Evidence of disulfide bond scrambling during production of an antibody-drug conjugate

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ABSTRACT
Antibody-drug conjugates (ADCs) that are formed using thiol-maleimide chemistry are commonly produced by reactions that occur at or above neutral pHs. Alkaline environments can promote disulfide bond scrambling, and may result in the reconfiguration of interchain disulfide bonds in IgG antibodies, particularly in the IgG2 and IgG4 subclasses. IgG2-A and IgG2-B antibodies generated under basic conditions yielded ADCs with comparable average drug-to-antibody ratios and conjugate distributions. In contrast, the antibody disulfide configuration affected the distribution of ADCs generated under acidic conditions. The similarities of the ADCs derived from alkaline reactions were attributed to the scrambling of interchain disulfide bonds during the partial reduction step, where conversion of the IgG2-A isofrom to the IgG2-B isofrom was favored.

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Introduction
Antibody-drug conjugates (ADCs) are a rapidly growing class of targeted therapeutics that utilize a recombinant antibody to deliver a highly potent cytotoxic molecule to the target cells, most of which are developed for oncology indications. The mechanism of action of an ADC relies on the internalization of the antibody-drug complex, followed by intracellular degradation and release of the cytotoxic drug that disrupts an essential cellular process and leads to cell death.

Endogenous cysteine and lysine residues are currently the most popular conjugation locations for ADCs in clinical development, but other locations such as glycans and engineered antibodies with single amino acid substitutions are also being developed. Currently, four ADCs have gained US Food and Drug Administration market approvals (Adcertis®, Kadcyla®, Mylotarg®, and Besponsa®) and over 50 ADCs were in active clinical trials in 2016. The majority of ADCs utilize monoclonal antibodies (mAbs) of the IgG1 subclass, and therefore the majority of published information relates to physicochemical and process characterization pertains to IgG1 ADCs. However, at least 9 clinical-stage ADCs use an IgG2 or IgG4 mAb, and knowledge gained from IgG1 ADCs may not apply to these molecules.

Cysteine-linked ADCs that target drug-linker conjugation to endogenous cysteines involved in interchain disulfide bonds constitute approximately half of all active clinical ADCs. Thiol-maleimide coupling is a common approach for attaching the drug-linkers (DL) to the free cysteines generated by a controlled partial reduction of the antibody interchain disulfide bonds. This approach results in a heterogeneous mixture of molecules with variable levels of conjugation, ranging from 0–8 drugs for IgG1 and IgG4 ADCs or 0–12 drugs for IgG2 ADCs. Furthermore, the main conjugation locations differ based on the antibody subclass, with IgG1s preferentially conjugating at cysteines in the antigen-binding fragment (Fab), but at the hinge cysteines in IgG2s. The distribution of drug-loaded species and the locations of drug-linker attachment are critical quality attributes (CQAs) because these properties affect the drugs product stability and their pharmacokinetic/pharmacodynamics (PK/PD) profiles.

The four IgG subclasses (IgG1, IgG2, IgG3, IgG4) differ structurally with respect to their conserved amino acid sequences, the length of the core hinge region, and the number and configurations of interchain disulfide bonds. IgG1 mAbs have been the most popular subclass used for therapeutic mAbs because they possess favorable biological and physicochemical characteristics that enhance their developability as clinical drug candidates. The cumulative knowledge of therapeutic IgG1 mAbs and their structural simplicity likely led to their selection as the preferred isotype for ADC development. However, IgG1 mAbs can elicit strong effector functions that conflict with the desired mechanism of action (MOA) of an ADC.

IgG2 and IgG4 mAbs are weak complement cascade activators and have weaker affinities for the Fcγ receptors than IgG1s, and therefore are potentially more compatible with the MOA of an ADC due to their increased possibility of cellular internalization. The IgG2 subclass includes the A, A/B, and B disulfide isoforms that are formed under common large-scale bioprocess conditions, where the isoform distribution depends on the cell culture parameters and the length of exposure of the antibody to the cell culture
Each IgG2 disulfide isoform differs in the linkages between the light chain-heavy chain (LC-HC) interchain disulfide, thereby affecting the higher-order structures, overall charge, hydrophobicity, and binding affinities. The higher-order structural differences at the Fab/C_\text{H2} interface of the IgG2 isoforms affect the solvent accessibilities of the interchain disulfides.

One consideration 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 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.

We previously reported that the IgG2-A isoform interchain disulfide bonds reduce and conjugate at a faster rate than the IgG2-B isoform. This earlier experiment was conducted at low pH to prevent disulfide bond scrambling during the conjugation process, and therefore preserve the original antibody disulfide configuration. The aim of this investigation was 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.

### Results

#### Influence of process pH on ADC conjugation profiles

IgG2-A and IgG2-B mAbs were selectively enriched and purified from a mixture of A, A/B, and B disulfide isoforms (see Supplementary Information S1). 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 8–9 followed by the addition of 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 used 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 resulting in similar average DAR values. The conjugation distributions of each ADC pool produced at each TCEP:mAb ratio were evaluated in reduced, intact, and denatured forms.

**Figure 1** compares the HC and LC drug-loading from ADCs with average DAR of ~2, 4, and 7 generated under alkaline or acidic conditions. The x-axis lists 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. 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.
acidic conditions. Both acidic and alkaline conditions resulted in predominance of conjugated HCs, indicating that the hinge cysteines were the primary conjugation sites as previously reported. ADCs generated under alkaline conditions were largely composed 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 isomers were not as pronounced compared to the ADCs generated under acidic conditions. The IgG2-A ADCs derived using acidic conditions were composed of higher amounts of multiply conjugated heavy chains (H3–H5) compared to the IgG2-Bs and the starting material. Higher DAR of ~6 was only achieved using the IgG2-A and the starting material under low pH conditions.

The average DAR was calculated from the distribution of thevariably-conjugated LC and HC. The average DARs of the A and B isoform ADCs produced under alkaline conditions were more similar compared to the ADCs produced at pH 5.3 (Figure 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.

Intact ADC analyses using hydrophobic interaction chromatography (HIC) also showed similarities between IgG2-A and IgG2-B ADCs produced under alkaline conditions, and significant differences from ADCs generated under acidic conditions (Figure 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 we were unable to quantify this species due to the poor resolution of this peak and the sloping baseline. Nevertheless, the distribution of drug-loaded species in the IgG2-A and IgG2-B ADCs generated under alkaline conditions are largely similar compared to the ADCs generated under acidic conditions (Table S2 in Supplementary Information). 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 composed 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 S2 of Supplementary Information).

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)2L0), whereas the hinge-conjugated D4 isomer would be detected as the intact antibody (LHHL) with four drug-linkers (Figure 4).

The dissociated components showed that the alkaline process yielded ADCs with similar conjugation isomers, which are mainly composed of hinge-conjugated D4 species for an average DAR ~4 using a TCEP:mAb ratio of 4 for both IgG2-A and IgG2-B. Moreover, specific chromatographic features such as the D2 doublet, the predominance of the early-eluting (HH)5L0 peak, intact D8, and (HH)10 were detected in both disulfide isomers. Contrasting with the results described previously, IgG2-A and -B ADCs generated under acidic conditions were composed of different conjugated species; however, both achieved average DARs of ~3.8 using TCEP:mAb ratios of 24 for the A isoform and 40 for the B isoform. 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 isomer generated under acidic conditions. The profiles of the IgG2-B ADCs generated at low pH were similar to the chromatograms for the IgG2-A and IgG2-B ADCs derived from reactions at pH 8.9.

The distributions of non-covalently associated species for high average DAR ADCs are shown in Figure 4(c). 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, 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 in the high average DAR samples. 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 2. Average DAR of ADCs generated under alkaline (top) and acidic (bottom) conditions as a function of TCEP:mAb ratios. Gray circles indicate IgG2-A; black circles, IgG2-B; white circles, IgG2 starting material containing a mixture of A, A/B, and B isoforms.
Characterization of intact and partially-reduced mAbs

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 generate similar conjugation profiles and average DARs for both A and B isoforms, and that profiles of both IgG2-A and IgG2-B ADCs produced at high pH demonstrated comparability to the conjugation profile of the IgG2-B ADC derived from the low pH reaction. This suggests that disulfide bond scrambling leading towards B-form conversion occurred during the alkaline process, and we therefore evaluated which reaction step could allow for scrambling. Thiol exchange between a thiol-maleimide adduct and the native disulfide bonds has been reported to be kinetically unfavorable without an excess of a thiol compound, and we thus reasoned that disulfide scrambling would be unlikely in the formulated ADC. Therefore, our focus for this inquiry were the mAbs incubated in reaction buffer with and without added reducing reagent.

Previous LC-MS studies have demonstrated that IgG2 disulfide isomers exhibit differences in hydrophobicity and can be separated using RP-HPLC. The distribution of disulfide isomers was directly evaluated for the non-reduced mAbs via a mildly-denaturating RP-UHPLC method (Figure 5), whilst the partially-reduced mAbs were analyzed using a strongly denaturing RP-UHPLC method (Figure 6). In agreement with previous observations, the B isoform eluted earlier than the A isoform in both the non-reduced and partially-reduced mAbs generated at low pH.

Incubation in reaction buffer alone did not result in conversion of disulfide isoforms (Figure 5). Therefore, in agreement with previous in vitro and in vivo studies, the rearrangement of interchain disulfides in IgG2 antibodies requires the presence of a reducing agent. The mAbs treated with three concentrations of reducing reagent are shown in Figure 6 to represent the trend throughout the entire TCEP-HCl titration curve. The profiles of the IgG2-A and IgG2-B mAbs reduced at alkaline pH were comparable after partial reduction, with respect to both retention time of the intact mAb peak and distribution of components. Moderate differences attributable to the disulfide configuration became apparent only at the higher TCEP-HCl concentrations (≥8 molar equivalents), detected as increased LC and HC with concomitant decreased mAb peak. However, the retention times of the components remained the same between the A and B isoforms.

In contrast to the partially-reduced mAbs generated at pH 8.9, differences in both the mAb peak retention time and rates of reduction were observed between the A and B isoforms treated under acidic conditions. The retention time of the intact mAb peak of the A isoform was delayed compared to the B isoform (Supplementary Figure S2), which agrees with the reported hydrophobicity differences between disulfide isoforms. A lower amount of intact mAb was detected in the IgG2-A isoform compared to the IgG2-B isoform, which reflects differences in the rate of reduction. Additionally, a higher amount of LC was detected in the A isoform at all TCEP-HCl concentrations at low pH. Disulfide scrambling in IgG therapeutics have been shown to accelerate under alkaline conditions and minimized under acidic conditions,
Figure 4. Comparisons of nRP-UHPLC chromatograms obtained from A and B isoform mAbs at pH 8.9. Left panel (A) compares the IgG2-A (gray) and IgG2-B (black) ADCs at average DAR ~3.5 and Panel B compares the IgG2-A and IgG2-B ADCs generated at low pH to a DAR ~3.8, Panel C compares the ADCs with DAR ~ 7.0 (solid lines are pH 8.9 reactions, and dotted line in (C) is the IgG2-A from the pH 5.3 reaction). The cartoon representations of the non-covalently associated components are shown in the lower right corner, where only the IgG2-A is depicted for simplicity purposes (lines represent disulfide bonds and stars represent vcMMAE). The hinge conjugations are also shown for illustrative purposes only, as the specific conjugation locations have not been determined.

Figure 5. Disulfide isoform distribution of IgG2-A (gray) and IgG2-B (black) mAb incubated in the reaction buffer for 3 hours at 37°C. The solid lines are the mAbs incubated at pH 5.3 and dashed lines are the mAbs incubated at pH 8.9.
further supporting our inference that conversion from IgG2-A to IgG2-B occurred during the alkaline conjugation process.

**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 → B conversion is revealed in the similarities of the HIC and nrRP-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.\(^{21,25,49}\)

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 7 and Supplementary Figure S3. The mAb disulfide isoforms yielded unique melting curves, similar to previous reports.\(^{21,50}\) The B isoform showed separate transitions for the C_{H2}, C_{H3}, and Fab domains, in contrast to the overlapping transitions observed in the A isoform. The largest enthalpy peak observed in both disulfide isoforms is attributed to the unfolding of the Fab domain, and the T\(_m\) of the B-isofrom Fab was 1–2 °C higher than the A isoform (Table 1). The melting temperatures (T\(_m\)) of each domain depend on the disulfide configurations, and DL conjugation significantly affected the stability of the C\(_{H2}\) domain. Additionally, a second melting transition of the C\(_{H2}\) was observed in both IgG2-A and IgG2-B ADCs. Increased drug loading resulted in destabilization of the lower-temperature C\(_{H2}\) transition for both isoforms, and increased stabilization of the higher-temperature C\(_{H2}\) transition for the A isoform only. The Fab and C\(_{H3}\) 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 (Figure S3 of Supplementary Information). It is important to note that the current data set was derived from ADCs composed of a

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**Figure 6.** nrRP-UHPLC chromatograms of partially-reduced IgG2-A (gray) and IgG2-B (black) mAbs generated by reactions at pH 8.9 (left column) and pH 5.3 (right column).
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).

**Discussion**

The conjugate distribution, average DAR, and conjugation locations are 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 affects the ADC conjugation profile is important for the design of the ADC manufacturing process.

In this study, we demonstrated that alkaline, reducing conditions similar to standard cysteine-linked manufacturing processes can lead to interchain disulfide bond rearrangement. Our characterization of the IgG2-A and IgG2-B partially-reduced mAbs and ADCs found evidence of conversion of the A → B form occurring during the partial reduction step. Specific chromatographic similarities were detected between the A and B isoforms treated at high pH and the B isoform treated at low pH. For example, fully hinge-conjugated species such as intact D8 and (HH)10 were detected under denaturing conditions in both IgG2-A and IgG2-B ADCs produced at high pH, and the IgG2-B ADC generated at low pH. Detection of these species after disruption of all hinge disulfides indicates the presence of strong non-covalent forces between the HCs, in alignment with the extensive Fab-Fab and Fab-C3H2 interactions previously reported for the B isoform.26,51 Our observed disulfide isoform conversion directionality is in agreement with previous studies that have reported conversion of the A → A/B → B forms in a reducing environment similar to physiological conditions.24,25 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. Rearrangement of the mAb to the B configuration suggests that the disulfide isoform distribution of the mAb intermediate may not necessarily be a critical attribute for IgG2 cysteine-linked ADCs generated at higher pHs. However, the pKa of the cysteinyi thiol can vary based on the local environment, and thus the 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. The ADCs generated under both conditions showed prevalent HC conjugation in the reduced chromatographic analysis and significant destabilization of the C12 domain in the thermal analysis. Drug-linker conjugation at the LC-HC cysteines would have instead resulted in higher presence of the L1 species in the reduced ADC analyses, as well as a thermal shift of the Fab domain. While alterations to the Fab thermal stability were not detected in the IgG2-A ADCs, minor Fab domain destabilization (−0.5°C) was observed in the IgG2-B ADCs. We reasoned that the post-conjugation decrease of the Fab Tm in the B isoform was due to disruptions to the non-covalent interactions between the Fab and C12 resulting from hinge conjugations, rather than an indication of the breakage of the LC-HC disulfides. Although the drug attachments were directed to the hinge region, we have not been able to determine which specific cysteines are conjugated, and reason it would be possible for the A and B isoforms to exhibit different hinge conjugation sites due to differences in local solvent accessibility.

Our findings about disulfide isoform conversion during the conjugation process could apply to other molecules that are prone to disulfide scrambling. For instance, 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. 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 and can be very challenging in heterogeneous samples, particularly if in-source deconjugation and drug-linker fragmentation are expected to occur. Nonetheless, the results of our study suggest that process characterization for ADCs using proteins that are susceptible to disulfide scrambling should evaluate the relationship between process conditions and disulfide configurations.

Materials and methods
Materials and equipment

Human IgG2k monoclonal antibody was obtained as a 25 mg/mL frozen formulated bulk in acetate/sucrose buffer produced by the Process Sciences and Manufacturing group at Agensys, Inc. High purity vcMMAE (drug-linker) was provided by the ADC Chemistry group at Agensys, Inc. as a 10 mM solution in dimethyl sulfoxide.

All chromatographic analyses were performed on a Thermo Scientific Ultimate 3000RS UHPLC with a quaternary pump, split-loop autosampler with 100 µL injection system, forced-air column compartment with pre-column heater and post-column cooler, and a diode-array detector with an analytical flow cell. Thermo Scientific Chromelone v. 6.8 software was used for data analysis. Trifluoroacetic acid (TFA, A116-10X1AMP), formic acid (FA, A11710X1-AMP), acetonitrile (CAN, BDH86340.400), and water used in the mobile phases were all HPLC or LC/MS grade and purchased from either Fisher Scientific or VWR.

Antibody partial reduction and conjugation with vcMMAE

Partial reduction and conjugation of the antibody were carried out using buffers composed of either 25 mM sodium acetate, 150 mM NaCl, 1 mM Na2EDTA, pH 5.3 (acidic buffer), or 25 mM sodium borate, 150 mM NaCl, 1 mM Na2EDTA, pH 8.9 (alkaline buffer). The antibodies were diluted to 5 mg/mL in the reaction buffer and incubated for 3 hours at 37°C in defined TCEP-HCl (Thermo Pierce, PI20491) concentrations. TCEP-HCl was removed prior to addition of vcMMAE using Amicon Ultra-0.5 10 K MWCO centrifugal devices (MilliporeSigma, UFC5010BK). The estimated free cysteine concentrations were 1 mole SH:mol TCEP-HCl at pH 8.9, and 0.5 moles SH:mol TCEP-HCl at pH 5.3. Five-fold molar excess of vcMMAE per estimated mole of SH was reacted for 1 hour at 25°C in an Eppendorf ThermoMixer at 300 RPM. The ADCs were purified by first quenching the unreacted vcMMAE with N-acetyl-L-cysteine (Sigma Aldrich, A2750) for 30 minutes at room temperature, followed by buffer exchange into acetate/sucrose buffer at pH 5.0.

Hydrophobic interaction chromatography

A Tosoh Bioscience TSKgel Butyl-NPR column (4.6 mm ID x 35 mm L) was used for HIC analysis. ADC samples were diluted in 5 M NaCl (50:50 v/v) and 50 µg were loaded onto the 35°C column. ADCs with increasing molar ratios of drug-linker to antibody eluted via a gradient from 0%–100% B over 18 minutes at 0.8 mL/min, where mobile phase A (MPA) was composed of 25 mM sodium phosphate, 1.1 M ammonium sulfate, pH 6.7, and MPB was composed of 25 mM sodium phosphate, pH 6.7 ± 25% IPA. Peak elution was monitored by UV absorbance at 214 nm and 280 nm.

Reversed-phase chromatography of mAbs and ADCs

Analyses of both partially-reduced mAbs and ADCs under reducing and non-reducing conditions were performed using an Agilent Technologies Zorbax RRHD 300SB-C8 column (2.1 mm ID x 50 mm L). Mobile phases were composed of 0.1% TFA in water (MPA) and 0.08% TFA in 90% ACN (MPB). UV detection was set to λ1 = 280 nm, λ2 = 214 nm, and λ3 = 248 nm. Reduced ADC analysis (rRP-UHPLC) was achieved by incubating the ADCs adjusted to pH – 8 in 20 mM DTT for 15 minutes at 37°C. Reduced ADCs were diluted with equal volumes of 2% FA in 50/50 ACN/water prior to loading 7.5 µg onto the 70°C column. A multi-step gradient with a flow rate of 0.8 mL/min started with a 34.5% B 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. The average DAR was calculated using the equation below based on the number of drug-linkers attached (n) to each LC or HC:
Analyses of the partially-reduced mAbs and non-reduced ADCs via nrRP-UHPLC utilized 5 µg of neat protein loaded onto a 75°C column. A gradient from 30%–60% B was applied at 0.5 mL/min over 14 minutes.

**Determination of disulfide isoform distribution**

Five micrograms of mAb diluted in 20 mM sodium acetate, 5% (w/v) sucrose, pH 5.0 were injected onto an Agilent Technologies Zorbax RRHD 300SB-C8 column (2.1 mm ID x 50 mm L), previously equilibrated at 82°C. MPA was 0.1% TFA, 2% IPA, 98% water, and MPB was 0.1% TFA, 25% ACN, 75% IPA. Disulfide isoforms were separated at 0.4 mL/min using a gradient of 25.0%–27.5% B over 6 minutes, 27.5%–28.0% B over 1 minute, and finally from 28.0%–30.0% B over 1 minute. The eluant was cooled to 30°C prior to the DAD, with UV detection set at λ = 280 nm.

**Differential scanning calorimetry**

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.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was reported by the authors.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ADC          | antibody-drug conjugate |
| DAR          | drug-to-antibody ratio |
| DL           | drug-linker |
| IgG          | immunoglobulin G |
| HC           | heavy chain |
| LC           | light chain |
| mAb          | monoclonal antibody |
| TCEP         | tris(2-carboxyethyl)phosphine hydrochloride |
| vcMMAE       | maleimidocaproyl valine citrulline para-aminobenzoic acid monomethyl auristatin E |

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