A \( \rightarrow \) B form occurring during the partial reduction step. This directionality is in agreement with previous studies that have reported conversion of the A \( \rightarrow \) A/B \( \rightarrow \) B forms in a redox environment similar to physiological conditions.\textsuperscript{32-33} The higher thermal stability of the IgG2-B mAb and ADCs observed in this study provides further evidence that IgG2 disulfide scrambling favors the thermodynamic product. The rearrangement of the mAb to the B configuration suggest that the disulfide isoform distribution of the mAb intermediate may not necessarily be a critical attribute for IgG2 cysteine-linked ADCs generated at high pH. However, the pKa of the cysteiny1 thiol can vary based on the local environment and thus relationship between process pH, disulfide configuration, and conjugation profile should remain a factor to consider during conjugation process development.
Although drug-loading differences in the ADCs were linked to the reaction pH, the primary conjugation sites were the hinge cysteines for both A and B isoforms and were unaffected by process pH. Drug-linker conjugation at the LC-HC cysteines would result in higher presence of the L1 species in the reduced ADC analyses and in a thermal shift of the Fab domain. The ADCs generated under both conditions showed prevalent HC conjugation. While alterations to the Fab thermal stability were not detected in the IgG2-A ADCs, Fab domain destabilization was observed in the IgG2-B ADCs. We reasoned that the post-conjugation decrease of the Fab Tm in the B isoform is due to disruptions to the non-covalent interactions between the Fab and C\textsubscript{H}2 that result due to hinge conjugation, rather than an indication of the breakage of the LC-HC disulfides.

In the case of IgG4s, the hinge interchain and intrachain disulfides are believed to be in equilibrium and the formation of half-antibodies could be accelerated under redox conditions\textsuperscript{14}. Therefore, standard cysteine-linked ADC conjugation processes may affect the equilibrium between hinge interchain and intrachain disulfides, which may influence the amounts of full and half-molecules present in the solution. To our knowledge, characterization of the post-conjugation disulfide linkages is not a typical activity during clinical process development. The findings presented herein suggest that disulfide configuration should perhaps be included in the process characterization for proteins that are susceptible to disulfide scrambling.
Chapter 6 - Future Directions

6.1 Introductory Remarks

Originally, the dissertation research goals included localizing the conjugated hinge cysteines and obtaining a high-resolution structure of a full-length, fully glycosylated IgG2 and IgG2 ADC. As research progressed, it became clear that these aims would take considerable more effort. This section discusses the progress made towards these stretch goals. The experiments discussed in this section lay the foundation for future explorations in this field.

6.2 Approaches to Localize the Conjugated Hinge Cysteines in IgG2 ADCs

The identification of the hinge conjugation sites can be elucidated using various approaches, two of which are discussed herein. Conjugated hinge cysteines can be directly detected via LC-MS or LC-MS/MS methods, or indirectly by deduction based on the remaining disulfide bonds characterized via LC-MS/MS with collision-induced dissociation (CID), electron-transfer dissociation (ETD), or electron-capture dissociation (ECD). As the molecular weight of deglycosylated cysteine-linked ADCs are in the range of 145 – 160 kDa, the large size can complicate top-down characterization via LC-MS/MS. Therefore, the first step in this study was to evaluate suitable proteinases for bottom-up or middle-down characterization of ADCs.
6.2.1 Development of an Approach for the Direct Identification of Conjugated Hinge Cysteines via LC-MS

6.2.1.1 *In-silico* Digestion using Conventional Proteinases

Several proteinases are commercially-available, commonly used for protein mass spectrometry, and yield peptides fragments between 2,000 – 4,000 Da, which are suitable for bottom-up characterization using LC-MS. The *in-silico* digests were performed using PeptideCutter from the ExPASy server (http://web.expasy.org/peptide_cutter/) on the heavy chain of the model IgG2 using common enzymes as depicted in Figure 6.1. Traditional enzymes such as Lys-C, Asp-N, and trypsin yield fragments containing all four hinge cysteines in a single peptide, which makes localization of the conjugation position difficult via LC-MS. Endoproteinase Glu-C can cleave at the C-terminus of Glu residues with high specificity in ammonium bicarbonate and ammonium acetate buffers, and would be a good choice for subsequent MS/MS analysis. Preliminary experiments using Glu-C in an 20 – 100 mM ammonium bicarbonate buffer generated extensive protein aggregation during digestion for the IgG2 mAb, even in the presence of 8 M urea or 6 M guanidine-HCl. Since the mAb digestion was problematic, digestion of the ADC was not evaluated. Future work could focus on optimizing the digestion conditions to improve protein solubility while preserving enzymatic activity.
Figure 6.1. *In silico* digestion schematic near the IgG2 hinge region.
Depicted is the primary amino acid sequence of the model IgG2 heavy chain near the hinge region, with the hinge residues in red. The cleavage sites and fragment sizes generated by each enzyme are listed below the primary sequence. The K/C cleavage site is common for Lys-C and trypsin, but each enzyme generates a different size fragment. Similarly, the E/C position is common for Lys-N and Arg-C.

### 6.2.1.2 Site-Specific Proteinases for Middle-Down Characterization

Separation of the antibody into Fab and Fc fragments has been traditionally achieved using papain or pepsin.\textsuperscript{102} Papain is a broadly-specific enzyme that exhibits selectivity at amino acids with a large hydrophobic side chain, and in the case of an IgG has been described to cleave above the hinge to generate Fab fragments and a single Fc fragment.\textsuperscript{103-105} Pepsin preferentially cleaves at the C-terminus of Phe and Leu residues, and has been used to generate (Fab')\textsubscript{2} fragments from IgGs as it cleaves below the hinge.\textsuperscript{104, 106-109} One drawback of using either papain or pepsin is that careful limited digestion optimization is required to prevent protein precipitation, preserve enzyme activity, and reduce non-specific digestion outside the hinge region. Recent availability of site-specific proteases developed by Genovis (Lunden, Sweden) are able to generate clean Fab and Fc fragments under neutral conditions that are compatible with human monoclonal IgGs.\textsuperscript{110} The enzymes that are able to digest IgG2 antibodies are IdeS (trade name FabRICATOR\textsuperscript{TM}) and SpeB (trade name FabULOUS\textsuperscript{TM}).
Per the manufacturer, IdeS can be used under non-reducing conditions with specificity under the IgG2 hinge at ERKCCVECPPCPAPPVA // GPSVF, generating a (Fab’)$_2$ and a Fc fragment (Figure 6.2).$^{111}$ IdeS is an IgG-specific cysteine protease derived from *Streptococcus pyogenes* of approximately 37 kDa.$^{111-113}$ The published digestion site was confirmed via LC-MS using the model IgG2 mAb and IgG2-vcMMAE ADC in both glycosylated and deglycosylated forms, as shown in Figure 6.3 and Table 6.1 for the mAb, and Figure 6.4 and Table 6.2 for the ADC. The Fc portion in the mAb analysis was detected as three species with slight mass differences, and correlate with different post-translational modifications previously reported$^{114-115}$, such as oxidation (+16 Da) and reduced disulfide (+2 Da).
Figure 6.3. RP-UHPLC chromatograms of IdeS-digested IgG2 mAb. The top panel (A) shows the mAb that incubated sequentially with IdeS → PNGaseF, and bottom panel (B) shows mAb incubated with PNGaseF → IdeS. IdeS enzyme was detected as three similarly abundant species of 37.6, 37.1, and 36.6 kDa.

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Table 6.1. Identification of peaks from Figure 6.3 by LC-MS. Species are listed in order of abundance. Theoretical masses were calculated using the ExPASy server and the primary sequence without glycans or C-terminus HC lysines (unless indicated). Disulfide bonds and N-terminus HC pyroglutamate (pE) formation are included in the theoretical masses. Delta (Δ) mass refers to the value of observed mass – theoretical mass.

One of the advantages of using IdeS is to separate the ADC into smaller components that is more compatible with MS analysis. Also shown in Figure 6.4 are the protein A fractions of the IdeS-digested and deglycosylated ADC, demonstrating that the conjugated (Fab’)₂ species do not bind the protein A resin and are recovered in the wash fraction. Protein A purification in this work used the
Amicon Pro system, which takes only ~1.5 hours from resin equilibration to final UF/DF of the purified sample. If necessary, the Amicon Pro conditions could be further optimized to increase the binding of the Fc fragment to the resin. For the purpose of this investigation, however, some co-purification of the Fc fragment with the Fab fragments is a minor concern since the LC conditions provide baseline resolution of the Fc fragment for this ADC.

Identification of the RP-UHPLC peaks demonstrates that elution of the IdeS-digested ADC fragments is driven by hydrophobic contributions from both the mAb component and the vcMMAE molecule. This is an important observation since it diverges from common perception of the traditional ADC assays (such as HIC and reduced RP), where elution of conjugated species is driven solely by the number of vcMMAE molecules. A comprehensive catalog of all possible ADC structures was made a priori, which facilitated the deconvolution of the more complex components by matching the observed to the theoretical masses. This approach is able to separate the hinge-conjugated and Fab-conjugated species. For instance, a hinge-conjugated D6 molecule would be detected as (Fab’)_2 + 6 vcMMAE, whereas the isomer containing two drug-linkers on the LC-HC cysteines and 4 drug-linkers on the hinge would be detected as Fab + Fd + 5 vcMMAE after dissociation of the L1 component. While the current method was unable to determine the hinge conjugation sites, this could be possible via analysis of the y-ions derived from the MS/MS spectra of the Fab fragments.
Figure 6.4. RP-UHPLC chromatograms of IdeS-digested IgG2-vcMMAE ADC with an average DAR of 3.7 and its protein A wash and eluate fractions. (A) shows the ADC that was sequentially digested with IdeS→PNGaseF, and (B) shows the minor peaks detected in the range of 10.5 – 18.0 minutes. (C) is the ADC digested in the sequence of PNGaseF→IdeS, and (D) and (E) are the protein A wash and eluate fractions, respectively.
### Table 6.2. Identification of peaks from Figure 6.4 by LC-MS.

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<td>13.7</td>
<td>(Fab')_2 + 16 vcMMAE</td>
</tr>
<tr>
<td>15.9</td>
<td>31161.3</td>
<td>31159.2</td>
<td>2.1</td>
<td>Fd + 3 vcMMAE</td>
</tr>
<tr>
<td>16.3</td>
<td>84240.1</td>
<td>84231.7</td>
<td>8.4</td>
<td>(Fab')_2 + 17 vcMMAE</td>
</tr>
<tr>
<td>17.4</td>
<td>62325.5</td>
<td>62318.3</td>
<td>7.2</td>
<td>2 * (Fd + 5 vcMMAE)</td>
</tr>
</tbody>
</table>

*aThe retention times listed in this table are from the total ion chromatogram and may be ±0.1 minutes off compared to the LC chromatogram in the figure.

*bTheoretical masses were calculated from the primary sequence without glycans or C-terminal lysines. Disulfide bonds and N-terminal pyroglutamate (pE) formation are included in the theoretical masses. The value used for vcMMAE in the theoretical masses was 1316.5 Da.

*cDelta (Δ) mass refers to the value of observed mass – theoretical mass.

*dThe conjugated (Fab + Fd) fragments result from dissociation of L1 from the (Fab')_2

*eH5 is the heavy chain fully conjugated at all interchain cysteines

*fThe nomenclature indicates a dimeric aggregate of the conjugated Fd fragment.

A second IgG2 mAb conjugated with mcMMAF was digested with IdeS to evaluate if this enzyme can be used for different types of cysteine-linked IgG2 ADCs. The mcMMAF drug-linker is ~400 kDa smaller than vcMMAE (MW = 925.2 Da) and contains a terminal carboxylic acid group that confers the molecule an
overall -1 charge (Figure 1.7). Therefore, cysteine-linked mcMMAF ADCs contain a distribution of molecules with different hydrophobicities and total charges that are modulated by the drug-linker. Compared to an IgG2-vcMMAE ADC, the charge on the mcMMAF molecule could lead to electrostatic repulsion that limits the number of drug-linkers attached at the hinge. Electrostatic interactions due to mcMMAF conjugation at hinge cysteines can also potentially affect the substrate binding ability of IdeS. This experiment used the same digestion and LC-MS conditions as discussed for the vcMMAE conjugate. The RP-UHPLC chromatogram in Figure 6.5 shows successful digestion of the IgG2-mcMMAF ADC using IdeS. However, the LC conditions require further optimization to provide suitable resolution of the conjugated fragments. The peak identifications via LC-MS are listed in Table 6.3 and demonstrate that all expected species were detected in this analysis. However, an unidentified species of 24.1 kDa was detected under the same peak as the glycosylated Fc.

Figure 6.5. RP-UHPLC chromatogram of IdeS-digested IgG2-mcMMAF ADC with an average DAR of 4.0.
Another site-specific enzyme available from Genovis is the SpeB enzyme (FabULOUSTM), which is a cysteine protease derived from Streptococcus pyogenes that cleaves IgG1 at KTHT / CPPCPAPE.110, 116 Genovis has not identified the digestion site in IgG2s and this information was also not available after a literature search. Three different IgG2 mAbs and their respective ADCs were evaluated to determine both the SpeB digestion efficiency and the cleavage location. Analysis of the mAb fragment masses via LC-MS indicates that the IgG2 cleavage site is ERKCCVE // CPPCP in all three mAbs (Table 6.4). Activation of SpeB involves reduction of the catalytic cysteine,116 which also leads to the reduction of the interchain disulfides in the antibody. The expected fragments

<table>
<thead>
<tr>
<th>Ret. Time (min)a</th>
<th>Observed Mass (Da)</th>
<th>Theoretical Mass (Da)b</th>
<th>∆ Mass (Da)c</th>
<th>Peak ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.93</td>
<td>25237.1</td>
<td>25233.9</td>
<td>3.2</td>
<td>½ Fc</td>
</tr>
<tr>
<td></td>
<td>24140.2</td>
<td></td>
<td></td>
<td>Not identified</td>
</tr>
<tr>
<td>6.58</td>
<td>24521.8</td>
<td>24522.4</td>
<td>-0.6</td>
<td>L1</td>
</tr>
<tr>
<td></td>
<td>99222.3</td>
<td>99225.6</td>
<td>-3.3</td>
<td>(Fab')2 + 2 mcMMAF</td>
</tr>
<tr>
<td>9.79</td>
<td>101070.6</td>
<td>101076.0</td>
<td>-5.4</td>
<td>(Fab')2 + 4 mcMMAF</td>
</tr>
<tr>
<td>11.45</td>
<td>101079.0</td>
<td>101076.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>102926.4</td>
<td>102926.4</td>
<td>0.0</td>
<td>(Fab')2 + 6 mcMMAF</td>
</tr>
<tr>
<td>11.87</td>
<td>78401.1</td>
<td>78408.0</td>
<td>-6.9</td>
<td>(Fab + Fd) + 5 vcMMAEd</td>
</tr>
<tr>
<td>13.15</td>
<td>101079.0</td>
<td>101076.0</td>
<td>3.0</td>
<td>(Fab')2 + 4 mcMMAF</td>
</tr>
<tr>
<td></td>
<td>78401.1</td>
<td>78408.0</td>
<td>-6.9</td>
<td>(Fab + Fd) + 5 vcMMAEd</td>
</tr>
<tr>
<td>14.42</td>
<td>52386.4</td>
<td>52392.4</td>
<td>-6.0</td>
<td>Fab + 4 mcMMAF</td>
</tr>
<tr>
<td>16.28</td>
<td>29715.4</td>
<td>29716.4</td>
<td>-1.0</td>
<td>Fd + 5 mcMMAF</td>
</tr>
<tr>
<td>17.88</td>
<td>29716.4</td>
<td>29716.4</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3. Identification of peaks from Figure 6.5 by LC-MS.

aThe retention times listed in this table are from the total ion chromatogram and may be ±0.1 minutes off compared to the LC chromatogram in the figure.

bTheoretical masses were calculated from the primary sequence with G0F glycosylation at N297 and without C-terminal K. Disulfide bonds and N-terminal pyroglutamate (pE) formation are included in the theoretical masses. The value used for mcMMAF in the theoretical masses is 925.2 Da.

ΔMass (Δ) mass refers to the value of observed mass – theoretical mass.

dThe conjugated (Fab + Fd) fragments result from dissociation of L1 from the (Fab')2.
resulting from incubation with SpeB are the LC, Fd, and \( \frac{1}{2} \) Fc (Figure 6.6). The SpeB enzyme (28.1 kDa\(^{116}\)) and an unidentified 12.4 kDa species were detected in all three mAbs and ADCs. As the unidentified species was present in all the samples, it is likely an enzyme-related impurity rather than a secondary cleavage site in the antibody.

![Figure 6.6. Illustration of the IgG2 fragments detected via RP-UHPLC after SpeB digestion.](image)

Several issues were identified during the analysis of the SpeB digests, which complicated the interpretation of the MS data. The Fd species in mAb 1 and mAb 3 were detected as a mixture of truncated species that ranged from loss of 1 hinge residue at the C-terminus to complete loss of all hinge residues, all eluting under the same peak. Variable loss of amino acid residues on the N-terminus of the Fc fragment was also detected in mAb 3. This suggests that the loss of hinge residues is likely an artifact caused by in-source fragmentation, and tuning of the MS conditions could improve recovery of the full-length Fd and Fc fragments. Aggregation of digested fragments further complicated data analysis, and should be resolved in future investigations. Interestingly, all aggregates identified were dimers of the same species (e.g., LC-LC, Fd-Fd, and \( \frac{1}{2} \) Fc-\( \frac{1}{2} \) Fc). As several minor species that correspond to less than 20% of the total ion intensity could not
be assigned by mass alone, it is possible that heterodimers (e.g., Fd-LC) could also be present. Analysis of the mAb 2 fragments was straightforward in comparison to mAbs 1 and 3, and no dimeric species were detected, which indicates that optimization of the SpeB digestion and analysis conditions will need to be assessed on a case-by-case basis.

<table>
<thead>
<tr>
<th>Moleculea</th>
<th>Observed mass (Da)</th>
<th>Theoretical mass (Da)</th>
<th>Δ (Da)</th>
<th>Fragment ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 1</td>
<td>12371.7</td>
<td>26167.1</td>
<td>4.6</td>
<td>½ Fc⁶</td>
</tr>
<tr>
<td></td>
<td>22879.9</td>
<td>22923.6</td>
<td>-43.7</td>
<td>Truncated Fd⁷</td>
</tr>
<tr>
<td></td>
<td>46638.9</td>
<td>46622.2</td>
<td>16.7</td>
<td>Truncated Fd-Fd⁹</td>
</tr>
<tr>
<td></td>
<td>46297.4</td>
<td>46287.8</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46751.8</td>
<td>46721.4</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46410.2</td>
<td>46416.0</td>
<td>-5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46526.1</td>
<td>46463.8</td>
<td>62.3</td>
<td>LC-LC⑩</td>
</tr>
<tr>
<td></td>
<td>52345.3</td>
<td>52334.1</td>
<td>11.2</td>
<td>Fc</td>
</tr>
<tr>
<td></td>
<td>52458.1</td>
<td>52462.3</td>
<td>-4.2</td>
<td>Fc + K</td>
</tr>
<tr>
<td>mAb 2</td>
<td>12368.1</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23596.7</td>
<td>23597.3</td>
<td>-0.6</td>
<td>LC</td>
</tr>
<tr>
<td></td>
<td>26178.6</td>
<td>26167.1</td>
<td>11.5</td>
<td>½ Fc⁶</td>
</tr>
<tr>
<td></td>
<td>24157.0</td>
<td>24156.2</td>
<td>0.8</td>
<td>Fd</td>
</tr>
<tr>
<td>mAb 3</td>
<td>12370.1</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26168.7</td>
<td>26167.1</td>
<td>1.6</td>
<td>½ Fc⁶</td>
</tr>
<tr>
<td></td>
<td>23340.2</td>
<td>23339.0</td>
<td>1.3</td>
<td>LC</td>
</tr>
<tr>
<td></td>
<td>23758.3</td>
<td>23739.8</td>
<td>18.5</td>
<td>Fd⁹</td>
</tr>
<tr>
<td></td>
<td>47441.8</td>
<td>47479.7</td>
<td>-37.9</td>
<td>Fd-Fd⁹</td>
</tr>
</tbody>
</table>

Table 6.4. Masses of SpeB digest fragments detected in three IgG2 mAbs

aMab 1 is the model IgG2 used throughout this thesis. The three mAbs are different IgG2 mAbs engineered specific for different targets.
bTheoretical mass of ½ Fc assumes C-terminal K truncation and G0F glycosylation (+1445 Da)
cTheoretical mass of Fd fragment for mAb 1 with intrachain disulfides and N-term pE modification is 23642.5 Da. Observed masses suggest loss of 1 – 5 hinge residues (up to KCCVE).
dThese species were all detected under the same peak, correlating with theoretical masses of Fd-Fd aggregates with heterogeneous C-term losses of 1 – 5 hinge residues (up to KCCVE) on each Fd fragment.
eMab 1 LC was detected as an aggregated LC-LC. Minor amounts of LC monomer with a +32 Da modification was also detected (data not shown).
fTheoretical mass of full Fc fragment assumes C-term K truncation and N297 glycosylation with G0F/G0F.
gFull-length Fd fragment and full-length Fd-Fd dimer was detected in minor amounts in mAb 3. The majority of Fd-related fragments exhibited C-term losses of 1 – 7 hinge residues (up to ERKCCVE). The full list of truncated fragments is not listed for mAb 3.
As SpeB cleaves in the middle of the hinge, this enzyme could be useful for future LC-MS/MS development to identify the hinge conjugation locations. Digestion efficiency was evaluated using one IgG2-mcMMAF (conjugated from mAb 2) and two different IgG2-vcMMAE ADCs (conjugated from mAbs 1 and 3), all with similar average DAR of ~4. RP-UHPLC analysis of the IgG2-mcMMAF ADC is shown in Figure 6.7 and the peaks were matched to the theoretical species according to Table 6.5. Determination of the peak identities was straightforward for this ADC, since each peak was reasonably resolved and the digested fragments were found in full-length monomeric form.

Figure 6.7. RP-UHPLC chromatogram of SpeB-digested IgG2-mcMMAF ADC with an average DAR of 4.0.
<table>
<thead>
<tr>
<th>Ret. Time (min)</th>
<th>Observed Mass (Da)</th>
<th>Theoretical Mass (Da)</th>
<th>Δ Mass (Da)</th>
<th>Peak ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>12368.1</td>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
<tr>
<td>5.0</td>
<td>23597.7</td>
<td>23597.2</td>
<td>0.5</td>
<td>LC</td>
</tr>
<tr>
<td>5.3</td>
<td>26167.7</td>
<td>26167.0</td>
<td>0.7</td>
<td>½ Fc</td>
</tr>
<tr>
<td>5.9</td>
<td>28130.9</td>
<td>~28 kDa</td>
<td>N/A</td>
<td>Spe B</td>
</tr>
<tr>
<td>6.3</td>
<td>24522.3</td>
<td>24522.4</td>
<td>-0.1</td>
<td>L1</td>
</tr>
<tr>
<td>6.6</td>
<td>24522.3</td>
<td>24522.4</td>
<td>-0.1</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>27093.3</td>
<td>27092.2</td>
<td>1.1</td>
<td>½ Fc + 1 mcMMAF</td>
</tr>
<tr>
<td>8.0</td>
<td>24157.0</td>
<td>24157.2</td>
<td>-0.2</td>
<td>Fd</td>
</tr>
<tr>
<td>8.1</td>
<td>24157.0</td>
<td>24157.2</td>
<td>-0.2</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>25082.7</td>
<td>25082.4</td>
<td>0.3</td>
<td>Fd + 1 mcMMAF</td>
</tr>
<tr>
<td>9.1</td>
<td>25082.7</td>
<td>25082.4</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>9.8</td>
<td>25082.6</td>
<td>25082.4</td>
<td>0.2</td>
<td>Fd</td>
</tr>
<tr>
<td></td>
<td>24157.0</td>
<td>24157.2</td>
<td>-0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52157.2</td>
<td>52156.6</td>
<td>0.6</td>
<td>H2</td>
</tr>
<tr>
<td>10.7</td>
<td>28018.0</td>
<td>28017.4</td>
<td>0.6</td>
<td>½ Fc + 2 mcMMAF</td>
</tr>
<tr>
<td>11.0</td>
<td>26007.3</td>
<td>26007.6</td>
<td>-0.3</td>
<td>Fd + 2 mcMMAF</td>
</tr>
<tr>
<td>11.5</td>
<td>26938.9</td>
<td>26932.8</td>
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<td></td>
</tr>
<tr>
<td>12.5</td>
<td>26933.0</td>
<td>26932.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>13.3</td>
<td>26934.0</td>
<td>26932.8</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.5. Identification of peaks from Figure 6.7 by LC-MS

aThe retention times listed in this table are from the total ion chromatogram and may be ±0.1 minutes off compared to the LC chromatogram in the figure.
bTheoretical masses were calculated from the primary sequence with G0F glycosylation at N297 and without C-terminal K. Disulfide bonds and N-terminal pyroglutamate (pE) formation are included in the theoretical masses. The value used for mcMMAF in the theoretical masses is 925.2 Da.
cDelta (Δ) mass refers to the value of observed mass – theoretical mass.

The analysis using IgG2-vcMMAE ADCs derived from mAbs 1 and 3 also encountered hinge fragmentation, aggregation, and coelution issues observed during the mAb analysis. The RP elution profiles of the two vcMMAE ADCs are
shown in Figure 6.8, and the peaks were matched to the theoretical masses of expected components as listed in Table 6.6. Unlike the elution profile for the SpeB-digested mcMMAF conjugate, the elution of components appear to be driven by hydrophobic contributions from both the antibody fragment and the vcMMAE molecule. For an undetermined reason, conjugated species that correspond to the highly conjugated DAR species (D6 and above) were not detected using the present LC conditions, even though the HIC analysis demonstrated presence of these molecules (data not shown). Both conjugated and unconjugated LC were also not detected in the mAb 1 ADC.

Figure 6.8. RP-UHPLC chromatograms of SpeB-digested IgG2-vcMMAE ADCs with an average DAR of ~4.
Top panel (A) is the mAb 1 conjugate and bottom panel (B) is the mAb 3 conjugate.
<table>
<thead>
<tr>
<th>Ret. Time (min)</th>
<th>Observed Mass (Da)</th>
<th>Theoretical Mass (Da)</th>
<th>∆ Mass (Da)</th>
<th>Peak ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>12371.0</td>
<td>Unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>26171.7</td>
<td>26167.1</td>
<td>4.6</td>
<td>½ Fc</td>
</tr>
<tr>
<td>10.2</td>
<td>22879.9</td>
<td>22923.6</td>
<td>-43.7</td>
<td>Fd with loss of RKCCVE</td>
</tr>
<tr>
<td></td>
<td>52344.3</td>
<td>52322.8</td>
<td>21.5</td>
<td>(Fd-truncated Fd) + 4 vcMMAE&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>12.2</td>
<td>46297.4</td>
<td>46287.8</td>
<td>9.6</td>
<td>(truncated Fd-truncated Fd)</td>
</tr>
<tr>
<td></td>
<td>47459.6</td>
<td>47476.1</td>
<td>-16.5</td>
<td>(truncated Fd-truncated Fd) + 1 vcMMAE</td>
</tr>
<tr>
<td></td>
<td>23419.5</td>
<td>23414.3</td>
<td>5.2</td>
<td>Fd with loss of VE</td>
</tr>
<tr>
<td></td>
<td>49803.0</td>
<td>49788.9</td>
<td>14.1</td>
<td>(Fd-truncated Fd) + 2 vcMMAE</td>
</tr>
</tbody>
</table>

Table 6.6. Identification of peaks from Figure 6.8 by LC-MS

<sup>a</sup>The retention times listed in this table are from the total ion chromatogram of the ADC derived from mAb 1 and may be ±0.1 minutes off compared to the LC chromatogram in the figure.

<sup>b</sup>Theoretical masses were calculated from the primary sequence of mAb 1 with G0F glycosylation at N297 and without C-terminal K. Disulfide bonds and N-terminal pyroglutamate (pE) formation are included in the theoretical masses. The value used for vcMMAE in the theoretical masses is 1316.5 Da.

<sup>c</sup>Delta (∆) mass refers to the value of observed mass – theoretical mass.

<sup>d</sup>Several species corresponding to the Fd fragment with loss of 1 – 7 hinge residues detected

While the current LC-MS method was unable to determine the specific conjugated cysteines, some conclusions can be inferred based on the abundance of the conjugated Fd and Fc fragments. Previous characterization of IgG2 ADCs indicated that the hinge region is the primary conjugation location for the D2, D4, and D8 species (discussed in Chapter 4), and D4 is the most abundant form in an ADC sample with average DAR ~4. Comparison of the abundance of singly and doubly conjugated Fd and Fc fragments in the IgG2-mcMMAF ADC suggests that the lower half of the hinge (CPPC) could be the primary conjugation site. As
mentioned earlier, the negative charge on the mcMMAF molecules may cause electrostatic repulsion that directs conjugation to cysteines that are further apart. On the other hand, the top half of the hinge (CCVE) appears to be the primary conjugation target for the IgG2-vcMMAE ADCs. The LC-MS analysis of the D4 species purified via HIC fractionation (Figure 6.9) also shows prevalence of conjugated Fd fragments, supporting the hypothesis that vcMMAE conjugation on IgG2 antibodies starts at the first two hinge cysteines.

Figure 6.9. RP-UHPLC chromatogram of SpeB-digested D4 fraction from an ADC with an average DAR of ~4.
The D4 species was collected from HIC-fractionation of the vcMMAE conjugate derived from mAb 1.

Protein A purification of the IgG2-vcMMAE SpeB digests was evaluated to determine the feasibility of using the Amicon Pro purification system. IgG binding to protein A reportedly involves mainly hydrophobic interactions between the
protein A binding pocket and the Cα2-Cα3 interface of the IgG. Reduction of the interchain disulfide bonds alters the higher-order structure of the Fc region and may impact its ability to bind to protein A. The IgG2-vcMMAE derived from mAb 3 processed through the Amicon Pro system (Figure 6.10) confirmed that separation of the SpeB-digested fragments was unsuccessful with protein A purification. As expected, the majority of the Fd and LC fragments were recovered in the wash fraction, but a minor amount was also collected from the eluate fraction. Moreover, the ½ Fc fragment was not detected in either wash or eluate fractions, and will require further investigation.

Figure 6.10. IgG2-vcMMAE SpeB digest processed with Amicon Pro protein A purification system.
Top panel (A) shows the ADC derived from mAb 3 that was digested with SpeB and not protein A purified. Middle panel (B) shows the species recovered in the protein A chromatography wash fraction and bottom panel (C) shows the species recovered after elution from protein A.
Identification of the conjugated peptides via LC-MS\textsuperscript{E} was routinely performed in our lab using tryptic, Lys-C, or Asp-N digests of the reduced ADC. MS\textsuperscript{E} is a feature from Waters mass spectrometers that simultaneously collects mass spectra generated from cycling between low and high collision energies. The high collision energy results in fragmentation of the vcMMAE molecules that are attached to the peptides, allowing conjugated peptides to be identified by extracting the chromatogram showing peaks that also contain the MMAE fragment (718.4 Da [M+H]\textsuperscript{+}). Localization of conjugated hinge cysteines using LC-MS\textsuperscript{E} with SpeB/trypsin or SpeB/Lys-C digests could be attempted, but the variable fragmentation of the hinge portion observed for the IgG2-vcMMAE ADCs would severely complicate data analysis.

6.2.2 Development of an Approach to Identify Disulfide Bonds Remaining Post-Conjugation

Portions of this section were submitted for publication in mAbs, as a Report titled “Rapid characterization of complex IgG2 disulfide-bonded peptides via MALDI-TOF/TOF,” with Anja Resemann, Lily Liu-Shin, Guillaume Tremintin, Arun Malhotra, Adam Fung, Fang Wang, Gayathri Ratnaswamy, and Detlev Suckau as co-authors.

An indirect approach to pinpointing the hinge conjugation sites is to detect the remaining disulfide bonds in the ADC, with the assumption that all reduced cystines are conjugated with the drug-linker. Although characterization of IgG disulfide bonds via site-directed mutagenesis, or non-reduced peptide mapping via LC-MS have been reported, meticulous optimization of these methods is usually required. The heterogeneous disulfide configurations and the
large peptide sizes derived from the IgG2-A/B and IgG2-B isoforms can further complicate MS method development using Q-TOF or ion trap instruments. Large molecule analysis and mapping of complex disulfide bonds have been successful using ETD or ECD,\textsuperscript{92, 121-122} and these approaches could be explored for IgG2 conjugation site identification. One potential limitation of characterizing disulfide bond linkages in ADCs is that fragmentation of the conjugated drug-linker under the high energy could result in highly complex mass spectra that would necessitate extensive manual data analysis.\textsuperscript{76, 123} Site-directed mutagenesis was excluded as a possibility for this work, as it involves replacing the cysteine residues which would also remove the conjugation sites.

One potentially simpler approach to characterize the IgG2 disulfide linkages is using MALDI-MS. The advantages of using MALDI for IgG2 non-reduced peptide analysis is the higher mass range compared to Q-TOF or ion trap instruments, and the predominance of singly-charged ions in the MALDI spectra which simplifies data analysis for larger peptides.\textsuperscript{124-125} To explore this option, we entered into a collaboration with Bruker (Bremen, Germany and Billerica, MA) to evaluate a highly-automated LC-MALDI-TOF/TOF workflow that was validated for the semi-quantitative mapping of native and scrambled IgG1 disulfide bonds. The objective of this collaboration was to achieve proof-of-concept using IgG2s with a heterogeneous mixture of disulfide isoforms. This section describes the identification of signature disulfide-bonded (DSB) peptides that pertain to each IgG2 disulfide configuration when analyzed using the LC-MALDI-TOF/TOF workflow.
MALDI in-source decay (MALDI-ISD) is a fragmentation process known for more than 20 years which is spontaneously caused by ion-molecule reactions that occur during desorption/ionization in the MALDI plume. Takayama et al. described this mechanism as a hydrogen radical transfer from the matrix to the peptide bond carbonyl group, followed by radical ion rearrangements that predominantly yield c- and z+2 ions. Based on this concept, several matrices that enhance hydrogen radical donation, such as sDHB and 1,5-DAN, were developed specifically for MALDI-ISD protein sequencing. This earlier work observed that some matrices facilitated partial reduction, which proposed the use of MALDI-ISD for disulfide bond identification. Since then, MALDI-ISD has also been used for protein identification, localization of PTMs, N- and C-terminal sequence determination and detection of variants, and de novo sequencing of a 13.6 kDa protein.

The MALDI-TOF/TOF approach is based on the principle that in-source decay (ISD) of DSB peptides generate three unique signals (“the triplet rule”), illustrated in Figure 6.11. Briefly, partial reduction in the ion source by MALDI-ISD results in the detection of two reduced peptides (P1-SH and P2-SH) in addition to the original DSB peptide (P1-S-S-P2). The m/z of the DSB peptide is therefore the sum of the m/z values of P1-SH and P2-SH, minus the molecular weight of (H2+H)+. De novo sequencing of the reduced peptides is performed via collision-induced dissociation (CID), and the identity of the DSB peptide is matched to the expected peptides derived from in silico digestion of a provided sequence or by using a search engine such as Mascot. Validated DSB peptides based on a user-defined
DSB score threshold that meet the triplet rule are tabulated in the DisulfideDetect software and quantified based on the intensities of the LC-MALDI data. This validation step drastically reduces the number of false positive triplets and thus yields a reliable representation of the disulfide structure of a protein. The DSB score represents the abundance of all the 8 peaks expected from the 2 quartet patterns in the DSB-peptide MS/MS spectra, which are fragmentation components of the reduced peptides in the forms of MH+, [MH+]+S, [MH+]-H2, and [MH+]-SH2.

This section describes the identification of signature DSB peptides that pertain to each IgG2 disulfide configuration when analyzed using the MALDI-ISD-CID workflow. The signature peptides can be added to the Native Disulfide Bond reference table in the DisulfideDetect software for automatic detection and semi-quantitation of IgG2 disulfide isoforms in samples containing a heterogeneous isoform distribution.

![Figure 6.11. Principle of disulfide-bonded peptide identification via LC-MALDI-MS/MS](image)

Triplet Rule: \( MH^+(P_1) + MH^+(P_2) - 2(H^-) - H^+ = MH^+ \) (DSB-Peptide)

Figure 6.11. Principle of disulfide-bonded peptide identification via LC-MALDI-MS/MS
IgG2-A, -A/B, and -B disulfide isoforms were fractionated via RP-UHPLC at Agensys and directly frozen in the mobile phase mixture at -80 °C. The TFA/IPA/ACN mobile phase mixture differed for each disulfide isoform due to their elution times, with IgG2-B containing the least amount of ACN. The high temperature, low pH, high organic concentration of the LC conditions would cause irreversible antibody denaturation, which is not a concern for this approach as the next step involves trypsin digestion under non-reducing conditions. Bruker prepared and analyzed the digests as described in Section 3.12.

6.2.2.1 Automatic Assessment of the DSBs of the IgG2 Disulfide Isoforms

The first step in our analysis of the IgG2 A, B and A/B disulfide isoforms was applying the automatic software-based assessment of the disulfide organization using the canonical IgG1 disulfide configuration as the template for the intra-chain and LC-HC inter-chain disulfide bonds. Both the canonical and non-canonical DSB-peptides comprised of a single disulfide crosslink were detected via automated analysis (Figure 6.12). The DSB connectivity map obtained for each of the IgG2 disulfide isoform fractions showed significant differences in the DSB organization of the isoforms, as expected. For example, the LC212-HC129 bond was detected only in the A and A/B forms using this method, which is consistent with previous reports about the LC-HC connectivity in these isoforms.\textsuperscript{18-19} Most native intra-chain and inter-chain disulfide bonds (12 total for an IgG2) were detected in all isoforms. However, the intra-chain HC22-HC96 could not be detected in neither the A nor the B isoforms, suggesting possible trypsin missed cleavage at this site or low ionization of this particular DSB peptide. A limitation of
the automatic software detection is that the hinge disulfides in the B configuration were not detectable, as these non-reduced peptides are comprised of (LC-hinge-HC)_2 peptide with multiple disulfide bonds.

![Disulfide structure obtained by trypsin digestion of the denatured IgG2 mAb disulfide isoforms under non-reducing conditions](image)

**Figure 6.12.** Disulfide structure obtained by trypsin digestion of the denatured IgG2 mAb disulfide isoforms under non-reducing conditions
Native disulfide bonds are indicated as blue lines and marked by an “N” inside the bubbles, whereas scrambled disulfides are indicated in red. The size of each bubbles correspond to the prevalence of each disulfide bond.

In addition to the native disulfide bonds, scrambled disulfides and scrambling hot spots were also detected and quantified automatically by the software. Higher abundance of scrambling was observed in the A isoform, which elutes later than the B and A/B isoforms in the RP-UHPLC method used for separation of disulfide isoforms (Figure 4.1A). The amount of scrambling in the A isoform is consistent with its longer exposure to the 82 °C column temperature used during fraction collection. The small size of the blue bubbles in the disulfide map of the A and A/B isoforms indicate that molecules containing scrambled inter-chain disulfides were present in very minor amounts relative to the native configurations. The histogram in Figure 6.13 shows that HC129 and HC142 were involved in scrambling in all 3 isoforms, whereas LC212 scrambling was detected only in the A isoform. The
lack of scrambled disulfides involving LC212 in the A/B and B isoform is likely due to the software’s limitation in the detection of multiply cross-linked peptides.

6.2.2.2 Identification and Validation of IgG2-A Isoform Signature DSB Peptides

DSB peptides containing two asymmetric peptides connected by a single disulfide bond were automatically identified and quantified by the Bruker DisulfideDetect 1.2 software. Peptides with multiple disulfide bonds or singly DSB peptides comprised of two symmetric peptides required manual peak identification and validation based on the MS/MS spectrum. In the IgG2-A isoform, the LC212 residue is connected to the HC129 in the C_H1 region, in contrast to the HC224 in the C_H2 region as is the case in IgG1s. The MS/MS spectrum of the respective tryptic dipeptide LC212-HC129 with m/z 2039.943 is shown in Figure 6.14. One missed cleavage was observed in the LC212 peptide, leading to the detection of the heptapeptide rather than the expected tripeptide. This missed cleavage was
commonly observed throughout this set of experiments. The quartet of fragment ions generated by the CID fragmentation of the HC peptide GPSVFPLAPCSR (1230.6 Da) yielded ions with m/z of 1196.642 (MH+-SH2), 1228.614 (MH+-H2), 1230.630 (MH+), and 1262.602 (MH++S). The corresponding fragment ion quartet was also detected for the LC peptide SFNRGEC (812.3 Da), albeit at lower intensity than the HC peptide fragments. In addition, sequence-specific b- and y-ions from both peptides were detected.

Figure 6.14. MS/MS spectra of the LC-HC peptide in A configuration with mass of 2040 Da
Top panel shows the HC (top row) and LC (bottom row) sequences of the DSB-peptide and the MS/MS spectrum with the matching HC sequence-related fragment ions annotated. Bottom panel is same MS/MS spectrum with the matching LC-sequence related fragment ions annotated. A quartet peak pattern is well represented in the spectrum indicative for the free peptide Mr as it comprises its form –SH, -H, +H (MH+) and +SH. Both peptide MH+ signals indicative for the 2 peptides comprising the DSB-peptide can thus be obtained and used for their MS/MS based identification.
The predicted hinge peptide structure for the A isoform is a symmetrical DSB peptide spanning residues HC217 – HC224 of 5351.562 Da. The tryptic peptide sequence of the A isoform hinge is CCVEC PPCPA PPVAG PSVFL FPPKP K with all four Cys residues disulfide-bonded to the opposite HC peptide (Figure 6.15). The fragment ions in the MS/MS spectrum covered the peptide sequence across residues PA PPVAG PSVFL FPPKP (residues 9 – 26). Since the presence of multiple disulfide bonds can lead to unpredictable bond cleavages under CID, de novo sequencing is challenging for multiply-bonded peptides.\textsuperscript{141-142} However, the presence of MS/MS fragments such as the $b_4$, $y_{19}$, and $y_{21}$ ions are consistent with the CID fragmentation pattern of disulfide-bonded peptides. Taking all datasets together, the m/z 5351.5 Da detected via MALDI-MS can be assigned as a signature for the A isoform (HC217-224)$_2$ DSB hinge peptide.

Figure 6.15. MS/MS spectra of the hinge peptide in A configuration with mass of 5352 Da
6.2.2.3 Identification and Validation of IgG2-A/B Isoform Signature Hinge Peptide

The asymmetric hinge peptide with cysteines LC212a,HC129,(HC217-224)\textsubscript{2} with m/z 6886.253 is specific to the A/B isoform. This DSB peptide is comprised of the 2 hinge peptides linked via three disulfide bonds at HC217-224, and the HC216 residue is connected to either LC212a or to HC129 (Figure 6.16). The MH\textsuperscript{+} ion was observed as m/z 6886.23, which is in agreement with the proposed structure with a -3.4 ppm error. The HC peptide sequences involved were identified in the MS/MS spectrum with high confidence, with 100% sequence coverage for the 12mer peptide containing the C\textsubscript{H1} HC129 residue, and 89% coverage for the two hinge HC217-224 peptides. Similar to the results observed for the A hinge sequence, the multiple disulfide bonds in this region precluded the full coverage of the A/B hinge sequence. The LC tripeptide was not directly observed with fragment ions in the MS/MS spectrum, but its presence was inferred by detection of the peak quartets at m/z 5658.65 and m/z 1230.60, which add up to the expected mass of the A/B hinge DSB peptide. Loss of the LC tripeptide could potentially be an ISD-related artifact, as a single disulfide bond connects the LC to the HC.
Figure 6.16. MS/MS spectra of the hinge peptide in A/B configuration with mass of 6886 Da

The inset shows the structure of the asymmetric peptide with 5 disulfide bonds. Bottom: the fragment ion coverage of the hinge peptide and the linked HC 12mer peptide are shown which provided for the identification of the respective HC-peptide sequences. The peak quartet at m/z 1230.630 corresponds to the non-hinge HC-peptide and the quartet at m/z 5658.65 to the tripeptide deficient of this peptide (peak quartets are indicated by red arrows).

6.2.2.4 Identification and Validation of IgG2-B Isoform Signature Hinge Peptide

The IgG2 B hinge peptide has a symmetrical structure involving residues LC212a, HC129, and HC217-224, and has an expected mass of 8419.9 Da. The DSB peptide linkage pattern of the B isoform shows the LC212 bonded to HC218, and HC129 bonded to HC217 (Figure 6.17). The analysis of the B isoform digests required crystallization in sDHB matrix instead of HCCA to reduce the effect of metastable fragmentation on the molecular ion.\textsuperscript{134, 143} When initially analyzed in HCCA matrix, the MH\textsuperscript{+} ion of the B isoform hinge was not observed under standard MALDI peptide spectra acquisition conditions.
Four DSB peptides that correspond to the B isoform hinge were detected, including the symmetric hinge peptide with m/z 8421.15 (theoretical m/z 8419.936). The m/z 8421.15 observed suggests the possibility of Mr+H+, possibly related to a reduced disulfide. The additional peaks at m/z 8925.20 and m/z 9429.21 correspond to peptides containing one missed cleavage on one or both LCs, respectively, yielding LC heptamers. The m/z 8116.17 variant of the hinge peptide correlated to the loss of one LC tripeptide, and the loss of the singly-bonded LC was also observed in the A/B isoform hinge peptide analysis.

Figure 6.17. Deconvoluted MS spectra of the hinge peptide in B configuration with mass of 8421 Da
Reflector MALDI-MS spectrum close-up of the hinge peptide fraction from the LC-MALDI sDHB dataset of the IgG2 B subtype. The main peak at m/z 8421.15 corresponds to the symmetric hinge peptide of the B subtype. The peaks at m/z 8925 and 9429 correspond to the same peptide structure with 1 or 2 heptapeptides instead of tripeptides due to incomplete proteolytic digestion. The peak at m/z 8116 corresponds to the tripeptide-loss species.
Complex peptide structures consisting of multiple peptides connected by multiple disulfide bonds, as in the case of B-form hinge peptides, typically do not generate sufficient peptide backbone fragmentation for de novo sequencing by MS/MS. Validation of the observed m/z to the corresponding theoretical sequences was achieved by evaluating the precursor mass together with the quartet patterns (Figure 6.18). For the B isoform, the peak quartet at m/z 4211.34 matches the symmetrically cleaved between the hinge peptides (theoretical m/z 4211.984). This peptide point towards the possibility that one of the two hinge disulfides at the C-terminus could already be reduced in the undigested sample, leading to the ISD reduction of the only other remaining disulfide connecting the two hinge peptides. Careful evaluation of the charge envelopes from each species shows the presence of both [M+H]+ and [M+2H]2+ (data not shown), confirming the presence of a reduced disulfide. The peak quartet at m/z 7192.59 results from the loss of the HC129 12mer peptide (theoretical m/z 7191.321), which could be either an ISD artifact or reduced cysteines that were present before enzymatic digestion.

Most of the peaks detected in this LC-MALDI data subset were determined as peptides containing missed cleavages, or losses of singly-disulfide bonded fragments. The limited solvent accessibility of Fab-C_{H2} region of the B isoform revealed by HDX-MS could provide one explanation for the increased missed cleavages observed in this fraction. Losses of singly-disulfide bonded fragments could be a result of in-source decay or the presence of a reduced disulfide in the native sample. IgG2 mAbs have been reported to contain higher amounts of free thiol in comparison to IgG1s, and it is possible that the B isoform contributes
greatly to the reported values due to the conformational strain imparted by the crossed-disulfide configuration.

Altogether, the fraction pertaining to the B isoform hinge peptide in the LC-MALDI dataset contained over 40 peptides, spanning LC-fractionation retention times from 55.25 – 56.50 minutes and 5 – 10 kDa mass range. Further analysis determined that this fraction contained a heterogeneous mixture of DSB-peptides pertaining to the A, A/B, and B hinge (Figure 6.19). The heterogeneity was attributed to the cross-contamination of the neighboring peaks from the LC fractionation of the disulfide isoforms.
6.2.2.5 Catalog of Signature DSB Peptides for Each IgG2 Disulfide Isoform

All the peptides verified by their MS and MS/MS spectra are summarized in Table 6.7. The set of m/z below can be added to software databases for future automatic detection of the each IgG2 disulfide isoform. High sequence conservation near the interchain cysteines, regardless of allotype, implies that this collection of signature peptides can be widely applicable to other human IgG2s.10

The peptides validated in this work are not able to discern between different cross-linking configurations of nearby cysteines (i.e., canonical vs. scrambled hinge disulfides within the same peptide). The possibility of disulfide bond variants within the native B isoform has been reported elsewhere.84
Table 6.7. List of signature peptides identified for each IgG2 disulfide configuration with the corresponding disulfide bond patterns

<table>
<thead>
<tr>
<th>MH+</th>
<th>A</th>
<th>A/B</th>
<th>B</th>
<th>Disulfide-bonded residuesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>2039.9</td>
<td>+</td>
<td>+</td>
<td></td>
<td>LC212b-HC129a</td>
</tr>
<tr>
<td>5351.6</td>
<td>+</td>
<td></td>
<td></td>
<td>(HC217-224)2</td>
</tr>
<tr>
<td>5479.7</td>
<td>+</td>
<td></td>
<td></td>
<td>K(HC217-224)2</td>
</tr>
<tr>
<td>6886.3</td>
<td>+</td>
<td></td>
<td></td>
<td>LC212a, HC129a, (HC217-224)2</td>
</tr>
<tr>
<td>8420.9</td>
<td>+</td>
<td></td>
<td></td>
<td>(LC212a, HC129a, HC217-224)2</td>
</tr>
<tr>
<td>8925.2</td>
<td>+</td>
<td></td>
<td></td>
<td>LC212a, LC212b, (HC129a, HC217-224)2</td>
</tr>
<tr>
<td>9429.4</td>
<td>+</td>
<td></td>
<td></td>
<td>(LC212b, HC129a, HC217-224)2</td>
</tr>
</tbody>
</table>

Table 6.7. List of signature peptides identified for each IgG2 disulfide configuration with the corresponding disulfide bond patterns

a “H” and “L” denote the light chain and the heavy chain, respectively, and the number after each letter indicates the position of the cysteine residue. Lowercase “a” and “b” refer zero or one missed cleavages.

6.2.2.6 Use of Extracted Ion Chromatograms to Quantify Abundance of IgG2 Disulfide Isoforms

One of the advantages of using MALDI-TOF is its semi-quantitative potential, which has been widely examined using small and large molecules in a variety of matrices. Extracting the intensities of specific ions of interest from the LC-MALDI data can provide a semi-quantitative assessment of their concentrations, even in the absence of a concentration-based internal standard or calibration curve. An example is shown in Figure 6.20, where the intensities of the 2040 Da peak of the IgG2-A form LC-HC dipeptide in the extracted ion chromatogram (XIC) is in close agreement with the expectation that its abundance in the A/B isoform should be half of the abundance in the A isoform. The XIC intensities of the A isoform hinge peptide shows the highest abundance in the IgG2-A MALDI target, and baseline levels in the A/B and B plates which can be attributed to the co-elution from the RP-UHPLC fractionation. Interrogation of the LC-MALDI heat map for the m/z 6889 peptide detected the A/B hinge peptide in preparations from all three
isoforms, with the highest abundance detected in the A/B MALDI preparation. This cross-contamination of the A/B hinge peptide was expected as the A/B isoform is the least resolved species in the RP-UHPLC fractionation. The relative amounts of A/B hinge observed in the A and B isoform MALDI preparations agree with the tailing peak shapes of the RP-UHPLC elution profiles.

Figure 6.20. Examples of LC-MALDI heat maps of the A, A/B, and B digests (A) and extracted ion chromatograms (XIC, B-D) of the m/z of interest
The XICs of the IgG2-A LC-HC peptide (m/z 2040), IgG2-A hinge peptide (m/z 5352), and IgG2-A/B hinge peptide (m/z 6889) are shown in panels B, C, and D, respectively. The intensities of the peaks in the XIC correspond to the abundance of each peptide detected in the A, A/B, and B MALDI targets.

In summary, the LC-MALDI-TOF/TOF technique described in this section can be a powerful tool in the semi-quantitative analysis of heterogeneous IgG2 disulfide isoform mixtures. In theory, this approach could be used for the direct structural characterization of ADCs using the principle that conjugation positional isomers generate different DSB peptides. Manual validation would be necessary to interpret the MALDI-MS and MS/MS signals resulting from the various conjugated peptides and the fragmentation of the drug-linker. However, cataloging
the signature peptides that are related to each conjugation isomer that can be generated from the A, A/B, and B disulfide isoforms would be a one-time effort.

6.3 Experiments Performed Towards Understanding the Structure of a Full-Length IgG2 mAb and ADC

Solving the structure of a human IgG2 antibody and an IgG2-vcMMAE ADC are stretch goals for this dissertation research. Obtaining high-resolution structures of these molecules would be valuable contributions for future development of mAb and ADC therapeutics. Currently, only two crystal structures of the full length human IgGs are available in the PDB (1HZH, an IgG1; and 5DK3, an IgG4).149-150 The crystallization trials using an IgG2-B antibody and homology models obtained using the two full-length structures are described in this section.

6.3.1 Crystallization Screening using IgG2-B Isoform mAb

The scarcity of full-length crystal structures in the PDB reflects the difficulty in obtaining diffraction-quality crystals of IgGs. The large molecular size, heterogeneity of glycosylation variants, and flexibility of the Fab’ and Fc domains are some of the challenges to the crystallization of IgGs. The initial aims were to obtain high resolution structures of both IgG2 mAb and ADC, and the starting point used the IgG2-B mAb due to its presumed lower overall flexibility that may increase the probability of successfully obtaining crystals.

Attempts to crystallize an IgG2-B mAb were made using the Hampton Research crystallization screening kits. Preliminary experiments done for the preceding sections have confirmed the purity of the isolated B isoform, and that it remains >99% monomeric in solution for a minimum of 4 months at the maximum
tested concentration of 48 mg/mL (data not shown). The wide buffer screen was conducted using the Hampton Research Index screen with the redox-enriched IgG2-B (~80% isomeric purity), at 24 mg/mL in 20 mM sodium acetate, 1 mM Na2-EDTA, 150 mM NaCl, pH 5.3 buffer. Buffer conditions that resulted in immediate precipitation of the antibody were excluded from the second buffer screen. The second buffer screen used select buffers at pH ≤6.5 from four different kits with the 100% pure IgG2-B isolated from redox-CEX purification, at 9.4 mg/mL in 20 mM sodium acetate, 5% (w/v) sucrose, pH 5.0. The reagents that did not result in severe protein precipitation are listed in Table 6.8, but no condition resulted in successful protein crystallization.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Tray examination frequency</th>
<th>Reagents that did not induce gross protein precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index (HR2-144), all 96 buffers</td>
<td>Daily for days 1 - 5, then weekly up to 8 weeks</td>
<td>9. 0.1 M BIS-TRIS pH 5.5, 3.0 M NaCl 10. 0.1 M BIS-TRIS pH 6.5, 3.0 M NaCl 11. 0.1 M HEPS pH 7.5, 3.0 M NaCl 12. 0.1 M Tris pH 8.5, 3.0 M NaCl 13. 0.1 M BIS-TRIS pH 5.5, 0.3 M Magnesium formate dihydrate 14. 0.1 M BIS-TRIS pH 6.5, 0.5 M Magnesium formate dihydrate 15. 0.1 M HEPES pH 7.5, 0.5 M Magnesium formate dihydrate 16. 0.1 M Tris pH 8.5, 0.3 M Magnesium formate dihydrate 17. 1.4 M Sodium phosphate monobasic monohydrate/Potassium phosphate dibasic pH 5.6 22. 0.8 M Succinic acid pH 7.0 26. 1.1 M Ammonium tartrate dihydrate pH 7.0 31. 0.8 M Potassium sodium tartrate tetrahydrate, 0.1 M Tris pH 8.5, 0.5% w/v MPEG 5,000 32. 1.4 M Sodium phosphate monobasic monohydrate/Potassium phosphate dibasic pH 5.6 33. 0.2 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol 34. 0.2 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 20% v/v 2-Propanol 35. 0.2 M Ammonium acetate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol 36. 0.2 M Potassium chloride, 0.05 M HEPES pH 7.5, 35% v/v Pentaerythritol propoxylate (5/4 PO/OH) 37. 0.01 M Cobalt(I) chloride hexahydrate, 0.1 M Tris pH 8.5, 20% w/v Polyvinylpyrrolidone K 15 38. 0.1 M HEPES pH 7.0, 30% w/v Jeffamine M-600 pH 7.0 47. 0.2 M Magnesium formate, 0.1 M HEPES pH 7.5, 40% w/v Peg 3,350 48. 0.2 M Magnesium formate, 0.1 M HEPES pH 7.5, 40% w/v Peg 8,000 51. 0.2 M Magnesium formate, 0.1 M HEPES pH 7.5, 40% w/v Peg 8,000 52. 0.2 M Magnesium formate, 0.1 M HEPES pH 7.5, 40% w/v Peg 8,000 53. 0.2 M Magnesium formate, 0.1 M HEPES pH 7.5, 40% w/v Peg 8,000 56. 0.2 M Potassium chloride, 0.05 M HEPES pH 7.5, 35% v/v Pentaerythritol propoxylate (5/4 PO/OH) 57. 0.2 M L-Proline, 0.1 M HEPES pH 7.5, 10% w/v Peg 3,350</td>
</tr>
<tr>
<td>Crystal Screen (HR2-110), 15/50 buffers evaluated</td>
<td>Daily for days 1 - 4, week 1, week 8, week 10</td>
<td>1. 0.02 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol 24. 0.2 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 20% w/v 2-Propanol 26. 0.2 M Ammonium acetate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 30% w/v (+/-)-2-Methyl-2,4-pentanediol 32. 2.0 M Ammonium sulfate 33. 4.0 M Sodium formate 34. 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Sodium formate 37. 0.1 M Sodium acetate trihydrate pH 4.6, 8% w/v Peg 4,000 42. 0.05 M Potassium phosphate monobasic, 20% w/v Peg 8,000 43. 30% w/v Peg 1,500 44. 0.2 M Magnesium formate dihydrate</td>
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</tbody>
</table>
Table 6.8. List of crystallization buffers that did not induce protein precipitation

<table>
<thead>
<tr>
<th>Crystal Screen 2 (HR2-112), 24/48 buffers evaluated</th>
<th>Days 1, 3, 4, 10, week 8, week 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2.0 M NaCl, 10% w/v PEG 6,000</td>
<td></td>
</tr>
<tr>
<td>2. 0.5 M NaCl, 0.01 M Magnesium chloride hexahydrate, 0.01 M Hexadecyltrimethylammonium bromide</td>
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<tr>
<td>3. 25% v/v Ethylene glycol</td>
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</tr>
<tr>
<td>4. 35% v/v 1,4-Dioxane</td>
<td></td>
</tr>
<tr>
<td>5. 2.0 M Ammonium sulfate, 5% v/v 2-Propanol</td>
<td></td>
</tr>
<tr>
<td>7. 10% w/v PEG 1,000, 10% w/v PEG 8,000</td>
<td></td>
</tr>
<tr>
<td>8. 1.5 M NaCl, 10% v/v Ethanol</td>
<td></td>
</tr>
<tr>
<td>9. 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M NaCl</td>
<td></td>
</tr>
<tr>
<td>10. 0.2 M NaCl, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol</td>
<td></td>
</tr>
<tr>
<td>11. 0.01 M Cobalt(iii) chloride hexahydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 1.0 M 1,6-Hexanediol</td>
<td></td>
</tr>
<tr>
<td>12. 0.1 M Cadmium chloride hydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% w/v PEG 400</td>
<td></td>
</tr>
<tr>
<td>15. 0.5 M Ammonium sulfate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 1.0 M Lithium sulfate monohydrate</td>
<td></td>
</tr>
<tr>
<td>16. 0.5 M NaCl, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2% v/v Ethylene imine imine polymer</td>
<td></td>
</tr>
<tr>
<td>17. 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 35% v/v tert-Butanol</td>
<td></td>
</tr>
<tr>
<td>18. 0.01 M Iron(ii) chloride hexahydrate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 10% v/v Jeffamine M-600</td>
<td></td>
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<tr>
<td>19. 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2.5 M 1,6-Hexanediol</td>
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<tr>
<td>21. 0.1 M Sodium phosphate monobasic monohydrate, 0.1 M Potassium phosphate monobasic, 0.1 M MES monohydrate pH 6.5, 2.0 M NaCl</td>
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</tr>
<tr>
<td>22. 0.1 M MES monohydrate pH 6.5, 12% w/v PEG 20,000</td>
<td></td>
</tr>
<tr>
<td>23. 1.6 M Ammonium sulfate, 0.1 M MES monohydrate pH 6.5, 10% v/v 1,4-Dioxane</td>
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</tr>
<tr>
<td>25. 0.01 M Cobalt(iii) chloride hexahydrate, 0.1 M MES monohydrate pH 6.5, 1.8 M Ammonium sulfate</td>
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</table>

<table>
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<tr>
<th>Grid Screen (NH4)2SO4 (HR2-211), 12/24 buffers evaluated</th>
<th>Days 1, 3, 4, 12, week 2, week 8, week 10</th>
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</thead>
<tbody>
<tr>
<td>A1. 0.1 M Citric acid pH 4.0, 0.8 M Ammonium sulfate</td>
<td></td>
</tr>
<tr>
<td>A2. 0.1 M Citric acid pH 5.0, 0.8 M Ammonium sulfate</td>
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<tr>
<td>A3. 0.1 M MES monohydrate pH 6.0, 0.8 M Ammonium sulfate</td>
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<tr>
<td>B1. 0.1 M Citric acid pH 4.0, 1.6 M Ammonium sulfate</td>
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</tr>
<tr>
<td>B2. 0.1 M MES monohydrate pH 6.5, 12% w/v PEG 20,000</td>
<td></td>
</tr>
<tr>
<td>B3. 0.1 M MES monohydrate pH 6.0, 1.6 M Ammonium sulfate</td>
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<tr>
<th>Grid Screen PEG 6000 (HR2-213), 12/24 buffers evaluated</th>
<th>Days 1, 3, 4, 10, week 2, week 8, week 10</th>
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<tbody>
<tr>
<td>A1. 0.1 M Citric acid pH 4.0, 5% w/v PEG 6,000</td>
<td></td>
</tr>
<tr>
<td>A2. 0.1 M Citric acid pH 5.0, 5% w/v PEG 6,000</td>
<td></td>
</tr>
<tr>
<td>A3. 0.1 M MES monohydrate pH 6.0, 5% w/v PEG 6,000</td>
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</tr>
<tr>
<td>B1. 0.1 M Citric acid pH 4.0, 10% w/v PEG 6,000</td>
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</tr>
<tr>
<td>B2. 0.1 M Citric acid pH 5.0, 10% w/v PEG 6,000</td>
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<tr>
<td>B3. 0.1 M MES monohydrate pH 6.0, 10% w/v PEG 6,000</td>
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</tr>
<tr>
<td>B4. 0.1 M Citric acid pH 4.0, 20% w/v PEG 6,000</td>
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<tr>
<td>C1. 0.1 M Citric acid pH 4.0, 20% w/v PEG 6,000</td>
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<tr>
<td>C2. 0.1 M MES monohydrate pH 6.0, 20% w/v PEG 6,000</td>
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<tr>
<td>C3. 0.1 M Citric acid pH 4.0, 30% w/v PEG 6,000</td>
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6.3.2 Homology Modeling of the IgG2-A Antibody

Homology models of IgG antibodies reported in literature typically use PDB 1HZH (IgG1) structure as the template. The availability of the PDB 5DK3 (IgG4) structure showed that the conformation of IgGs can be vastly different. The 5DK3 structure is more compact than the 1HZH structure, with major differences in the conformations of the hinge and C\textsubscript{H}2 domains.\textsuperscript{150} Due to the hinge sequence similarities between the IgG2 and IgG4 antibodies, it is possible that the spatial arrangement of the Fab/C\textsubscript{H}2 of an IgG2 is better represented using the 5DK3 structure as the template. The homology model of the IgG2-A isoform generated
using the 5DK3 template is shown in Figure 6.21, and using the 1HZH template shown in Figure 6.22.

Sequence alignment of the IgG2 used in this research and the template IgG4 (via LALIGN program in the ExPASy server) showed 83% identity (92% similarity) of the light chain and 82% identity (91% similarity) of the heavy chain. The main conformational difference was observed as increased rigidity of the hinge loops in the IgG2 model, likely due to the pair of additional disulfides and the presence of Val-Glu instead of Pro-Pro in the middle of the hinge.

Alignment of the IgG2 to the 1HZH sequence showed 77% identity (88% similarity) of the heavy chain and 80% identity (92% similarity) of the light chain. Separately aligning the Fd and Fc sequences found the lowest identity in the Fd portion. To alleviate this, the Fab of the IgG2 was first modeled using PDB 4HAF, which has 90% sequence identity. Each modeled IgG2 Fab derived from 4HAF were then connected to the IgG2 Fc model derived from 1HZH. Extensive loop refinement was needed, and some hinge disulfides could not be properly connected in this model.
Figure 6.21. IgG2-A homology model using PDB 5DK3 (an IgG4) as template

Figure 6.22. IgG2-A homology model using PDB 1HZH (an IgG1) as template
REFERENCES


80. Vlasak, J.; Ionescu, R., Fragmentation of Monoclonal Antibodies. mAbs 2011, 3 (3), 253-263.


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Lily is also an avid car enthusiast and competes in the Sports Car Club of America (SCCA) Solo program, having earned multiple local and regional autocross trophies from 2009 - 2013. In September 2014, she became the 2014 SCCA Solo National Champion in the Street Touring Sport Ladies (STSL) category piloting a 1989 Honda CRX Si.