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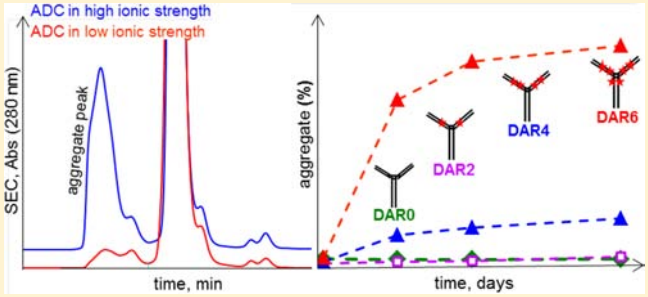
Auristatin Antibody Drug Conjugate Physical Instability and the Role of Drug Payload

Yilma T. Adem,*[,†] Kelly A. Schwarz,[†] Eileen Duenas,[‡] Thomas W. Patapoff,[†] William J. Galush,[†] and Osigwe Esue*[,†]

[†]Early Stage Pharmaceutical Development and [‡]Purification Development Department, Genentech, 1 DNA Way, South San Francisco, California 94080, United States

Supporting Information

ABSTRACT: The conjugation of hydrophobic cytotoxic agents such as monomethyl auristatin E (MMAE) to the interchain sulfhydryl groups of monoclonal antibodies (Mabs) through a protease-labile linker generates a heterogeneous drug load distribution. The conjugation process can generate high-drug-load species that can affect the physical stability of antibody−drug conjugates (ADCs). In this study, the mechanism of physical instability of ADCs was investigated by formulating the ADC pool as well as isolated drug load species in high and low ionic strength buffers to understand the effect of ionic strength on the stability of drug-conjugated Mabs. The results showed that the presence of high ionic strength buffer led to time-dependent aggregate and fragment formation of ADCs, predominantly ADCs with high-drug-load species under stress conditions. In addition, differential scanning calorimetry (DSC) results confirmed that there is a direct correlation between thermal unfolding and drug payload and that specific changes in the DSC thermogram profiles can be assigned to modifications by MMAE.



INTRODUCTION

The development of monoclonal antibodies (Mabs) has transformed the approaches of cancer treatments. In the past decades, several Mab-based therapies were approved for treating cancer patients, and their effectiveness is remarkable.[1] In recent years, drug developers have been looking into more effective and specific Mab-based cancer treatments using antibody−drug conjugates (ADCs), which consist of a cytotoxic agent linked to a monoclonal antibody. Lately, ADCs are getting special attention as treatments for various cancer types. This is mainly attributed to their specificity for target cells over normal cells, hence giving them the advantage of having a wider therapeutic index.[2,3] In recent years, two ADC molecules have been successfully approved, and many more are under development as treatments for cancer. Genentech's T-DM1 (Kadcyla) for the treatment of Her2-positive breast cancer patients and Seattle Genetics' brentuximab vedotin (ADCETRIS) for the treatment of relapsed Hodgkin and systemic anaplastic large cell lymphomas have shown great potential as part of the campaign to fight cancer.[4−6] Understanding the physicochemical properties of ADCs such as these is important, as the conjugation process and the presence of cytotoxic drugs could alter the stability of the Mabs.

The conjugation of cytotoxic drugs to antibodies could result in ADCs that are particularly susceptible to aggregate formation during manufacturing and storage. It is a challenge to keep proteins chemically and physically stable during manufacturing and during the life cycle of therapeutic drug products.[7,8] The chemical instability of proteins can be caused by oxidation, isomerization, deamidation, and disulfide reshuffling, whereas physical instability refers to degradation due to denaturation, adsorption to surfaces, and aggregation. Physical instability such as aggregation can be a limiting stability parameter for a product because it may have important efficacy and toxicity effects on patients.[9,10]

The exposure of hydrophobic surfaces of proteins is believed to cause protein−protein interactions that could lead to aggregate formation.[11,12] Conjugation of a hydrophobic cytotoxic moiety such as monomethyl auristatin E (MMAE) (calculated log $P \approx 3.1$)[13,14] to proteins may result in ADCs with an overall higher hydrophobic character [4] compared with an unconjugated antibody. In addition to protein interactions, factors such as protein concentration, temperature, agitation, and ionic strength can lead to protein aggregation.[15,16] Protein aggregation can affect the shelf life of a drug product as well as its in vivo pharmacokinetics.[9,17]

In addition to increased hydrophobicity, the conjugation of drugs to the interchain cysteines of antibodies generate ADCs that are susceptible to aggregate formation during manufacturing and storage.[18] The conjugation of large hydrophobic entities to the interchain cysteines can likely disrupt local secondary and tertiary structure, resulting in conformational

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changes. Antibodies conjugated to MMAE are generated by partially reducing the interchain disulfide bonds on the antibodies to create free sulfhydryl groups. MMAE with a valine−citrulline linker (vcMMAE) terminates in a maleimide moiety, which reacts with any free sulfhydryls on the protein, thereby covalently attaching the drug to the antibody. Since reduction of a disulfide bond produces two free cysteine residues, usually this class of ADC has a drug payload that is an even number. Depending on the conjugation process conditions, the conjugation of MMAE to Mabs' cysteine residues responsible for interchain disulfide bonds can result in a heterogeneous distribution of 0−8 drugs per antibody. Higher numbers of drugs, and thus fewer disulfide bonds to hold the quaternary structure together, can lead to physical instability of ADC molecules, especially under stress conditions.

In this work, we investigated the physical stability of ADCs by studying the relationship between the drug load distribution on Mabs and aggregate formation using ionic strength and temperature as stress conditions. We also used differential scanning calorimetry (DSC) to understand the relationship between drug load and thermal stability of Mabs. Knowledge gained from this study will help to elucidate the probable cause of physicochemical destabilization of ADC molecules during manufacturing and storage.

RESULTS

High Ionic Strength Induces Aggregate Formation of ADCs. In this study, two monoclonal antibodies conjugated with linker-attached drug (vcMMAE) were used to study the mechanism of aggregate formation and its impact on stability. The stability of Mab1-ADC (average drug-to-antibody ratio (DAR) of 3.5) formulated in both low (buffer at pH 5.5) and high ionic strength buffers (buffer at pH 5.5 with 100 mM arginine acetate) was assessed by size-exclusion chromatography (SEC) after samples were stored at 5, 30, or 40 °C for up to 8 weeks. Samples formulated in high ionic strength buffer and stored at 40 °C showed a substantial time-dependent increase in aggregate formation. Aggregation increased rapidly (~9%) within the first week and reached ~17% after 4 weeks at 40 °C. Samples stored at 30 or 5 °C showed minimal to no aggregate formation (Figure 1A). Additionally, samples formulated under low ionic strength conditions and stored at 5 or 30 °C showed no aggregate formation (data not shown), and samples stored at 40 °C showed little aggregate formation (Figure 1A,B). The SEC profile of the aggregate peaks contains dimers and significant amounts of other high-molecular-weight species (Figure 1B).

To investigate whether aggregate formation is either excipient- or ionic-strength-dependent, Mab1-ADC was dialyzed into buffer at pH 5.5 with various concentrations (0, 50, 100, and 500 mM) of either NaCl or arginine-HCl and incubated at 5, 30, or 40 °C. These results confirmed that ionic strength but not buffer species contributed to aggregation, although the extent of aggregation in NaCl-containing solutions was higher than in arginine-containing solutions (Figure 2A). Subsequently, another molecule, Mab2-ADC, was tested to elucidate whether the aggregation in high ionic strength buffer was solely restricted to Mab1-ADC. Mab2-ADC (average DAR of 3.6) showed aggregation behavior similar to that of Mab1-ADC (Figure 2B). As controls, unconjugated Mab1 and Mab2 were also dialyzed into similar buffers (buffer at pH 5.5 with 0, 50, 100, or 500 mM arginine-HCl) and incubated at 40 °C for up to 4 weeks. Small amounts of aggregate formation were

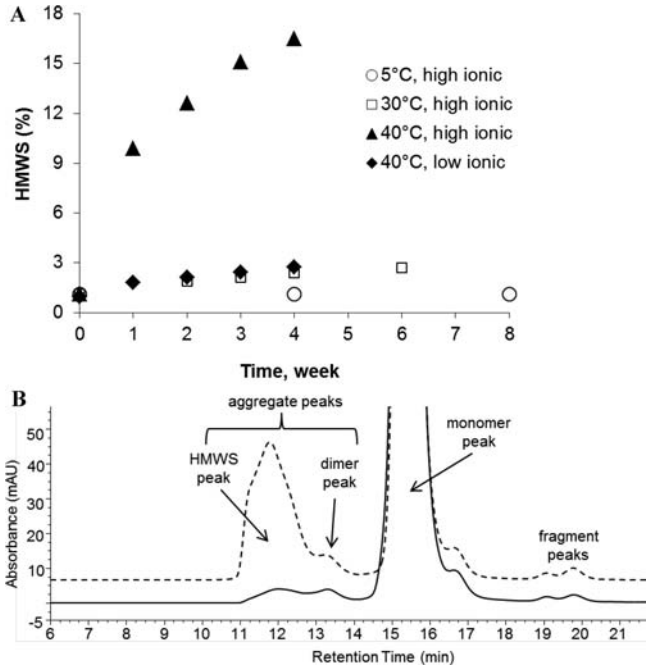

Figure 1. (A) Thermal stability of Mab1 ADCs formulated in low and high ionic strength buffers (100 mM arginine acetate) and then stored at 5, 30, or 40 °C for up to 8 weeks. (B) SEC chromatogram of Mab1-ADC formulated at low (solid line) and high (dashed line) ionic strength buffers and stored at 40 °C for 4 weeks. (HMWS = high-molecular-weight species).

observed for unconjugated molecules in comparison with Mab-ADC molecules. These results show that the enhanced aggregate formation observed is likely a general characteristic of MMAE-containing ADC molecules in high ionic strength solutions.

Despite significant differences in aggregate formation of Mab1-ADC under high and low ionic strength buffer conditions, there were similar changes in acidic charge variant formation (as assessed by imaged capillary isoelectric focusing (iCIEF) assay) for samples formulated at high versus low ionic strength (Figure 3). These results suggest that the presence of high ionic strength mainly affects the physical stability of ADC molecules.

High Drug Payload Affects the Overall Stability of ADCs. To assess the impact of each DAR species, fractions of Mab2-ADC based on DAR species were collected from semipreparative hydrophobic interaction chromatography (HIC) column runs and incubated at 40 °C for up to 14 days in buffer with no NaCl at pH 5.5 (low ionic strength) and buffer with 100 mM NaCl at pH 5.5 (high ionic strength) (Figure 4). Mab2-ADC with a mixture of DAR species ranging from 0 to 8 drugs per antibody (resulting in an average DAR of 3.6) was used as a control (Figure S1 in the Supporting Information). After 14 days at 40 °C, solutions containing isolated DAR0, 2, 4, or 6 species increased in aggregate content to 0.5%, 0.7%, 4.7%, and 18% respectively, while the aggregate content in control samples increased to 14% (Figure 4). The aggregate content of DAR8 samples increased from 1.3% to 7.7%, but the SEC profile of the monomer peak was atypical: broad, slightly split at the tip, and eluted early (Figure S2 in the Supporting Information). The control sample showed a higher percentage of aggregation than the DAR4 samples and a lower percentage than the DAR6 samples at all time points (Figure

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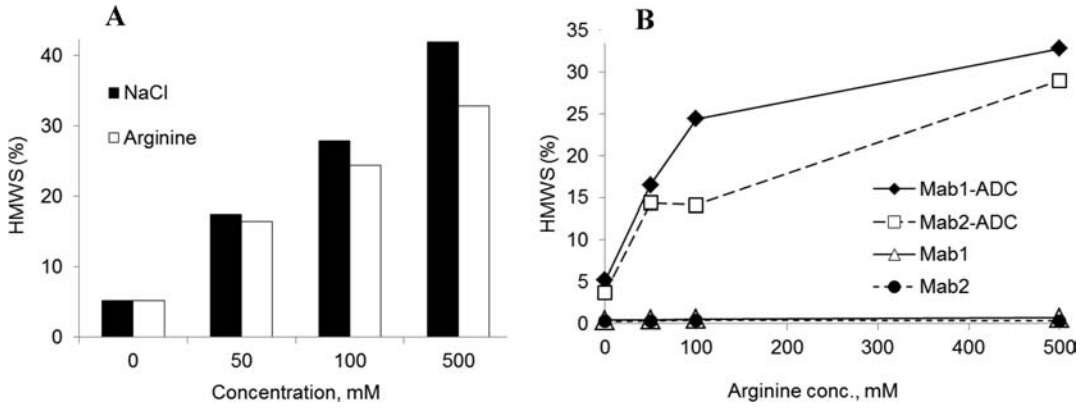

Figure 2. (A) Thermal stability of Mab1-ADC in buffer at pH 5.5 with various concentrations of either arginine-HCl or NaCl and stored at 40 °C for up to 4 weeks (SEC data). (B) Thermal stabilities of Mab1-ADC (average DAR = 3.5), Mab2-ADC (average DAR = 3.6), and unconjugated Mab1 and Mab2 formulated in various concentrations of high ionic strength buffer and stored at 40 °C for up to 4 weeks.

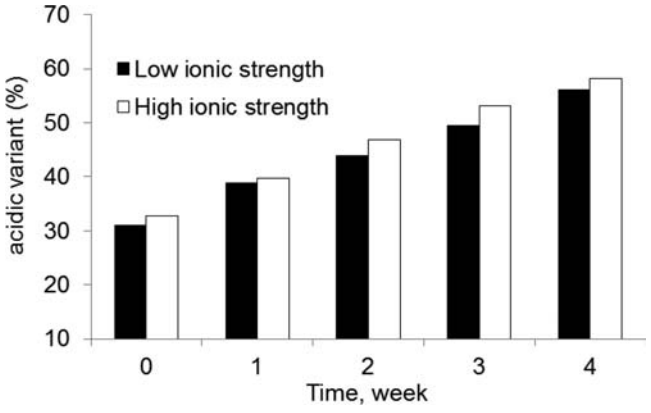

Figure 3. Charge variant analysis (as measured by iCIEF) of Mab1-ADC after formulation in either low or high ionic strength buffer and storage at 40 °C for up to 4 weeks.

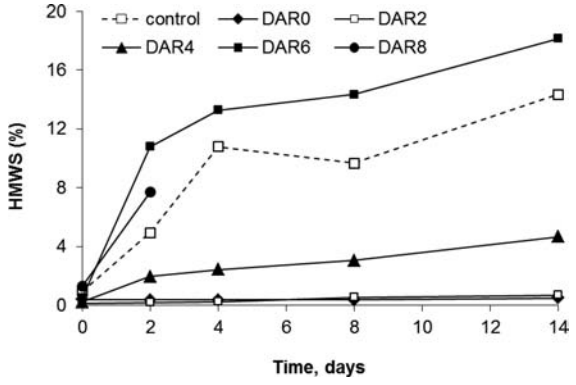

Figure 4. Thermal stabilities of isolated Mab2-ADC DAR species (DAR = 0, 2, 4, 6, and 8) formulated in high ionic strength buffer and stored at 40 °C for up to 14 days. The control was Mab2-ADC containing a mixture of 0−8 drug molecules per Mab (average DAR = 3.6).

4). The presence of NaCl resulted in higher aggregate formation in isolated DAR2 and DAR4 samples compared with DAR2 and DAR4 formulated in low ionic strength reagents (Table 1). For instance, the aggregate content of DAR2 samples increased from 0.1% to 0.5% and that of DAR4 samples increased from 0.2% to 3.1% after storage for 8 days at 40 °C when the samples were formulated in high ionic strength buffer. However, when the samples were formulated in low ionic strength buffer, the aggregate contents of DAR2 and DAR4 increased from 0.1% to 0.2% and from 0.1% to 0.5%, respectively, when the samples were stored at 40 °C for 8 days (Table 1).

Table 1. Mab2-ADC Containing DAR4 Forms More High-Molecular-Weight Species (HMWS) Than Mab2-ADC with DAR2 for Both High- (100 mM NaCl) and Low-Salt (no NaCl) Solutions; Aggregation Is More Pronounced in High Ionic Strength Solutions[a]

	HMWS (%)			
	DAR2		DAR4	
time (days)	without NaCl	with NaCl	without NaCl	with NaCl
0	0.1	0.1	0.1	0.2
2	0.1	0.2	0.4	2.0
4	0.1	0.2	0.4	2.4
8	0.2	0.5	0.5	3.1
14	–	0.7	0.6	4.7

[a]All samples were stored at 40 °C for up to 14 days.

In addition to aggregation, fragmentation or dissociation of chains of Mab2-ADC also increased with time and as the DAR increased from 0 to 8, which is consistent with decreased stability resulting from loss of disulfides due to drug conjugation. Significant fragmentation was observed in the DAR6 and 8 samples after storage at 40 °C, in contrast to DAR0, 2, and 4 samples, where fragmentation was mostly less than 1.8% (Table 2). The thermal stability of DAR6 and DAR8 samples was tested only in high ionic strength buffer because of limited sample supply. The fragmentation of DAR6 species

Table 2. SEC Data Showing Time- and DAR-Dependent Fragment Formation of Mab2-ADC Formulated in High Ionic Strength Buffer and Stored at 40°C for Up to 14 Days

	fragment (%)				
time (days)	DAR0	DAR2	DAR4	DAR6	DAR8
0	0.1	0.1	0.3	2.9	45.7
2	0.2	0.2	0.5	7.9	65.1
4	0.2	0.3	0.7	11.4	–
8	0.3	0.4	1.2	16.7	–
14	0.4	0.6	1.8	23.4	–

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increased from 2.9% to 23.4% after 14 days at 40 °C, while DAR8 fragmentation increased from 45.7% to 65.1% after only 2 days at 40 °C. Additionally, the HIC results for DAR6 samples showed a decrease in relative peak area from 82.6% to 50%, while the peak areas at the retention times of DAR0, 3, and 4 increased when samples were stored at 40 °C for up to 14 days (Table S1A in the Supporting Information). The HIC profile also showed a decrease in DAR8 peak area from 33% to 17% and an increase in the peak area at the retention time of DAR1 from 46% to 64% after 2 days of storage at 40 °C (Figure S3 and Table S1B in the Supporting Information). Furthermore, there was a significant amount of DAR8 sample loss during sample preparation.

ADC Melting Temperature Decreases with Increasing Density of Cytotoxic Drug. Differential scanning calorimetry measures the temperature-dependent unfolding of an antibody and often can separately resolve unfolding of the Fab and Fc domains. The formulation and composition of an antibody determines its thermal-induced unfolding.[19,20] Here we used DSC to monitor the thermal-induced unfolding of conjugated and unconjugated Mab1 formulated with various concentrations of either NaCl or arginine-HCl buffers. The thermogram profiles showed three melting transitions for unconjugated Mab1 and Mab1-ADC (Figure 5A). There are reports showing that the first transition peak represents the unfolding of the CH2 domain and the second and the third transition peaks represent unfolding of the Fab and CH3 domains, respectively.[19] The DSC results indicate that with increasing NaCl (or arginine-HCl, data not shown) concentration from 0 to 500 mM, the melting point transition temperature (T_m) of Mab1 (defined by the temperature at the peak maximum of the CH2 domain) decreased from 67 to 62 °C while that of conjugated Mab1-ADC decreased from 63 to 58 °C (Figure 5B). Overall, the unconjugated molecules with or without NaCl have higher T_m values compared with the conjugated molecules.

To further elucidate correlations between thermal-induced unfolding and drug payload, Mab2 with drug payloads from DAR0 to DAR8 were analyzed using DSC. The data showed that there is a DAR-dependent decrease in T_m of the CH2 domain (Figure 6A). In addition, samples formulated in solutions containing 100 mM NaCl had lower melting temperatures than samples formulated without NaCl (Figures 5B and 6A). The T_m of Mab2 samples formulated at high ionic strength decreased from 65 °C (DAR0) to 60 °C (DAR4) to 57 °C (DAR8), whereas Mab2 samples formulated at low ionic strength decreased from 67 °C (DAR0) to 62 °C (DAR4). DAR-dependent CH2 and Fab/CH3 domain DSC peak shifts and shoulder formations were observed for DAR2−8 samples at high ionic strength and DAR2 and DAR4 samples at low ionic strength (Figure 6B,C). However, the Fab/CH3 domain peak separation of DAR samples at high ionic strength showed poor resolution. The presumed origin of these shifts is discussed in the following section.

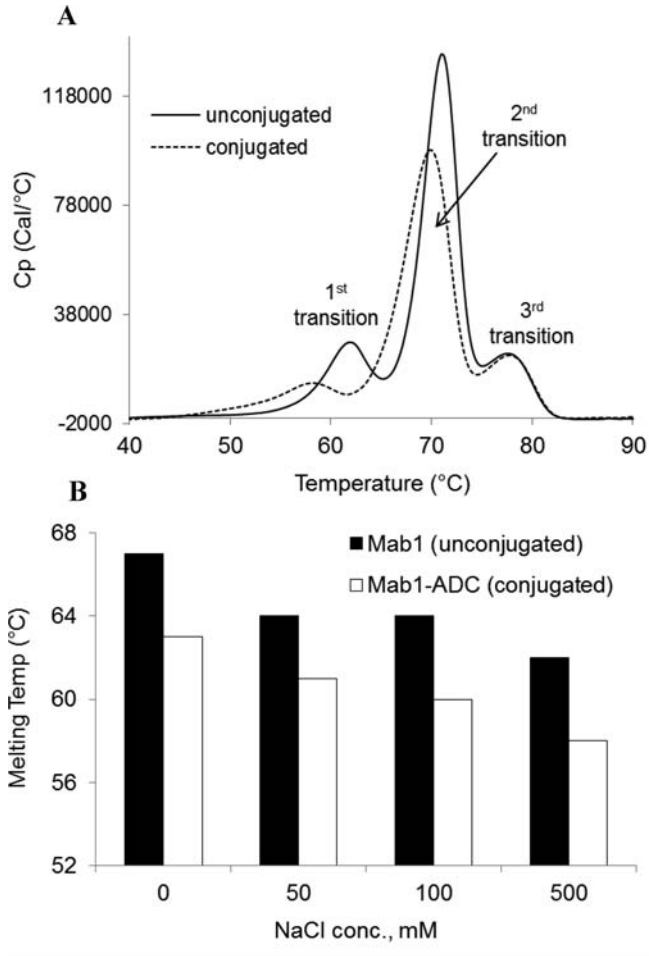

Figure 5. (A) DSC thermograms of unconjugated (solid line) and conjugated (dashed line) Mab1 formulated in high ionic strength buffer (500 mM arginine-HCl). (B) DSC data demonstrated that the melting temperature (T_m) decreased in an ionic-strength-dependent manner for both Mab1 and Mab1-ADC. T_m for Mab1-ADC was lower than T_m of Mab1 at all NaCl concentrations tested. Similar results were obtained for Mab1 formulated in arginine buffer (data not shown).

DISCUSSION

This study investigated the mechanism by which conjugation of cytotoxic drugs affects the physical stability of monoclonal antibodies. The conjugation of small-molecule drugs via the interchain disulfide bonds of Mabs as well as the introduction of hydrophobic drugs (e.g., MMAE) to the native cysteines of Mabs can trigger structural changes resulting in physical instability of Mabs.[4,17,21] The formulation of ADCs in high ionic strength solution resulted in physical instability, predominantly for samples with high DAR species. This could be caused by the decrease in the electrostatic repulsion between single molecules in the presence of high ionic strength buffer, inducing hydrophobic interactions between the molecules.[22,23] The aggregation of protein products during manufacturing and/or storage has a detrimental effect on their overall shelf life[9] and ultimately could induce an immune response in vivo.

Aggregation of Mab1-ADC and Mab2-ADC was apparent when they were incubated in high ionic strength buffers (i.e., arginine and sodium salts with salt concentrations of up to 500 mM) and thermally stressed. On the other hand, the incubation of these ADC molecules in low ionic strength buffers had minimal impact on their stability. Unlike the ADCs, when Mab1 and Mab2 (unconjugated Mabs) were incubated under high ionic strength conditions, aggregate formation was insignificant. This clearly suggests that ADCs are more susceptible to instability when formulated in high ionic strength buffers. These ADC molecules markedly aggregated when incubated in buffers containing either NaCl or arginine-HCl,

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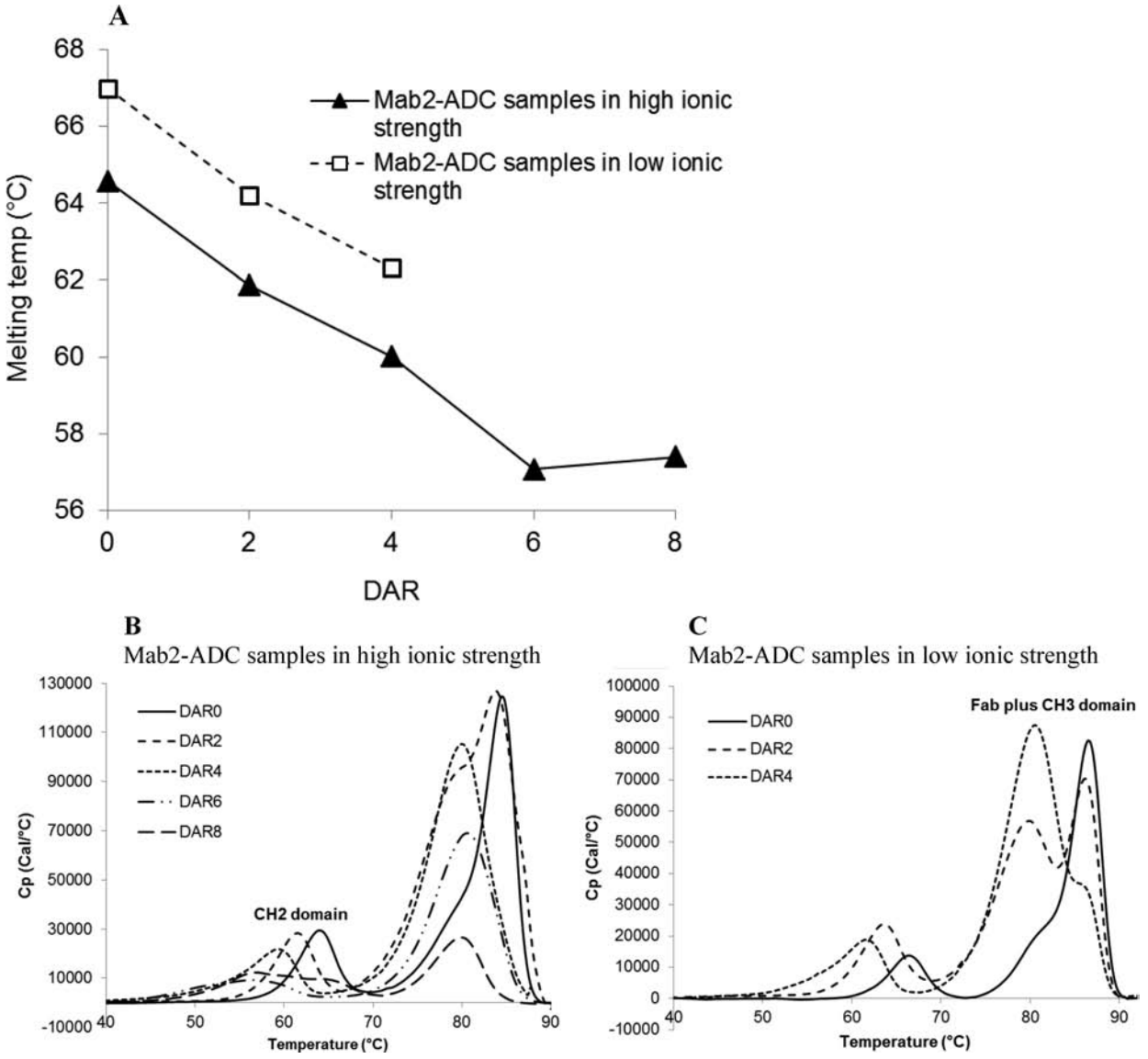

Figure 6. (A) DSC analysis of Mab2-ADC drug load fractions formulated in low and high ionic strength buffers, showing the T_m of the CH2 domain. (B, C) DSC thermograms of Mab2-ADC DAR fractions formulated at (B) high and (C) low ionic strength show T_m shifts of the CH2 domain as well as separation and/or shifts of the Fab/CH3 domains depending on the number of drug molecules attached to Mab2.

confirming that the dominant cause of aggregation is the high ionic strength and not the types of buffer species present. However, samples in arginine buffer aggregated less, mainly at high concentration, compared with samples in NaCl. This could be attributed to the preferential interaction of the guanidinium group of arginine with the protein surface.[24]

ADCs are manufactured by the reduction of the interchain disulfide bonds in Mabs, resulting in free cysteine residues that are then conjugated to the linker-containing drug (vcMMAE) through a reactive maleimide.[25] The conjugation process is known to produce a heterogeneous population of drug-loaded antibodies.[18] This knowledge initiated the investigation of the involvement of species with various drug loads in the physical stability of ADCs. The formulation of individual DAR0, 2, 4, 6, and 8 species elucidated further that the higher-DAR species can result in physical instability in ADCs. Fractions containing high-DAR species (i.e., DAR6 and 8) have higher propensity to aggregate,[26] whereas aggregation was much lower for low-DAR species (DAR2 and 4) and little aggregate formation was observed for the unconjugated Mabs. The SEC profiles of the aggregate peaks of various DAR species (not shown) were similar in size, except that the peaks broadened when DAR2 was compared with DAR4 and DAR6 following thermal stress, indicating that either oligomers formed or the aggregates interacted with the column resin matrix differently. In addition to in vitro instability, aggregated Mabs are unsuitable in vivo because of accelerated clearance, heterogeneity, and increased liver toxicity.[27] In vivo studies have shown that high-drug-load species (e.g., DAR8) have a relatively poor therapeutic index as they clear faster than low-DAR species.[28,29]

In this study, fractions with high-DAR species (DAR6 and DAR8) had higher fragmentation rates. This is not surprising since the covalently linked interchain cysteine residues that form disulfide bonds to hold the quaternary structure of an antibody together are replaced with hydrophobic cytotoxic drugs (here MMAE). In this case, attractive non-covalent interactions are vital to keeping the structure of the molecule intact. However, in a high ionic strength environment the

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attractive forces are disrupted, leaving the molecule susceptible to increased physical strain, which can result in the fragmentation of Mabs with high DAR (DAR6 and DAR8) species. In theory, high-DAR species with a fully conjugated hinge region where there are no covalent bonds to hold the molecule together could dissociate under stress conditions and form fragments such as light-chain, heavy-chain, and heavy-light-chain species.[30,31] This observation was further reinforced when HIC assay results showed a relative peak area decrease of DAR6 species over time with increases in the formation of other DAR species such as DAR0, 1, 3, and 4 (Table S1A in the Supporting Information). These HIC results also indicate that fragments of DAR6 and DAR8 elute at various retention times based on their drug payload content. This finding is in agreement with the SEC assay results showing a time-dependent increase in the fragmentation of DAR6 species (Table 2). The cause of fragmentation observed in the DAR8 (t = 0) sample is not fully understood, but it could have occurred during the conjugation or purification processes and/or could be due to the presence of high ionic strength. However, the fragmentation observed in the DAR6 species suggests that higher-DAR species are prone to physical instability, mainly under stress conditions. Overall, it can be concluded that the observed fragmentation phenomenon is consistent with the lost disulfide bonds of Mab2-ADC due to conjugation of the cytotoxic drug. Fragmentation of Mabs may change the biodistribution of a drug,[32] and this is mainly true if fragmentation occurs in immunoconjugate molecules, as it could result in loss of target binding potential of the Mab.[7] Additionally, the recovery of the Mab2 fraction containing DAR8 was lower than the other DAR fraction pools after protein concentration and handling; this is potentially due to the hydrophobic nature of DAR8 species, which could lead to binding of the protein to membranes and surfaces.[33,34]

The use of DSC to measure thermal unfolding of proteins is a tool to predict thermal stability, especially for thermally induced aggregation of proteins.[35] Excipients such as NaCl and arginine have been implicated in decreasing the melting temperature of an immunoglobulin G (IgG) molecule, which is an indicator of increased susceptibility of proteins to unfolding or aggregation.[20] This is consistent with our findings that the thermal stabilities of Mab1 and Mab1-ADC decreased with increasing ionic strength. Additionally, Mab1-ADC showed lower T_m across all the salt concentrations tested, suggesting that ADCs are structurally altered by conjugation of cytotoxic agents. Additional analysis revealed that there is a gradual decrease in T_m with increasing drug payload of Mab2 under both high and low ionic strength conditions. These data further strengthen the argument that there is a direct correlation between drug payload and T_m (and ultimately physical instability). The likelihood of changes in the secondary or tertiary structure of a Mab due to conjugation of drugs to the interchain cysteines could be small, but any structural effects could become more pronounced when samples are thermally stressed.[30] The unfolding of the CH2 domain of the high-DAR species (i.e., DAR6) at a lower T_m indicates that the secondary and tertiary structure is susceptible to change. The correlation between the unfolding of the CH2 domain of the high-DAR species, where the hinge region interchain cysteines are fully conjugated, at a lower T_m and the increase in aggregation and interchain fragmentation can be a plausible signal that structural changes have occurred.

In addition, it is possible to assign tentatively many of the features in the DSC thermograms of the different DAR fractions to location-specific modifications of Mab2. Although Mab2 lacks the three well-resolved transition peaks seen with Mab1, when it was formulated under both high and low ionic strength conditions, the peak representing the Fab/CH3 domain of the DAR2 sample split (Figure 6 B,C). Previous estimates have shown that DAR2 features approximately 95% of the drug in the Fab region,[31] implying that the left-hand portion of the split Fab/CH3 peak represents the destabilization of the Fab region due to the attachment of MMAE through the disulfide linking the heavy and light chains.

According to Le et al.,[31] approximately 57% of DAR4 molecules have the drug molecules exclusively in the Fab region, about 32% only in the hinge region, and the remainder a mixture of Fab- and hinge-linked drugs. Under low ionic strength conditions, the putative drug-containing Fab peak became larger, and the unmodified Fab/CH3 peak was reduced to a shoulder. This transition was less obvious in high ionic strength solutions, and the unmodified Fab/CH3 peak was not clearly resolved from the broad peak corresponding to drug-linked Fab. Almost all of the DAR6 molecules contained four drug molecules in the hinge region, with the other two in the Fab. DSC analysis of DAR6 samples under high ionic strength conditions revealed that the broad peak containing drug-conjugated Fab, unmodified Fab, and CH3 domains shifted slightly to higher temperature compared with DAR4. This is consistent with most of the molecules containing only a single conjugated Fab, though resolution is lacking.[31] The shift back to lower transition temperatures was seen with DAR8, in which all of the interchain hinge and Fab disulfides are conjugated with MMAE.

There was a considerable amount of change in the unfolding of the CH2 domain under both low and high ionic strength conditions. It is clear that the CH2 domain is destabilized even for DAR2 samples, and this implies that the presence of drugs in the Fab region somehow affects the CH2 domain stability, even though they are in different domains. This effect becomes more pronounced as additional pairs of drugs are added to the Mab, which is consistent with an additional destabilizing influence on the CH2 domain resulting from breaking and conjugation of the hinge disulfide bonds.

CONCLUSION

This study has demonstrated that ADC molecules degrade at higher rates than their parent molecules (unconjugated Mabs) when formulated in high ionic strength solution. Furthermore, isolated DAR fractions have different levels of physical instability that is exacerbated by the ionic strength conditions of the solution. Finally, these varied physical stability behaviors are correlated with the apparent thermal stability of the ADCs as measured by DSC. Analysis of the individual DAR species by DSC also allowed assignment of the thermal stability penalty prompted by drug conjugation to disulfides. Taken together, the results of this study depict a systematic characterization of the physical stability consequences of MMAE conjugation to MAbs. As a possible solution to the physical instability phenomena of ADCs with cytotoxic drugs conjugated to interchain cysteines, it is worth exploring the physical stability of Mabs where cytotoxic drugs are linked to engineered unpaired cysteines, such as THIOMABs.[36] This approach can provide a well-controlled drug load per antibody as well as avoid the use of the interchain cysteines, which play an

important role in keeping antibodies structurally intact. Overall, understanding of the physical stability of ADC molecules can aid in the formulation development and help the manufacture of well-characterized and controlled drug products.

MATERIALS AND METHODS

ADC Preparation and Formulation. Humanized monoclonal antibodies, Mab1 and Mab2, were constructed from an IgG1 human framework with kappa light chains having a molecular weight of approximately 150 kDa. The isoelectric points (pI's) of Mab1 and Mab2 were 8.9 and 8.5, respectively. The antibodies were expressed in Chinese hamster ovary (CHO) cell lines and purified. Both Mabs were conjugated with the cytotoxic agent MMAE through a protease-cleavable linker, maleimidocaproyl−valine−citrulline−*p*-aminobenzyloxycarbonyl (MC-VC-PAB). The Mab1-ADC was formulated in buffer at pH 5.5 either with or without 100 mM arginine acetate. Additional samples of conjugated and unconjugated Mab1 and Mab2 were dialyzed into a pH 5.5 buffer containing 0, 50, 100, or 500 mM arginine-HCl or NaCl using Slide-A-Lyzer cassettes (Thermo Scientific, Rockford, IL). Unconjugated Mab1 and Mab2 were used as controls. The samples were filled into sterile USP type-1 3 cm^3 glass vials stoppered with 13 mm FluoroTec-coated rubber stoppers and stored at 5, 30, or 40 °C for up to 4 weeks.

Isolation and Formulation of Individual DAR Species. Individual Mab2-ADC DAR species were purified with an AKTA Explorer 100 system (GE Healthcare, Pittsburgh, PA) by HIC using a 22 mL semipreparative column (1.6 cm × 11 cm) packed with Phenyl 5PW resin (20−30 μm, Tosoh Bioscience LLC, King of Prussia, PA). To prepare the load materials, batches of Mab2 were conjugated with vcMMAE to achieve a low or high average DAR (1.3 and 4.8 MMAE/Mab, respectively). To purify DAR2 species, the column was equilibrated with buffer A (25 mM sodium phosphate, pH 6.95), loaded with the low-average-DAR material diluted in buffer A, and washed with buffer A. The DAR species were eluted with an initial hold at 55% buffer B (25 mM sodium phosphate, pH 6.95, with 20% isopropyl alcohol (IPA)) for 10 column volumes (CVs) followed by a gradient to 90% buffer B over 12 CVs. The DAR0 sample used in this study was unconjugated Mab2, which was not subjected to the processing outlined above.

To purify DAR4, DAR6, and DAR8 species, the column was equilibrated with buffer C (50 mM sodium acetate, pH 5.5), loaded with the high-average-DAR material diluted in buffer C, and washed with buffer C. The DAR species were eluted with an initial hold at 55% buffer D (50 mM sodium acetate, pH 5.5, with 30% IPA) for five CVs followed by a gradient to 92% buffer D over 20 CVs.

The purifications were performed at room temperature at a linear flow rate of 150 cm/h, loaded to a density of 3−5 mg/mL of resin, and monitored at 280 nm. Fractions were collected during the elution phases, analyzed by HIC−HPLC and SEC−HPLC as described below, and pooled on the basis of the results of the analyses. Each of the individual DAR species samples was split in two and buffer-exchanged using Slide-A-Lyzer dialysis cassettes. DAR0, 2, and 4 samples were dialyzed into buffer at pH 5.5 or buffer at pH 5.5 with 100 mM NaCl. However, DAR6 and DAR8 samples were formulated only into buffer at pH 5.5 with 100 mM NaCl because of limited sample supply. After removal from the cassettes, the samples were concentrated using Amicon Ultra 15 (cat no. UFC901024) to approximately 5 mg/mL. The purity of each DAR fraction, following the aforementioned processing, was assessed by HIC and found to be 90% for DAR2 and DAR4 and 84% for DAR6. Despite its elution time, the DAR8 fraction consisted mostly of non-DAR8 species, specifically 46% DAR1, 17.5% DAR6, and 3.5% others. This may be due to instability of DAR8 molecules through this isolation procedure. Samples were filled into USP type-1 3 cm^3 glass vials stoppered with 13 mm FluoroTec-coated rubber stoppers and placed on stability at 40 °C for up to 14 days. Samples were removed at 0, 2, 4, 8, and 14 days for analysis. The MAb2 ADC (conjugated) sample with average DAR of 3.6 and unconjugated Mab2 were used as controls.

Protein Concentration Measurement. The protein concentration of Mabs was assessed using an Agilent 8453 diode array spectrophotometer with a 1 cm quartz cuvette. The concentration was calculated using an absorptivity of 1.7 or 1.74 cm^{-1} $(mg/mL)^{-1}$ for Mab1 or Mab2, respectively, as determined by quantitative amino acid analysis.

Size-Exclusion Chromatography. Size-exclusion assays of all samples were carried out using an Agilent 1200 HPLC system with a Tosoh TSK G3000 SW_{XL} column (7.8 mm × 30 cm, 5 μm particle size). All samples were injected without dilution. The mobile phase consisted of 0.2 M potassium phosphate and 0.25 M potassium chloride (pH 6.95) with 5% IPA. An isocratic elution at a flow rate of 0.5 mL/min for a run time of 30 min was applied. UV detection at a wavelength of 280 nm was used to trace protein peaks, and analysis was done using a Chromeleon version 6.8 chromatography data system (Dionex Corporation, Sunnyvale, CA).

Charge Variant Analysis by Imaged Capillary Isoelectric Focusing. iCIEF was performed on an iCE280 Analyzer (ProteinSimple, Toronto, ON) equipped with a fabricated cartridge consisting of a 5 cm × 100 μm I.D. fluorocarbon-coated capillary connected to electrolytes through hollow dialysis membranes. The anolyte was 80 mM phosphoric acid and the catholyte was 100 mM NaOH, each prepared with 0.1% methylcellulose. Mab1-ADC samples were prepared by dilution in deionized water. An ampholyte mixture containing 2.5% (v/v) Pharmalytes, 2 M urea, 0.35% (v/v) methylcellulose, and pI 7.65 and 9.77 markers was combined to obtain a final concentration of 0.25 mg/mL. Final solutions were mixed and centrifuged at 11200*g* for 3 min. Samples were introduced to the capillary through a PrinCE microinjector and focused for 1 and 10 min at 1500 and 3000 V, respectively. Electropherograms were imaged with a UV detector at 280 nm.

Hydrophobic Interaction Chromatography. Analysis of the DAR distributions of conjugated Mab1-ADC and Mab2-ADC fractions was accomplished using an Agilent 1200 HPLC system with a butyl-NPR column (4.6 mm I.D. × 3.5 cm, 2.5 μm particle size) (Tosoh Bioscience, Montgomeryville, PA). The method consisted of a linear gradient from 100% mobile phase A (1.5 M ammonium sulfate, 25 mM sodium phosphate, pH 6.95) to 100% mobile phase B (25 mM sodium phosphate, pH 6.95, with 25% IPA) over 18 min. The flow rate was 0.8 mL/min, the column temperature was 25 °C, and detection was followed at 280 nm. A maximum of 5 μL (50 μg) of each sample was loaded on the column. A Chromeleon version 6.8 chromatography data system (Dionex Corporation) was used to analyze chromatograms.

Differential Scanning Calorimetry. Thermal analysis of unconjugated Mab1 and Mab2, Mab1-ADC, and Mab2-ADC fractions with DARs of 0, 2, 4, 6, and 8 was performed using a high-throughput VP Capillary differential scanning calorimeter

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(MicroCal, Northampton, MA). Mab1, Mab1-ADC, Mab2, and Mab2-ADC fractions were diluted to 1 mg/mL (DAR2 and DAR4), 0.8 mg/mL (DAR6), and 0.5 mg/mL (DAR8) in their respective buffers. Samples were loaded into a 96-well plate, alternating with wells containing 500 μL of formulation buffer. The instrument scanned each sample−buffer pair over the temperature range 15−95 °C at a rate of 1 °C/min. Data analysis was performed using Origin software (OriginLab, Northampton, MA).

■ ASSOCIATED CONTENT

Supporting Information

Table S1 and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: (650) 467-2389. Fax: (650) 225-8951. E-mail: adem.yilma@gene.com.

*Phone: (650) 467-6112. Fax: (650) 225-8951. E-mail: esue.osigwe@gene.com.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADC, antibody drug conjugate; DAR, drug-to-antibody ratio; IgG, immunoglobulin G; vcMMAE, valine−citrulline−monomethyl auristatin E; HIC, hydrophobic interaction chromatography; SEC, size-exclusion chromatography; DSC, differential scanning calorimetry; Mab, monoclonal antibody; Mab1-ADC and Mab2-ADC, conjugated Mab1 and Mab2; CH2, constant heavy chain domain 2; CH3, constant heavy chain domain 3; Fab, fragment antigen-binding; T_m, melting temperature.

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