

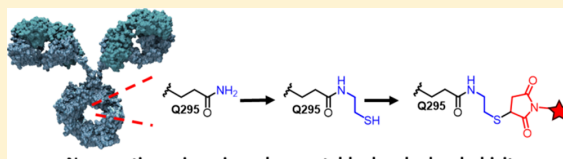
Thiolation of Q295: Site-Specific Conjugation of Hydrophobic Payloads without the Need for Genetic Engineering

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Supporting Information

ABSTRACT: Site-specific conjugation technology frequently relies on antibody engineering to incorporate rare or non-natural amino acids into the primary sequence of the protein. However, when the primary sequence is unknown or when antibody engineering is not feasible, there are very limited options for site-specific protein modification. We have developed a transglutaminase-mediated conjugation that incorporates a thiol at a “privileged” location on deglycosylated antibodies (Q295). Perhaps surprisingly, this conjugation employs a reported transglutaminase inhibitor, cystamine, as the key enzyme substrate. The chemical incorporation of a thiol at the Q295 site allows for the site-specific attachment of a plethora of commonly used and commercially available payloads via maleimide chemistry. Herein, we demonstrate the utility of this method by comparing the conjugatability, plasma stability, and in vitro potency of these site-specific antibody–drug conjugates (ADCs) with analogous endogenous cysteine conjugates. Cytotoxic ADCs prepared using this methodology are shown to exhibit comparable in vitro efficacy to stochastic cysteine conjugates while displaying dramatically improved plasma stability and conjugatability. In particular, we note that this technique appears to be useful for the incorporation of highly hydrophobic linker payloads without the addition of PEG modifiers. We postulate a possible mechanism for this feature by probing the local environment of the Q295 site with two fluorescent probes that are known to be sensitive to the local hydrophobic environment. In summary, we describe a highly practical method for the site-specific conjugation of genetically nonengineered antibodies, which results in plasma-stable ADCs with low intrinsic hydrophobicity. We believe that this technology will find broad utility in the ADC community.



No genetic engineering, plasma stable, low hydrophobicity

KEYWORDS: antibody drug conjugate, conjugation, ADC, transglutaminase, hydrophobicity, HIC, plasma stability, brequinar

INTRODUCTION

There are currently four antibody–drug conjugates (ADCs) that have been approved for marketing by the FDA and more than 80 additional ADCs in clinical trials.¹ While there have been setbacks in recent years due to unexpected toxicity,^{2–4} interest in the field continues to grow as new modes of payload release, new mechanisms of internalization, and new payload classes are explored.^{5–7} The success of immuno-oncology drugs is beginning to influence ADC design, and early reports of ADCs that deliver immune-activating agents are emerging.^{8,9} Furthermore, the technological advancements that have enabled the current cohort of oncology ADCs have facilitated the preclinical development of ADCs that target other therapeutic areas such as autoimmune disorders, rare diseases, ophthalmology, infectious diseases, and even pain management.^{10–14} As the field grows, there continues to be a need to identify new technologies that can be rapidly deployed for the preparation of ADCs to explore these possibilities.

ADC technology relies on an antibody to selectively deliver a payload of interest to an antigen-expressing cell type. Upon binding to the antigen, the ADC is internalized by clathrin-mediated uptake and trafficked to the lysosome wherein the

ADC linker and/or backbone is degraded, thus releasing the free payload. Upon lysosomal escape, the payload is able to engage with its intracellular target.^{1,15,16} This elegantly simple mechanism is greatly complicated by a number of factors including antigens that are poorly internalized, heterogeneous and/or low expression of antigen, poor lysosomal escape, linkers that are inefficiently cleaved, payloads that are actively effluxed from the cell, and payload metabolism and deconjugation in circulation. These are areas of active exploration in current ADC research and are significant factors that are taken into consideration during the design of potential ADC therapeutic agents.

One ADC design factor that has seen rapid development over the past 10 years is the site of conjugation. Traditional (first generation) ADCs typically relied on heterogeneous methods of conjugations to endogenous cysteine and lysine residues. Such ADCs, while therapeutically useful, have

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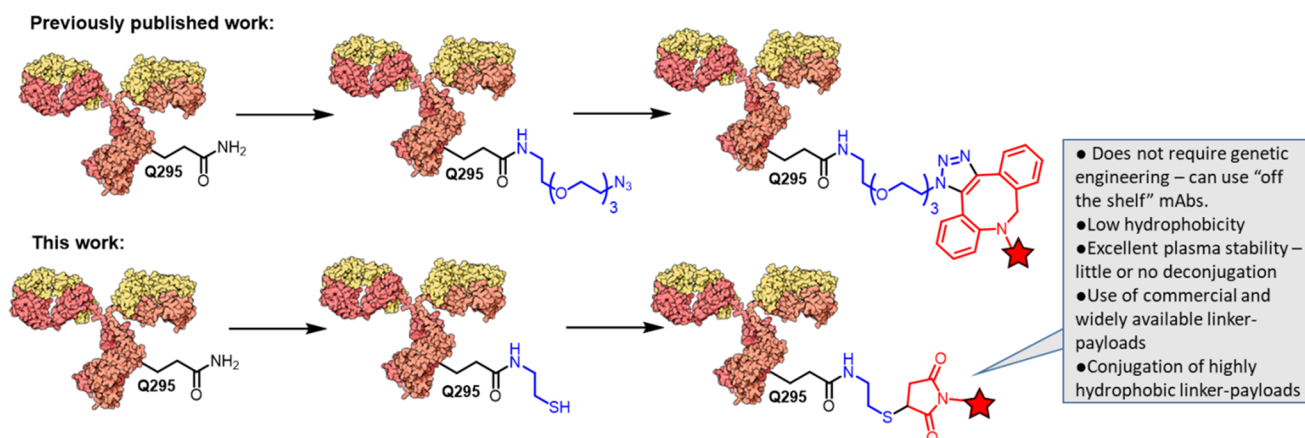


Figure 1. Comparison of the present work to previously published site-specific conjugation at Q295.

limitations due to product heterogeneity and instability. The variously loaded species in the drug product typically have different pharmacological and pharmacokinetic properties that complicate therapeutic development.¹⁷ Thus, there have been tremendous research efforts into site-specific conjugation technology that can result in a single homogeneous ADC species. Typically, such site-specific conjugation relies on antibody engineering to introduce an exposed cysteine residue,^{18–20} an unnatural amino acid,^{21,22} or a short peptide that is recognized by a ligation enzyme.^{23,24} These methods have been successfully employed in a variety of clinical and preclinical ADC programs.

However, the disadvantage of such antibody-engineering approaches is that they require site-directed mutagenesis and/or other recombinant DNA technology to express a modified antibody of interest. This can be time-consuming, resource-intensive, and outside the technical skill sets of certain research labs. Given the plethora of antibody therapeutics that have been clinically explored and the ready availability of high-quality antibodies from commercial sources, it seems that site-specific conjugation technology that does not require protein engineering would be of tremendous benefit. Indeed, there have been several of such approaches that have been reported in the past few years including disulfide rebridging,²⁵ glycosyl group modifications,²⁶ and selective lysine conjugations.^{27,28}

One particularly attractive conjugation approach for the site-specific modification of native antibodies has been the microbial transglutaminase-mediated attachment of amine-containing payloads to the Q295 position of deglycosylated antibodies.^{29,30} Antibody glycosylation at Asn297 sterically conceals a reactive Q295 glutamine group, which can be exposed by deglycosylation with PNGase F, thus enabling facile and highly selective conjugation at this residue. Contrary to early speculation, antibody glycosylation has been clearly demonstrated to be inconsequential for ADC pharmacokinetics.^{31,32} Thus, a number of deglycosylated Q295 conjugates have shown excellent activity in *in vivo* efficacy models.^{32,33} However, attachment of large hydrophobic payloads at the Q295 position has proven to be problematic.²⁹ In particular, the transglutaminase reaction is often slow and is highly sensitive to organic cosolvents. Hydrophobic payloads often require 10–20% of organic cosolvent to maintain solubility during the conjugation step, thus preventing efficient employment of transglutaminase-mediated reactivity. For this reason, there have been several reports of “click chemistry”-enabled

conjugations at the Q295 position wherein a small azide or alkyne tag is first attached to the Q295 position using transglutaminase chemistry, and the hydrophobic payload is subsequently attached using a higher percentage of organic cosolvent (Figure 1). One particularly elegant example of this chemistry was reported by Tsuchikama wherein a dendritic azide-containing linker was introduced by transglutaminase chemistry, and multiple payloads were subsequently “clicked” into place resulting in an ADC with four payloads attached at the Q295 positions.³⁴

While this approach is useful, it necessitates the presence of a vestigial “click” structural element that remains in the linker that may impart unnecessary and perhaps detrimental hydrophobicity (Figure 1, top). Moreover, this chemistry requires the synthesis of click chemistry-enabled payloads, a feat that may be time-consuming and beyond the technical capabilities of many labs. We envisioned attaching a short-chain thiol at the Q295 position to facilitate the direct site-specific conjugation of widely available maleimide linker-payloads. In an earlier attempt described by Schibli *et al.*,²⁹ an *S*-acetyl protecting group was employed during the conjugation, which was then followed by a hydroxylamine-mediated deprotection step. However, this led to incomplete deprotection and poor drug loading.²⁹ Herein, we report the successful site-specific incorporation of a thioethyl moiety at the Q295 position using a previously reported transglutaminase inhibitor, cystamine.

■ EXPERIMENTAL SECTION

General Methodology. Tritosomes were obtained from XenoTech, mouse serum from Equitech-Bio, and MMAE from BroadPharm. Biosimilar rituximab, adalimumab, and trastuzumab were purchased from SydLabs. Coltuxumab was a gift from Immunogen. The trastuzumab cysteine mutants were gifts from Pfizer. Microbial transglutaminase was obtained from Modernist Pantry (Ajinomoto, Activa TI) and consists of ~0.75–1% protein by mass; PNGase F was obtained from Bulldog Bio. Raji and Ramos cell lines were purchased from ATCC. The SKBR3 cell line was a generous gift from Dr. Susan Bane and was originally purchased from ATCC.

LCMS Analysis. Small molecule and protein LCMS was performed as described in the [Supporting Information](#).

Size Exclusion Chromatography (SEC). SEC analysis was performed on a Waters Acquity H-class with a TUV detector using a BEH200 SEC column (4.6 mm × 150 mm,

1.7 μM). Analysis was performed at room temperature using an isocratic gradient of phosphate buffer (50 mM, pH 7.4) containing 15% acetonitrile at 0.30 mL/min. The elute was monitored by UV at both 220 and 280 nm. Under these conditions, the antibody eluted at 4.15 min, and any aggregate eluted at ~ 3.8 min. Excess small molecules eluted at approximately 6.9 min.

Hydrophobic Interaction Chromatography (HIC). HIC analysis was performed on a Bio-Rad NGC Quest 10 Plus Pro. Samples (typically in PBS) were diluted 1:1 with 3 M ammonium sulfate buffer. Separation was achieved using a TOSOH TSKgel Butyl-NPR (4.6 mm $\times$ 10 cm) column using a gradient from 100% 1.5 M ammonium sulfate (pH 8) to 100% PBS (pH 7.4) containing 15% isopropanol. Flow rate = 0.7 mL/min; gradient volume = 10 mL. The eluent was monitored at 220, 254, and 280 nm.

Typical Q295 Thiolation and Conjugation Conditions.

Step 1: Deglycosylation. The antibody (10 mg) was treated with 2.5 μg of PNGase F (Bulldog Bio) and diluted to a final concentration of 10 mg/mL in PBS. The antibody was incubated at 37 $^{\circ}\text{C}$ overnight. LCMS analysis indicated that the deglycosylation was complete.

Step 2: Conjugation with Cystamine. The deglycosylated antibody was diluted with 2.4 mL of 50 mM phosphate buffer (pH 6.0) and treated with 200 μL (100 equiv) of 30 mM aqueous cystamine solution followed by 700 mg of trans-glutaminase. The reaction was vortexed extensively until the TG powder was completely dissolved. The reaction was allowed to stand at 37 $^{\circ}\text{C}$ for 48 h. The reaction was loaded onto 10 prewashed protein A SpinTrap columns (~ 1 mg/column) following the manufacturer's instructions. After washing extensively with PBS to remove the excess TG and cystamine, the loaded antibody was eluted with 400 μL of glycine buffer (pH 2.7) and immediately neutralized with 30 μL of 1 M Tris buffer (pH 9.0). The combined samples were buffer exchanged into PBS by ultrafiltration. Loading was evaluated by LCMS.

Step 3: Disulfide Reduction. The antibody from step 2 was treated with 50 mM TCEP (50 equiv) and left at room temperature for 2 h. The excess TCEP was removed by ultrafiltration with PBS, and the resulting material was allowed to stand at 4 $^{\circ}\text{C}$ for 16 h in order to reoxidize the endogenous disulfide bonds. A small aliquot (20 μL) was removed and treated with a maleimide payload (1 μL of 20 mM stock). After 1 h, the aliquot was treated with TCEP and evaluated by LCMS to determine when the reoxidation was complete. When the above analysis showed that the reoxidation was complete (typically ~ 16 h), the samples were either used immediately in a conjugation reaction (step 4) or were frozen for preservation at -80 $^{\circ}\text{C}$. The typical yield for the 3-step process is 50–70%. Note: In cases where the antibody was underloaded due to overoxidation, step 3 was repeated with a shorter reoxidation period.

Step 4: Conjugation with a Maleimide Linker-Payload. The prepped antibody (in PBS) from step 3 was treated with 10 equiv of a maleimide linker-payload (LP) (as a 10 mM stock solution in DMA). Sufficient DMA was added to bring the composition of the reaction buffer to $\sim 10\%$ DMA. After standing for 2 h at rt, the material was buffer-exchanged via a sephadex column to remove excess LP. The resulting material was filter-sterilized, and the protein concentration was determined by nanodrop, aggregation content was determined

by SEC, and loading and mass shifts were determined by LCMS.

General Conjugation Conditions for Engineered Cysteine ADCs. The engineered cysteine antibody was treated with 100 equiv of 50 mM TCEP. After allowing the solution to stand at 37 $^{\circ}\text{C}$ overnight, the excess TCEP was removed by sephadex filtration, and the resulting mAb was treated with 1% by volume of 50 mM DHA solution (~ 50 equiv). After standing at 4 $^{\circ}\text{C}$ overnight, the antibody was buffer-exchanged into PBS using sephadex filtration. The prepped antibody was treated with 10 equiv of a maleimide LP (as a 10 mM stock solution in DMA). Sufficient DMA was added to bring the composition of the reaction buffer to $\sim 10\%$ DMA. After standing for 2 h at rt, the material was buffer-exchanged via a sephadex column to remove excess LP. The resulting material was filter-sterilized, and the protein concentration was determined by nanodrop, aggregation content was determined by SEC, and loading and mass shifts were determined by LCMS.

General Conjugation Conditions for Endogenous Cysteine ADCs. The antibody (in PBS) was treated with 4 equiv of 5 mM TCEP. After heating for 1 h at 37 $^{\circ}\text{C}$, the antibody was treated with 15 equiv of a maleimide LP (as a 10 mM stock solution in DMA). Sufficient DMA was added to bring the composition of the reaction buffer to $\sim 10\%$ DMA. After standing for 2 h at rt, the material was buffer-exchanged via a sephadex column to remove excess LP. The resulting material was filter-sterilized, and the protein concentration was determined by nanodrop, aggregation content was determined by SEC, and loading and mass shifts were determined by LCMS.

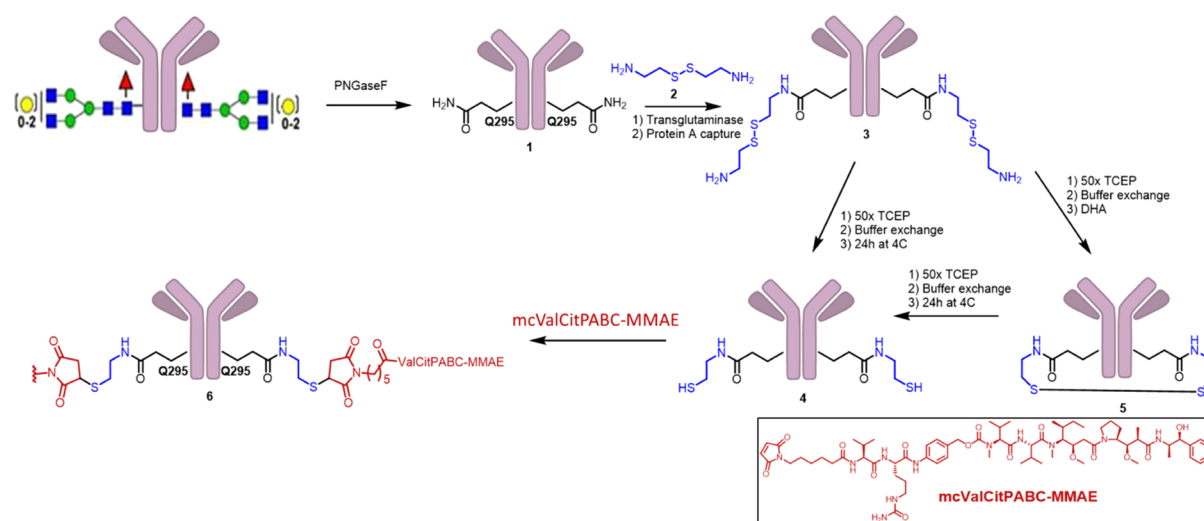
Cytotoxicity Studies in Raji, Ramos, and SKBR3 Cells.

The Raji and Ramos cell lines were cultured in RPMI1640 media (Sigma-Aldrich) supplemented with 10% FBS (Corning). The SKBR3 cell line was cultured in McCoy 5A media (Lonzo) supplemented with 10% FBS (Corning). Cells were harvested for cytotoxicity studies during the exponential growth period.

The Raji and Ramos cell lines were seeded in a 96-well plate with a density of 1 million cells per milliliter, $\sim 100,000$ cells per well. A serial dilution of the ADC was performed (2.5 times per step), and the resulting ADCs were spiked into the cells giving a typical maximum test ADC concentration of 100 $\mu\text{g/mL}$. For the SKBR3 cell line, cells were digested from the vessel using Trypsin–EDTA solution (0.25% Trypsin/0.53 mM EDTA, VWR) then seeded into 96-well plates with a seeding density of 0.5 million cells per milliliter ($\sim 50,000$ cells/well). Cells were allowed to attach for 6 h, unattached cells were removed, and the attached cells were treated with ADCs as above. All ADC-treated cells were incubated at 37 $^{\circ}\text{C}$ under 5% CO_2 for 72 h then assessed using an XTT kit (Biotium). Absorbance results were obtained using a SPECTROMAX microplate reader at 460 and 630 nm. IC_{50} results were generated using GraphPad Prism 7 software, the log (inhibitor) against the response with a variable slope (three or four parameters) formula.

Plasma Stability and Glutathione Stability Experiments. BODIPY FL maleimide (Thermo Fisher) was conjugated to the engineered or thiolated antibodies using the aforementioned methods. Under sterile conditions, 0.04 mg of each ADC was placed in 613 μL of mouse serum or 0.5 mM glutathione solution. The resulting solution was diluted to 800 μL with PBS to give a final concentration of 0.05 mg/mL.

Scheme 1. Synthetic Scheme Illustrating Typical Reaction Conditions Used for Conversion of Native Antibodies to Thiolated Q295 Conjugates



The solutions were incubated at 37 °C under 5% CO₂ for 6 days. Aliquots (200 μ L each) were removed at 0, 1, 2, 3, and 6 days and immediately frozen at -80 °C. The transfer of the BODIPY tag to albumin or glutathione was determined by SEC with fluorescence detection (λ_{ex} = 480 nm, λ_{em} = 520 nm). GSH–BODIPY eluted as two peaks (isomers) at ~ 7 –9 min while the antibody–BODIPY conjugate eluted at ~ 4 min.

Plasma Release of MMAE. The following site mutants of site-specific anti-Her2 ADCs containing an mcValCitPABC-MMAE linker-payload were assessed for mouse serum stability: Q295-thiol (DAR = 1.7), 334C (DAR = 1.5), 114C (DAR = 2.0), 421C (DAR = 1.9), 388C (DAR = 1.9), 347C (DAR = 2.0), and 443C (DAR = 1.7).¹⁹ The ADC (20 μ g) was diluted in 308 μ L of mouse serum and diluted to 400 μ L with PBS to achieve a final concentration of 50 μ g/mL. The samples were incubated at 37 °C in a 5% CO₂ atmosphere. One-hundred-microliter aliquots were taken at 0, 72, and 168 h and frozen immediately at -80 °C to prevent further reaction. Ten-microliter aliquots of these stocks were treated with 40 μ L of ACN, vortexed, and then centrifuged at 10,000 rpm for 5 min. The supernatant (40 μ L) was diluted with 40 μ L of water prior to LCMS analysis (20 μ L injection) using the aforementioned LCMS conditions. MMAE was detected by MS using selected ion recording (SIR) under electrospray positive ionization (ESI+) for the 718.5 m/z ($M + H$) ion. The AUC of the MMAE peak was obtained for each ADC at each timepoint. MMAE release is expressed as a percentage of the maximal observed AUC versus time.

Quantitative Analysis of 7-Day (168-Hour) Mouse Serum Samples. Ten-microliter aliquots of the 168 h samples above were reprocessed using the above protein-precipitation sample preparation. A serial dilution of 10 mM MMAE (in DMSO) was performed into 1:1 ACN/water to permit quantitation. MMAE was detected using the SIR method outline above (20 μ L injection). The standard curve was found to be linear in the range of 10 μ M to 10 nM. (Figure S2, R^2 = 0.99). Using this standard curve, the AUC of each plasma sample was converted to an MMAE concentration. Samples were injected in triplicate, and the mean MMAE concentration (nM) $\pm$ standard deviation was reported for each ADC. The theoretical maximum release of MMAE for each ADC was ~ 60 nM assuming 100% linker cleavage.

Tritosomal Release of MMAE. Tritosomes (XenoTech) are lysosomal enzymes isolated from rat hepatocytes by density centrifugation. The following site mutants of site-specific anti-Her2 ADCs containing an mcValCitPABC-MMAE linker-payload were assessed for tritosomal stability: Q295-thiol (DAR = 1.7), 334C (DAR = 1.5), 114C (DAR = 2.0), 421C (DAR = 1.9), 388C (DAR = 1.9), 347C (DAR = 2.0), and 443C (DAR = 1.7).¹⁹ Ten micrograms of each ADC was buffer-exchanged into 300 μ L of pH 4.7 NaOAc buffer and concentrated to a volume of 30 μ L (~ 0.333 μ g/ μ L). The tritosomes (1 μ L of 2.5 mg/mL stock) were activated by treatment with 11 μ L of 2 mM DTT (in pH 4.7 NaOAc buffer) and 8 μ L of pH 4.7 NaOAc buffer. After incubation for 15 min at 37 °C, the activated tritosomes (2.5 μ g) were added into 30 μ L of ADC to achieve a total reaction volume of 50 μ L. The reactions were performed in parallel in a 96-well plate format. The plate was placed into an incubating shaker at 37 °C. Five-microliter aliquots were obtained at 1, 5, 15, 30, 60, 120, and 240 min. The aliquots were directly pipetted into 45 μ L of ACN (in a separate 96-well plate) to quench the reaction. After all aliquots were obtained, the samples were evaporated by SpeedVac and stored at -80 °C until analysis. Prior to analysis, the samples were reconstituted in 50 μ L of 90% ACN in water. The plate was sealed and centrifuged at 1000 rpm for 5 min. The supernatant (40 μ L) was removed from each sample, and the relative MMAE concentration in each sample was determined using the method described above. MMAE release was expressed as a percentage of the maximal observed AUC versus time.

Extrinsic Fluorescence Studies. Twenty-five microliters of each site-specific ADCs or mAb (220–800 μ g/mL) was treated with 50 μ L of bis-ANS (160 μ M in water) and 25 μ L of PBS in a 96-well plate. After 30 min of incubation in the dark, the fluorescence was recorded (λ_{ex} = 385 nm, λ_{em} = 500 nm) using a SpectraMax i3x. Background fluorescence was subtracted from each well. The data was normalized by dividing by the protein concentration as determined by nanodrop.

Normalized Dansyl Fluorescence. mc–ODansyl was conjugated to the engineered or thiolated antibodies using the aforementioned methods. SEC was conducted as previously described, and the fluorescence AUC of the monomeric peak

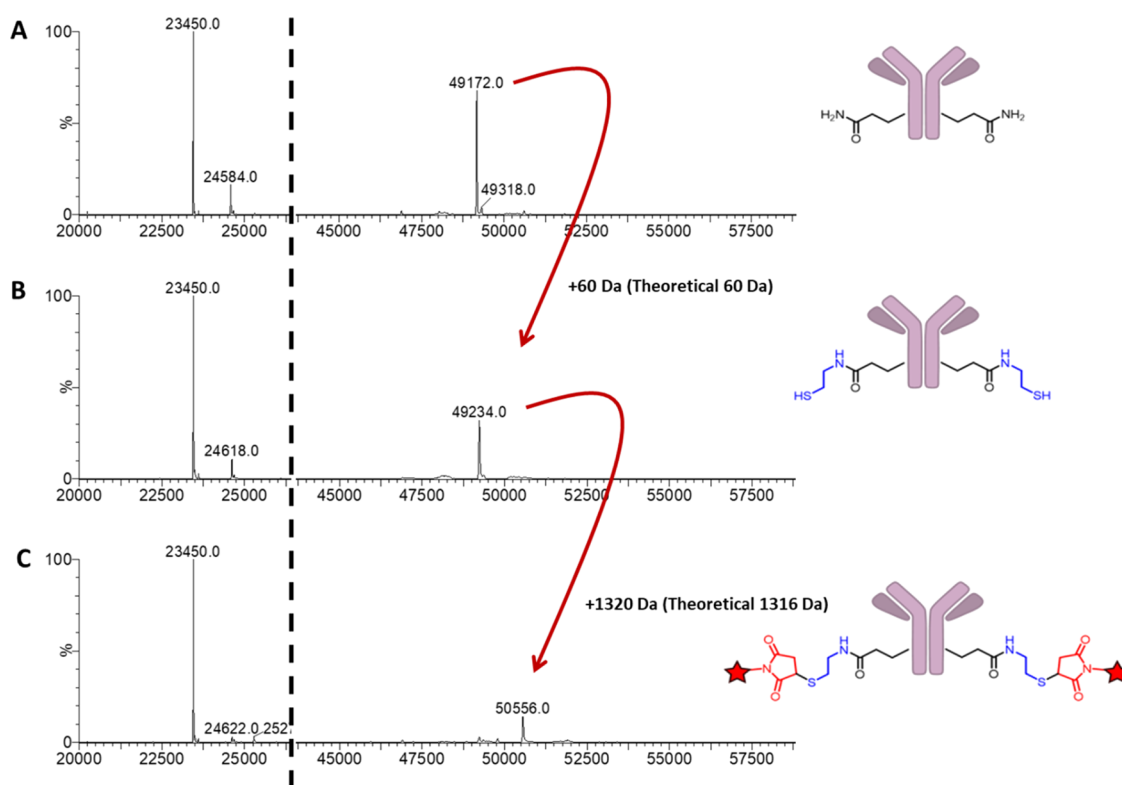


Figure 2. LCMS data demonstrating clean conversion from (A) the deglycosylated antibody to (B) the cystamine-modified antibody to (C) the vcMMAE-modified antibody.

($\lambda_{\text{ex}} = 335 \text{ nm}$, $\lambda_{\text{em}} = 518 \text{ nm}$) was divided by the AUC of the same 280 nm UV peak.

RESULTS

The generation of site-specific ADCs using bacterial transglutaminase was first reported by Schibli in 2010.³⁰ Removal of the glycosyl group from Asn297 exposes a previously concealed glutamine at position 295, which can subsequently undergo a transamination reaction in order to attach a linker and/or payload. We reasoned that a protected thiol residue with a short amine tail could be conjugated to the Q295 position and that subsequent deprotection would result in a “thiolated” glutamine moiety poised for site-specific conjugation with any of the multitude of readily available maleimide linker-payloads (Figure 1, bottom). Importantly, this approach allows for direct comparison of the resulting ADCs with engineered cysteine ADCs. We and others have previously shown that conjugation at carefully selected sites can protect payloads from metabolism, prevent antibody aggregation, maximize PK exposure, enable the conjugation of hydrophobic payloads, and prevent maleimide deconjugation.^{19,35,36} Thus, we envisioned to develop convenient methodology to “chemically engineer” a cysteine-like functionality at Q295 and then to compare and contrast the conjugates at this site with analogous site-specific engineered cysteine conjugates.

Cystamine (2, Scheme 1) proved to be a convenient source of S-protected 2-aminoethanethiol. Interestingly, cystamine (2) has long been reported to be an inhibitor of transglutaminase.^{37,38} Thus, not surprisingly, early attempts in our labs to use 2 as a transglutaminase substrate in a conjugation with deglycosylated trastuzumab were largely unsuccessful. However, hints of reactivity combined with the inexpensive availability of food-grade bacterial transglutaminase (TG)

prompted us to further investigate the use of cystamine as a TG substrate. We found a dependency of reaction pH, with reactions at pH 6 giving superior loading as compared to reactions performed at pH 7.5 and pH 8.1 (data not shown). Moreover, increasing the equivalents of cystamine to 100 equiv (mol:mol) and increasing the amount of the TG enzyme further increased the conversion, eventually resulting in complete modification of the Q295 position, as observed by LCMS under reducing conditions (Scheme 1 and Figure 2).

Engineered cysteine residues in antibodies are typically expressed as disulfide “capped” species, and thus the thiol must be liberated by selective reduction or (more commonly) by global reduction followed by reoxidation.³⁶ In a similar manner, the cystamine-modified trastuzumab (3) was treated with a large excess of TCEP (50X) to globally reduce the endogenous disulfides and cystamine disulfide. However, subsequent reoxidation with 1 mM DHA resulted in an antibody that was unable to be modified by a maleimide payload. Interestingly, attempts to enzymatically cleave the backbone with IdeS for LCMS studies resulted in no backbone cleavage, suggesting a global conformational change had taken place during the reoxidation step. We suspected that the thioethyl moiety at the Q295 position may have formed a “cross-link” with the adjacent Q295 residue (i.e., 5). Modeling studies of a glycosylated mAb (Figure 5) indicated that the two Q295 residues are spatially adjacent to one another (~30 Å apart). Given the removal of the glycosylation, it is likely that the two heavy chains will partially collapse and move even closer to one another, thus enabling the ready formation of an interchain disulfide between the two thioethyl moieties. With this in mind, we attempted a more mild air reoxidation to slow down the putative cross-linking. After the global reduction, the excess TCEP was removed by buffer exchange, and the

Table 1. Loading, Mass Shift, and % Aggregation for vcMMAE and mcMMAF Conjugates Using the Q295-Thiol Method in Scheme 1

ADC#	antibody	linker-payload	loading	expected mass shift	observed mass shift	% aggregation
ADC1	trastuzumab	mc-MMAF	1.8	925	924	1%
ADC2	trastuzumab	mcValCitPABC-MMAE	1.8	1316	1316	1%
ADC3	rituximab	mc-MMAF	1.8	925	930	<1%
ADC4	rituximab	mcValCitPABC-MMAE	1.7	1316	1316	<1%
ADC5	adalimumab	mcValCitPABC-MMAE	2.0	1316	1311	ND
ADC6	coltuximab	mcValCitPABC-MMAE	2.0	1316	1318	ND

Table 2. Cytotoxicity of Q295-Thiolated ADCs Demonstrating Selective Delivery and Comparable Activity to Native-Cysteine Conjugates^a

antibody	conjugation method	payload	DAR	IC ₅₀ Raji (μg/mL) (CD20+/Her2-)	IC ₅₀ Ramos (μg/mL) (CD20+/Her2-)	IC ₅₀ SKBR3 (μg/mL) (CD20-/Her2+)
trastuzumab	Cys	vcMMAE	5.8	NA	21 (12–36)*	0.026 (0.016–0.032)
	Q295-thiol		1.9	>7.76	26 (11–86)*	0.042 (0.021–0.061)
rituxumab	Cys	vcMMAE	4.9	0.31 (0.23–0.41)	0.43 (0.29–0.64)	11 (6.2–18)
	Q295-thiol		1.7	0.54 (0.12–1.1)	4.7 (4.1–58)*	26 (18–47)*
trastuzumab	Cys	mcMMAF	NA	NA	NA	NA
	Q295-thiol		1.8	>7.76	>39	0.034 (0.026–0.041)
rituxumab	Cys	mcMMAF	4.4	1.5 (0.71–8.7)	NA	NA
	Q295-thiol		1.8	1.5 (0.81–2.8)*	1.8 (1.1–2.6)	32 (17–73)*

^aActivity is reported as the inhibitor concentration 50% (IC₅₀, μg/mL) ± 95% confidence limits. IC₅₀ results were generated using GraphPad Prism 7 software, the log (inhibitor) against the response with a variable slope (four parameters) formula. Data marked with asterisk (*) were generated using a three-parameter variable slope formula (for improved curve fitting). Raw data is shown in Figure S4.

resulting antibody was allowed to stand at 4 °C for ~16 h. An aliquot was removed and treated with excess vcMMAE wherein full loading (DAR = ~1.9) was observed on the heavy chain, and no loading was observed on the LC. The mass shift (1320 Da) was consistent with the addition of vcMMAE (1316 Da) within the resolution of the single-quadrupole instrument employed for these studies. The location of the conjugation was further confirmed by treating the thiolated mAb (3) with IdeS. As can be seen in Figure S1, the loading took place exclusively on the Fc domain of the HC, and no loading was observed on the Fab domain of the LC or the HC.

Having optimized the thiolation and conjugation conditions using deglycosylated trastuzumab, we next set out to demonstrate that the optimized conditions were translatable to other antibodies of interest. Table 1 illustrates the loading, mass shift, and aggregation levels for a number of commercially available antibodies with two widely used linker-payloads. For reasons that are unknown at this time, this method failed to provide any loading on a commercial mouse IgG1 antibody (data not shown). Note that the aligned 292–300 consensus sequences are REEQYNSTY for human and REEQFNSTF for mouse. The subtle differences in sequence in this region (noted in bold) may render the Q295 (underlined) less reactive with bacterial TG. Further investigation and optimization for nonhuman antibodies will be performed in due course.

To verify that the modifications at the N297 and Q295 sites did not interfere with antigen binding and internalization, we evaluated a number of ADCs for their ability to block the proliferation of Raji cells (CD20+/Her2-), Ramos cells (CD20+/Her2-), and SKBR3 cells (CD20-/Her2+). As anticipated, the rituximab (anti-CD20) conjugates exhibited potent cytotoxicity against CD20 expressing cell lines (Raji and Ramos) while having little or no activity against a CD20-null cell line (SKBR3). In contrast, the trastuzumab (anti-Her2)

conjugates exhibited potent cytotoxicity against the Her2-expressing cell line (SKBR3) but showed little or no activity against Her2-null cell lines (Raji and Ramos). In most cases, the endogenous cysteine conjugate was slightly more potent than the site-specific conjugate, likely due to the differences in loading (DAR = ~4–6 vs DAR = ~2), as would be expected based on various examples from the literature³⁹ (see Table 2 for detailed results). Taken together, this data strongly suggests that conjugation at the Q295 site has little or no impact on ADC internalization and lysosomal processing. This, of course, is also in agreement with several reports demonstrating that Q295 conjugates exhibit potent *in vivo* and *in vitro* activity.^{29,34,40}

Numerous studies over the past five years have demonstrated that the site of conjugation plays an important role in plasma stability, including payload metabolism and maleimide deconjugation. Maleimide deconjugation takes place via a retro-Michael mechanism and results in the slow transfer of payload to serum albumin and, to a lesser extent, glutathione and cysteine.⁴¹ There have been three general mechanisms employed for overcoming such deconjugation: (1) maleimide ring-opening,^{42,43} (2) nonmaleimide electrophiles,⁴⁴ and (3) site-specific cysteine conjugation.^{19,36} The latter approach relies on the unusual observation that certain site-specific cysteine conjugates appear to be resistant to retro-Michael reactions. Early reports of this phenomenon suggested that the resistance to deconjugation was the result of spontaneous maleimide ring-opening.³⁶ However, later reports described multiple sites (such as 334C and 114C) that exhibited very little ring-opening but were still shown to be resistant to deconjugation.^{19,45} A very recent report presents compelling evidence that the resistance to retro-Michael deconjugation is correlated with the pK_a of the cysteine residue.⁴⁵

Regardless of the mechanism, it was imperative that we evaluate whether or not the thiolated Q295 conjugates were prone to deconjugation. With this in mind, a model conjugate

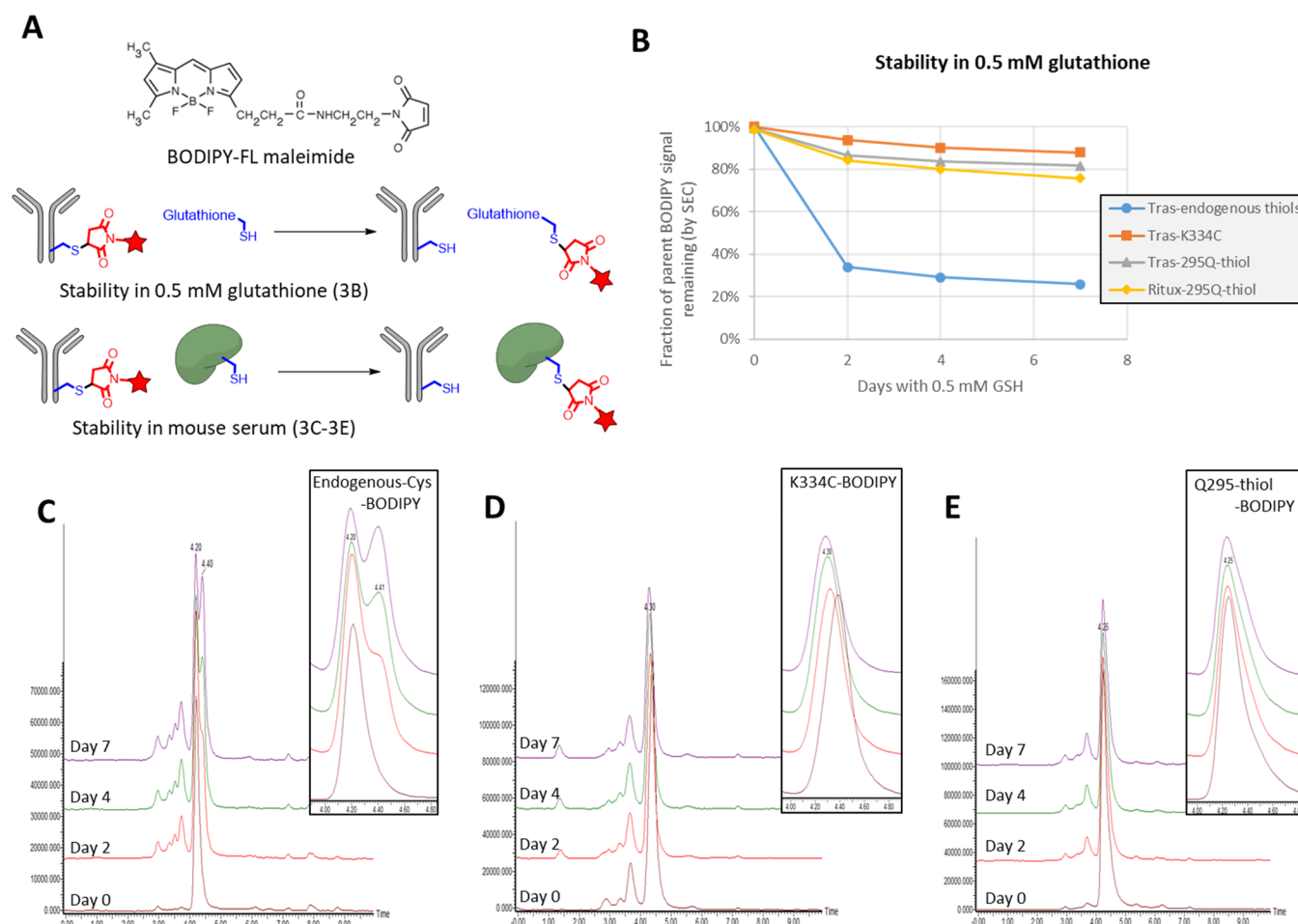


Figure 3. (A) Plasma stability and glutathione (GSH) stability study of a maleimide–BODIPY conjugate of the trastuzumab–Q295 thiolated antibody as compared to an analogous endogenous Cys conjugate and HC-K334C conjugate ($\lambda_{\text{ex}} = 480$ nm, $\lambda_{\text{em}} = 520$ nm); (B) fraction of the parent IgG BODIPY signal remaining at various timepoints following incubation with 0.5 mM GSH; (C) size exclusion chromatogram of the Tras endogenous Cys–BODIPY conjugate showing that a significant serum–albumin peak (retention time = 4.4 min) rapidly forms over the course of the incubation; (D and E) size exclusion chromatogram of Tras-334C–BODIPY and Tras-Q295-thiol–BODIPY showing that little or no serum–albumin transfer takes place over the course of the incubation.

was prepared in which a maleimide–BODIPY (Figure 3A) was attached to the Q295 position using the chemistry shown in Scheme 1. The stability of this conjugate in 0.5 mM glutathione (GSH) and in mouse serum was directly compared with an analogous stochastic cysteine conjugate (positive control) and an analogous HC-334C conjugate (negative control) (Figure 3B–E). Stability in GSH has been reported as a model system for studying deconjugation.^{19,42} The extent of deconjugation in both matrices was assessed over the course of 7 days by size exclusion chromatography (SEC), as shown in Figure 3. Previous reports have demonstrated that endogenous cysteine conjugates rapidly undergo deconjugation in plasma and in the presence of GSH while conjugates at the HC-334C site do not.^{19,35} Gratifyingly, the Tras-Q295-thiol BODIPY conjugate and Ritux-Q295-thiol BODIPY conjugate exhibited minimal deconjugation ($\leq 20\%$ loss) in 0.5 mM GSH (comparable to the 334C BODIPY conjugate) while the endogenous Cys conjugate underwent rapid deconjugation ($>70\%$ loss) (Figure 3B). Likewise, the Tras-Q295-thiol BODIPY and Tras-334C BODIPY proved to be stable in mouse plasma while the stochastic Cys conjugate resulted in rapid transfer of the BODIPY linker–payload to serum albumin, as evidenced by the rapid increase in the peak at 4.4 min in

Figure 3C. Interestingly, the GSH-mediated deconjugation of the endogenous Cys ADC (Figure 3B) seems to max out after ~ 3 days (as demonstrated elsewhere),⁴² suggesting that the maleimide ring has opened during the incubation and that the remaining ADC in solution is now resistant to further degradation.

To further probe the stability of the Q295-thiolated vcMMAE ADC, we prepared a series of known site-specific vcMMAE ADCs as direct comparators in a plasma stability study.¹⁹ The vcMMAE ADCs were incubated in mouse plasma (50 $\mu\text{g/mL}$) under 5% CO_2 . Aliquots were removed at 0, 72, and 168 h and treated with acetonitrile to precipitate plasma proteins. The supernatant concentration of MMAE at each timepoint was quantitated using single ion recording (SIR) ESI-MS (718.5 m/z). Several site mutants, including A114C, L347C, K388C, and (interestingly) K334C, exhibited significant ($>50\%$) loss of MMAE over the course of the incubation. In contrast, the Q295-thiolated ADC and L443C ADC exhibited minimal ($<20\%$) loss of payload over the same time course (Figure 4A,B). Unfortunately, the specific mechanism for the loss of payload is not readily apparent from this data. For example, the ValCit-PABC linker may be subject to direct cleavage by carboxylesterase 1C (CES1c), as

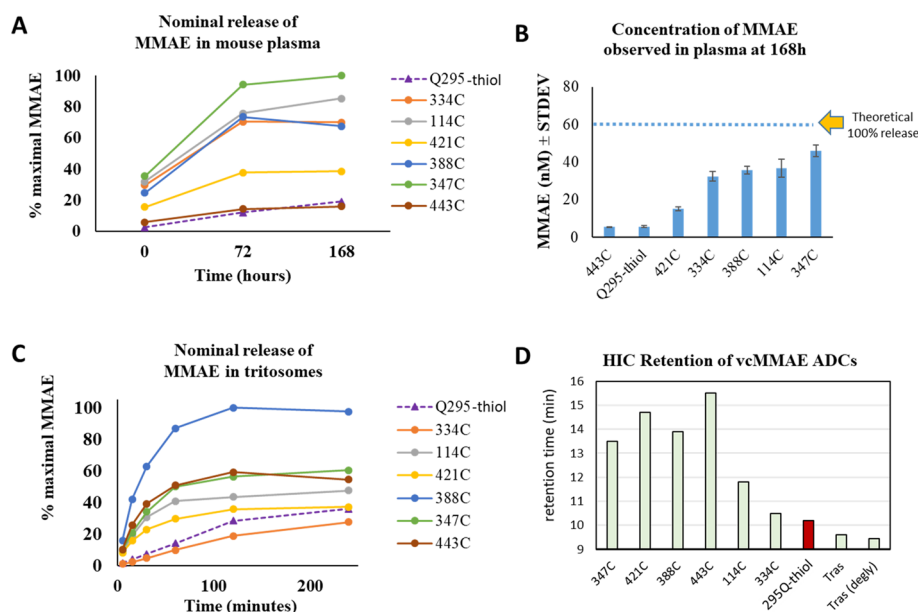


Figure 4. Comparison of the MMAE release kinetics in (A, B) mouse plasma and (C) tritosomes. The two conjugates with the slowest release rate in tritosomes were the least hydrophobic ADCs (334C and Q295-thiol), suggesting that these linker-payloads are located in a sterically inaccessible region of the antibody.

previously shown.⁴⁶ Alternatively, the vcMMAE may undergo maleimide transfer to serum albumin (or other plasma thiols) followed by CES1c or proteolytic cleavage. Regardless, it is clear from this data that the Q295-thiolated vcMMAE ADC exhibits comparable or improved plasma stability as compared to commonly used cysteine mutants.

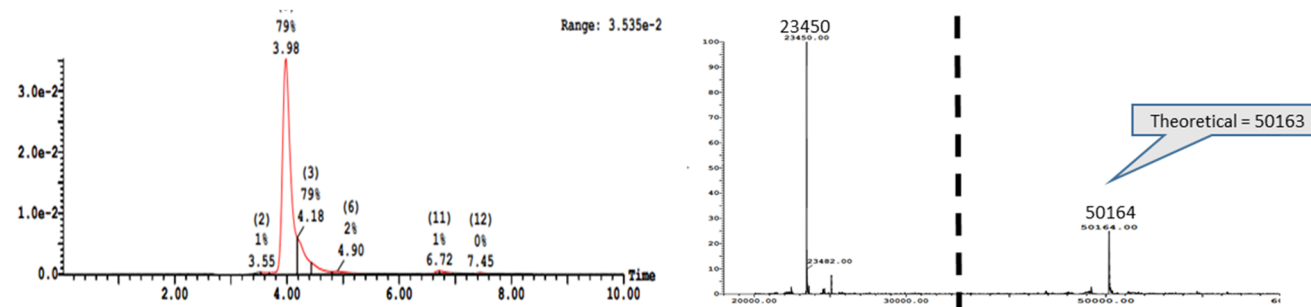
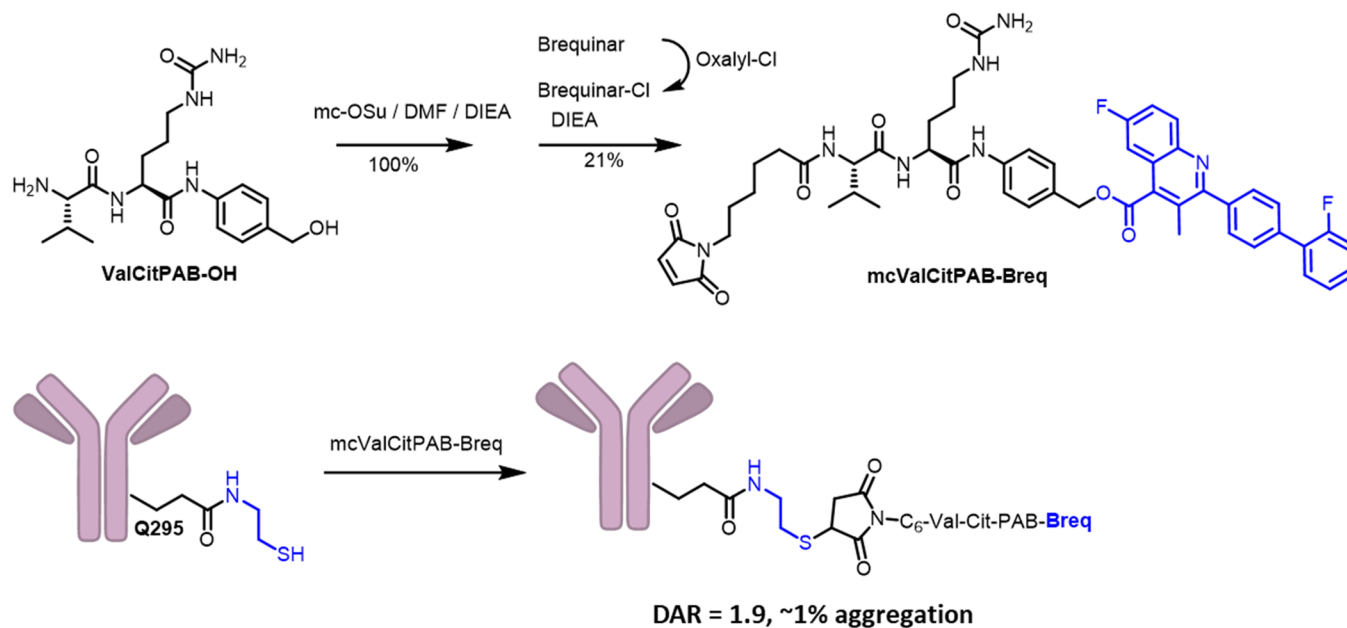
We have recently demonstrated that the susceptibility of an ADC to enzymatic metabolism is frequently correlated with its hydrophobicity.^{19,35} With this in mind, we undertook a direct comparison of the hydrophobicity of the Q295-thiolated vcMMAE ADC with analogous cysteine mutant ADCs. The results are illustrated in Figure 4D. As measured by hydrophobic interaction chromatography (HIC), the hydrophobicity of the Q295-thiolated vcMMAE ADC was only marginally higher than unmodified trastuzumab, suggesting that the Q295 is in a sterically “protected” region of the antibody. To further verify the implications of this observation, we studied the rate of MMAE release in the presence of isolated lysosomal enzymes as shown in Figure 4C. In short, 10 μ g of each ADC was incubated at pH 4.7 with 2.5 μ g of activated tritosomes (Xenotech). Aliquots (5 μ L) were removed at various timepoints and subjected to protein precipitation and ESI-MS quantitation using SIR analysis (718.5 m/z). Consistent with our previous report,¹⁹ the conjugates with the lowest HIC retention time (including the thiolated Q295 conjugate) exhibited the slowest payload release kinetics (Figure 4C and Figure S3). This further supports the notion that the Q295 site is in a sterically protected region of the antibody and that payloads attached in this region are likely to be resistant to enzymatic degradation. Importantly, the in vitro cytotoxicity data above (Table 2) illustrates that the lysosomal payload release kinetics, while slow, are sufficient to result in a functional activity.

As the structural diversity of payloads continues to increase, there is a growing need for site-specific conjugation methods that can readily accommodate hydrophobic payloads. One payload of interest in our lab is brequinar, an inhibitor of dihydroorotate dehydrogenase (DHODH), a key enzyme in

the de novo pyrimidine biosynthesis pathway⁴⁷ (Scheme 2). Brequinar is a low-nanomolar inhibitor of DHODH and, like other DHODH inhibitors, has been studied for its utility in the treatment of various autoimmune disorders.⁴⁸ In spite of its potency, it is exceptionally hydrophobic ($\log P = \sim 6.4$), which we suspected may cause problems during the conjugation reaction. Payloads such as this are often tethered to complex linkers that contain various polar functional groups to mask the payload hydrophobicity.^{7,49,50} We prepared a simple mcValCit-PAB ester-linked brequinar linker-payload (Scheme 2) as a model system for studying the conjugatability and utility of this payload. Perhaps not surprisingly, based on the above reports, we found that conjugation of this linker-payload to either endogenous cysteine residues or to commonly used cysteine mutants, including Tras-A114C, Tras-K334C, Tras-421C, Tras-347C, and Tras-388C, failed to give any measurable loading. Intriguingly, however, we found that conjugation to thiolated Q295 trastuzumab and thiolated Q295 rituximab proceeded smoothly and resulted in a conjugate with acceptable loading (~ 1.7 – 1.9), minimal hydrophobicity (HIC retention time = 11.2 min; naked Tras retention time = 9.6 min), and no significant aggregation ($\sim 1\%$).

To better understand why the brequinar linker-payload conjugated so efficiently to the Q295-thiol site but not to other sites, we began to explore the hydrophobicity of the region surrounding the Q295 residue. As shown in Figure 5, modeling the N297 glycosylation site indicates that a number of hydrophobic residues such as L233, F237, L238, F239, and Y296 are masked. Removal of the glycosyl group at N297 likely leads to the exposure of these sites to the solvent. The resulting hydrophobic cavity is an ideal location for the attachment of large hydrophobic payloads such as brequinar. We hypothesize that this cavity can be easily accessed by conjugation at the Q295 site, thus explaining the facile conjugation of brequinar even when this conjugation failed at numerous other sites.

ADC hydrophobicity is typically estimated using hydrophobic interaction chromatography (HIC). HIC retention is generally believed to be directly driven by interactions between

Scheme 2. Preparation of Initial Brequinar Linker-Payload and ADC Using the Thiolated Q295 Antibody^a

^aAnalogous conjugation with various engineered Cys mutant antibodies failed to give any meaningful loading. (DAR = <0.2) The SEC trace on the lower left shows that the brequinar conjugate had little or no aggregation while the MS trace on the lower right shows clean addition of brequinar to the HC and no addition to the LC. (See Figure 2A for the LCMS of the corresponding naked mAb.)

the hydrophobic stationary phase and nonpolar patches or residues on the protein. However, hydrophobic residues that are located in sterically occluded pockets of the protein are unlikely to be able to directly interact with the HIC stationary phase. Such is the case with the hydrophobic pocket shown in Figure 5. Indeed, even though deglycosylation clearly results in the exposure of numerous hydrophobic residues, the HIC retention of the deglycosylated antibody is essentially equivalent to the fully glycosylated antibody (Figure 4D). Thus, we turned to a complementary method that uses extrinsic fluorescence of a dye, 8-anilino-1-naphthalenesulfonic acid (ANS), that is known to have a quantum yield that is highly dependent upon the hydrophobicity of the solvent.⁵¹

ANS fluorescence has been used to estimate the hydrophobicity of variously loaded endogenous cysteine ADCs,^{52,53} but to our knowledge, this method has not been used to compare the hydrophobicity of various site-specific conjugates. No direct comparison of HIC hydrophobicity and ANS-hydrophobicity has been reported to date. ANS and its companion dye, bis-ANS, are fluorescent dyes that are highly sensitive to the local environment in which they bind. The literature reports clearly that ANS “lights up” as hydrophobic patches form on the surface of proteins, such as during the

process of heat-induced aggregation. However, it was unclear if the ANS or bis-ANS would be able to distinguish between the hydrophobicity of vcMMAE bound at various sites of an antibody. With this in mind, we treated the various vcMMAE site mutants (~0.25 mg/mL) with a large excess of bis-ANS (80 μM). After a short period of equilibration, the fluorescence intensity at 500 nm was measured (excitation at 385 nm) and normalized based on the protein concentration. Interestingly, we found that there was a significant difference between the hydrophobicity as measured by extrinsic fluorescence and the hydrophobicity as measured by HIC (Figure 6A,B). For example, the 443C conjugate, the most hydrophobic ADC as measured by HIC, proved to have equivalent intrinsic fluorescence to the 334C conjugate, one of the least hydrophobic ADCs. In contrast, the most hydrophobic ADC by extrinsic fluorescence proved to be the Q295-thiol ADC. The deglycosylated trastuzumab proved to be only marginally more fluorescent than its glycosylated counterpart. Importantly, this indicates that the high fluorescence of the Q295-thiol ADC was not simply due to “unmasking” of hydrophobic residues by deglycosylation. Rather, we believe that the increase in hydrophobicity is driven by a hydrophobic–hydrophobic interaction between the payload (vcMMAE) and

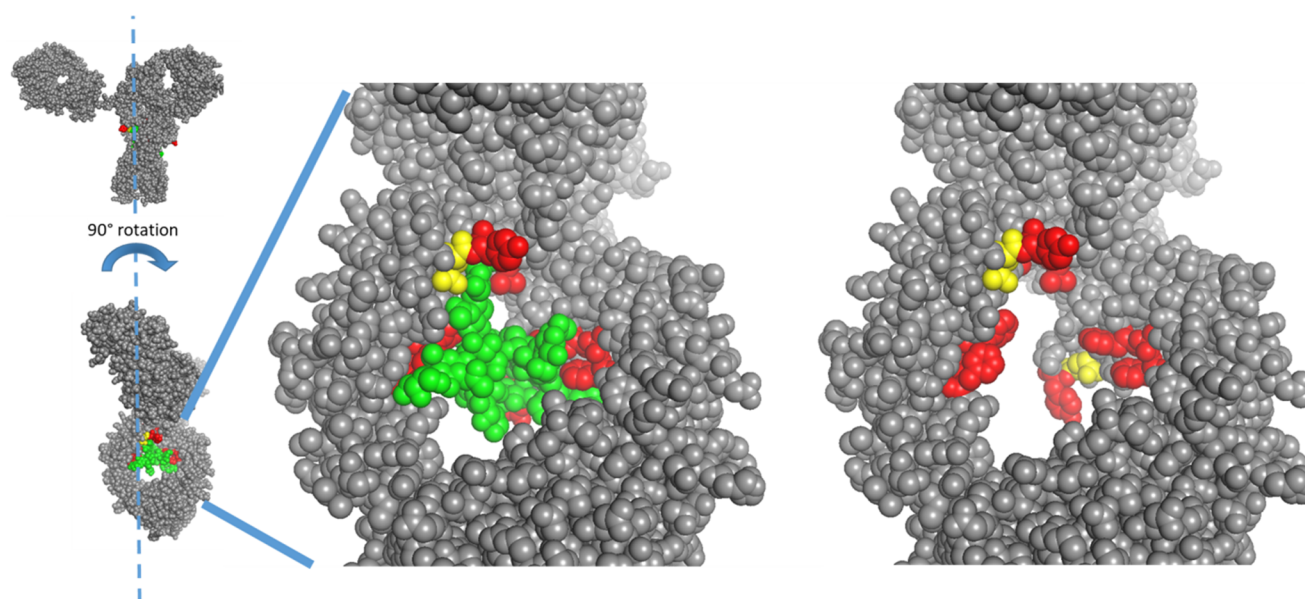


Figure 5. Deglycosylation of an IgG1 exposes numerous hydrophobic residues that were previously blocked by the glycosyl group (shown in green). The resulting cavity (right) is lined by a number of hydrophobic residues such as L233, F237, L238, F239, and Y296, highlighted in red. The Q295 residue (highlighted in yellow) is ideally positioned to access this cavity.

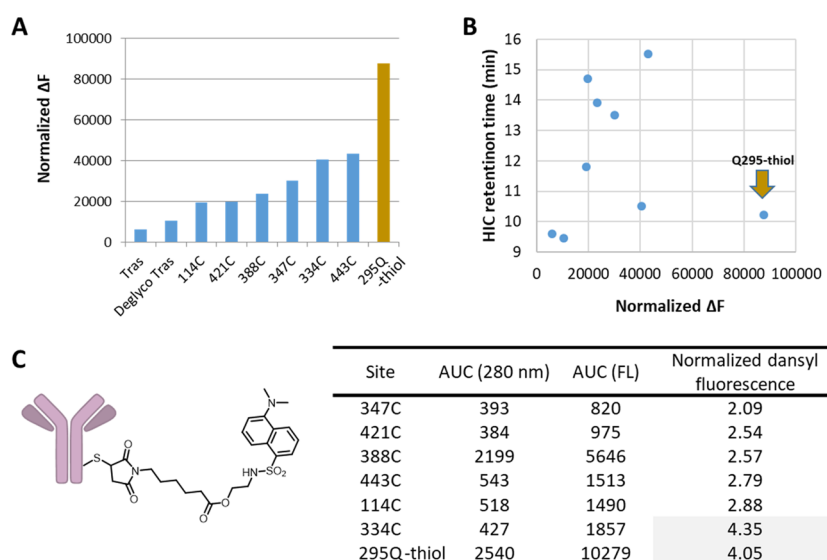


Figure 6. (A) The hydrophobicity of the various vcMAAE trastuzumab site mutant ADCs was evaluated using a bis-ANS extrinsic fluorescent probe; (B) there was little or no correlation between HIC retention time and the normalized extrinsic fluorescence with bis-ANS; (C) the local hydrophobicity of each site was estimated using a polarity-sensitive dansyl probe. The Q295-thiol and the 334C sites proved to be in a more hydrophobic environment than other sites of conjugation.

the aforementioned hydrophobic residues (L233, F237, L238, F239, and Y296). ANS dyes are known to bind to such interphases (as it happens during protein aggregation), thus resulting in an increase in fluorescence.⁵¹

In one final effort to understand the local environment around each conjugation site, we prepared a dansyl–maleimide probe shown in Figure 6C. The dansyl fluorophore is known to be sensitive to the local polarity of the solvent. As such, we evaluated a series of dansyl–maleimide conjugates using SEC with a fluorescence detector. The fluorescent emission of each ADC (AUC for $\lambda_{\text{ex}} = 350$ nm, $\lambda_{\text{em}} = 525$ nm) was corrected by the AUC at 280 nm to normalize the fluorescence to the protein concentration. In doing so, it was clear that not all conjugates were equally fluorescent. In fact, the fluorescence of

the Q295-thiol conjugate and the 334C conjugate were approximately 2-fold higher than most of the other sites, suggesting that the dansyl probe was located in an exceptionally hydrophobic region of the protein. This is consistent with the hypothesis that Q295-thiol conjugates are binding into the hydrophobic pocket liberated by deglycosylation of the N297 site.

DISCUSSION

Site-specific conjugation of payloads to antibodies is playing an increasingly important role in the design of ADCs for oncology, immunology, and infectious disease applications. While a wide variety of methods are available, each has limitations, and there continues to be a need to identify new

technologies that can be used for the incorporation of next-generation linker-payloads into site-specific ADCs. Site-specific conjugation to native (genetically nonengineered) antibodies is particularly attractive due to the fact that “off-the-shelf” antibodies can be employed for ADC discovery and development efforts. Herein, we describe a new site-specific conjugation method that takes advantage of the exquisite selectivity imparted by an enzymatic reaction between the Q295 residue and a protected thiol, cystamine. Liberation of the thiol by reduction results in an antibody that is poised for conjugation with any of the plethora of maleimide-containing linker-payloads that are commercially available or reported in the literature. We demonstrate that this method is useful for a variety of IgG1 antibodies and that it results in conjugates that are stable in mouse plasma. Our comparisons show that ADCs resulting from this method have similar activity and stability to ADCs generated using best-in-class cysteine-mutant technology.

Importantly, we demonstrate that this method is particularly applicable for the generation of ADCs from hydrophobic linker-payloads. Attachment of hydrophobic linker-payloads often results in aggregation issues and/or poor loading. Using brequinar as a model payload, we demonstrate that the Q295-thiol conjugation method results in efficient conjugation even in cases where conjugation has failed using other methodologies. Furthermore, we show that a possible mechanism for this enhanced reactivity with hydrophobic payloads involves binding into a hitherto unexplored hydrophobic pocket that is sandwiched between the two heavy chains of the IgG1 and is normally occluded by antibody glycosylation at the N297 residue. Using an extrinsic fluorescence method (bis-ANS) and a polarity-sensitive probe (dansyl-maleimide), we show that the Q295-thiol conjugates are far more hydrophobic than they appear by hydrophobic interaction chromatography. We do not believe that this is an inherently deleterious feature, as the hydrophobicity is “masked” in a region that is inaccessible to typical IgG interactions. To our knowledge, the disconnection between various measures of ADC hydrophobicity has not been explored in the published literature.

In summary, we describe a site-specific method for the conjugation of maleimide-containing linker-payloads to genetically nonengineered antibodies. This method overcomes typical limitations of other such methods, namely the multistep preparation of specialized conjugation handles (as in the case of disulfide rebridging) and the necessity to incorporate hydrophobic vestigial click handles (as in DBCO click chemistry and glycoengineering approaches). Importantly, the conjugation method we describe results in an antibody attachment that is resistant to maleimide deconjugation and is in a region of the antibody that is protected from plasma metabolism. Finally, we demonstrate that conjugation using this methodology appears to be particularly advantageous for the attachment of hydrophobic linker-payloads. Together, we believe that these features render this conjugation method to be a particularly attractive addition to the current repertoire of site-specific conjugation approaches.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.9b00323.

Details of the LCMS analysis methods, the preparation of the brequinar linker-payload, the preparation of the mc-ODansyl probe, figures supporting the tritosomal release assay and cytotoxicity assays (PDF)

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■ ABBREVIATIONS

TG, transglutaminase; HIC, hydrophobic interaction chromatography; SEC, size exclusion chromatography; LC, light chain; HC, heavy chain; DAR, drug-antibody ratio; mAb, monoclonal antibody; ACN, acetonitrile; MMAE, monomethyl auristatin E; mc, maleimide caproyl; vcMMAE, maleimide caproyl valine citruline para-aminobenzylcarbamate MMAE; TCEP, tris(2-carboxyethyl)phosphine

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