Improving the Serum Stability of Site-Specific Antibody Conjugates with Sulfone Linkers

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Ⓢ *Supporting Information*

ABSTRACT: Current routes for synthesizing antibody–drug conjugates commonly rely on maleimide linkers to react with cysteine thiols. However, thioether exchange with metabolites and serum proteins can compromise conjugate stability and diminish *in vivo* efficacy. We report the application of a phenyloxadiazole sulfone linker for the preparation of trastuzumab conjugates. This sulfone linker site-specifically labeled engineered cysteine residues in THIOMABs and improved antibody conjugate stability in human plasma at sites previously shown to be labile for maleimide conjugates. Similarly, sulfone conjugation with selenocysteine in an anti-ROR1 scFv-Fc improved human plasma stability relative to maleimide conjugation. Kinetically controlled labeling of a THIOMAB containing two cysteine substitutions was also achieved, offering a strategy for producing antibody conjugates with expanded valency.
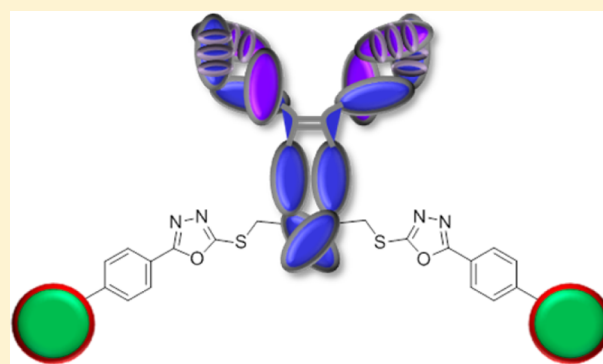
INTRODUCTION

Antibody–drug conjugates (ADCs) enable the delivery of cytotoxic payloads to tumors.[1–3] A significant advantage of ADCs over traditional chemotherapeutic agents is that ADCs impart specificity for drug delivery by selectively recognizing cancer cell antigens while avoiding normal tissues. For instance, ADCs bearing anti-mitotic drugs have demonstrated increased therapeutic efficacy.[4] Nonetheless, developing successful ADCs requires many considerations including antigen target, antibody, linker design, and payload.[5–9] Additionally, conjugation strategies, which dictate factors such as drug loading and stability, can greatly influence therapeutic outcomes. In particular, ADCs synthesized by conjugation to reduced interchain cysteine (Cys) sulfhydryl groups or lysine side-chain amines yield heterogeneous conjugates that have been associated with aggregation, inconsistent pharmacokinetic profiles, and toxicity.[10–13] One approach that can potentially overcome many of these limitations is the site-specific conjugation of antibodies that have been engineered to contain Cys residues within the heavy or light chains (THIOMABs).[14] These THIOMABs can be labeled to homogeneity without disruption of the immunoglobulin architecture.[12,14–19]

Antibody engineering methods have been required to identify suitable maleimide–thiol linkage sites because local structural and chemical environments dictate reactivity and conjugate stability.[15,16,19] Further, maleimide–thiol linkages are susceptible to hydrolysis and the retro-Michael reaction which can lead to thioether exchange with free Cys, glutathione, and the serum protein albumin.[6,16,20–23] For example, conjugation of monomethyl auristatin E (MMAE) to anti-HER2 (trastuzumab) THIOMABs has illustrated the importance of the relationship between conjugation site and therapeutic efficacy (Figure 1A).[16] These studies showed that THIOMAB conjugate stability was inversely correlated with predicted fractional solvent accessibility, as THIOMABs labeled in regions with high-predicted fractional solvent accessibility (Fc-S396C) demonstrated poor pharmacokinetics, with most MMAE conjugates being removed within 24 h. In contrast, residues with low-predicated fractional solvent accessibility (LC-V205C and HC-A114C) were stable for several weeks and exhibited improved *in vivo* efficacies.

We recently synthesized a panel of heteroaryl methylsulfone derivatives to compare their thiol reactivity and conjugate stability with standard maleimide conjugation.[21] Methylsulfonyl phenyloxadiazole compounds proved to be highly reactive with Cys residues under a variety of conditions allowing for conjugation with proteins. Sulfone modification of the maltose-binding protein containing a C-terminal Cys residue (MBP-Cys) was shown to be resistant to thioether exchange with albumin in human plasma, whereas MBP-Cys labeled with a maleimide compound readily underwent thioether exchange. Moreover, the half-life of the phenyloxadiazole sulfone
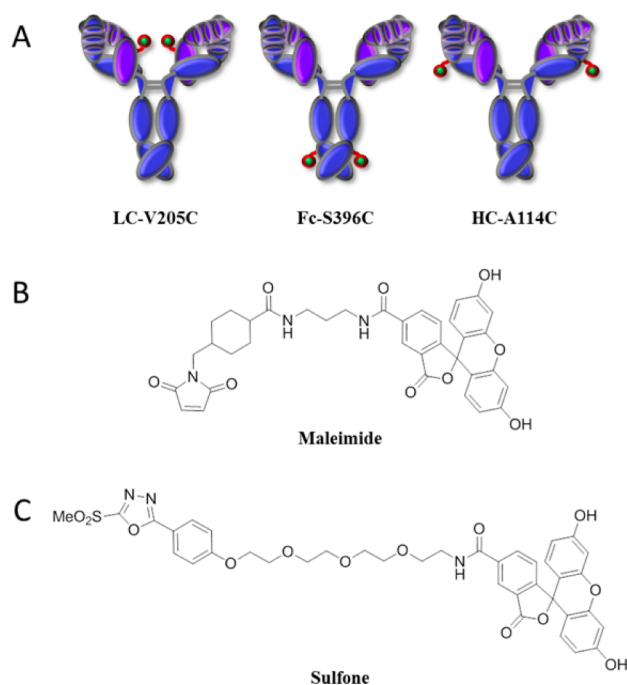
Figure 1. (A) Single Cys substitutions were introduced into the trastuzumab light or heavy chains providing THIOMABs LC-V205C, Fc-S396C, and HC-A114C. (B−C) Structures of the maleimide and phenyloxadiazole sulfone fluorescein compounds.

conjugate was doubled relative to the maleimide conjugate in human plasma. The application of our sulfone labeling chemistry for synthesizing ADCs thus seemed a viable route for making conjugates with improved serum stability. Here, we report the use of a phenyloxadiazole sulfone linker for preparing trastuzumab conjugates at engineered Cys residues as well as a ROR1-specific scFv-Fc conjugate at an engineered selenocysteine (Sec) residue. We show that this sulfone linker site-specifically labels engineered Cys and Sec residues and improves antibody conjugate stability in human plasma at sites known to be labile for maleimide conjugates.

■ RESULTS AND DISCUSSION

We began by synthesizing fluorescein derivatives with maleimide (Figure 1B) or phenyloxadiazole (ODA) sulfone (Figure 1C) linker functionalities for THIOMAB labeling, as benzothiazole (BTZ) and phenyltetrazole (PTZ) sulfone compounds were determined to have poor reactivity with THIOMABs (Supporting Information (SI) Figure S1).[21] To assess optimal reaction conditions and confirm antibody labeling, we performed a time course study. Neither the maleimide nor sulfone linkers conjugated to trastuzumab during the 8 h incubation period (Figure 2A). Hence, the sulfone linker maintains chemoselectivity, unlike vinyl sulfone compounds that have been reported to conjugate with lysine, histidine, and cysteine in proteins.[24] Rapid conjugation was observed between the maleimide compound and the light chain of LC-V205C, whereas the sulfone conjugate was not
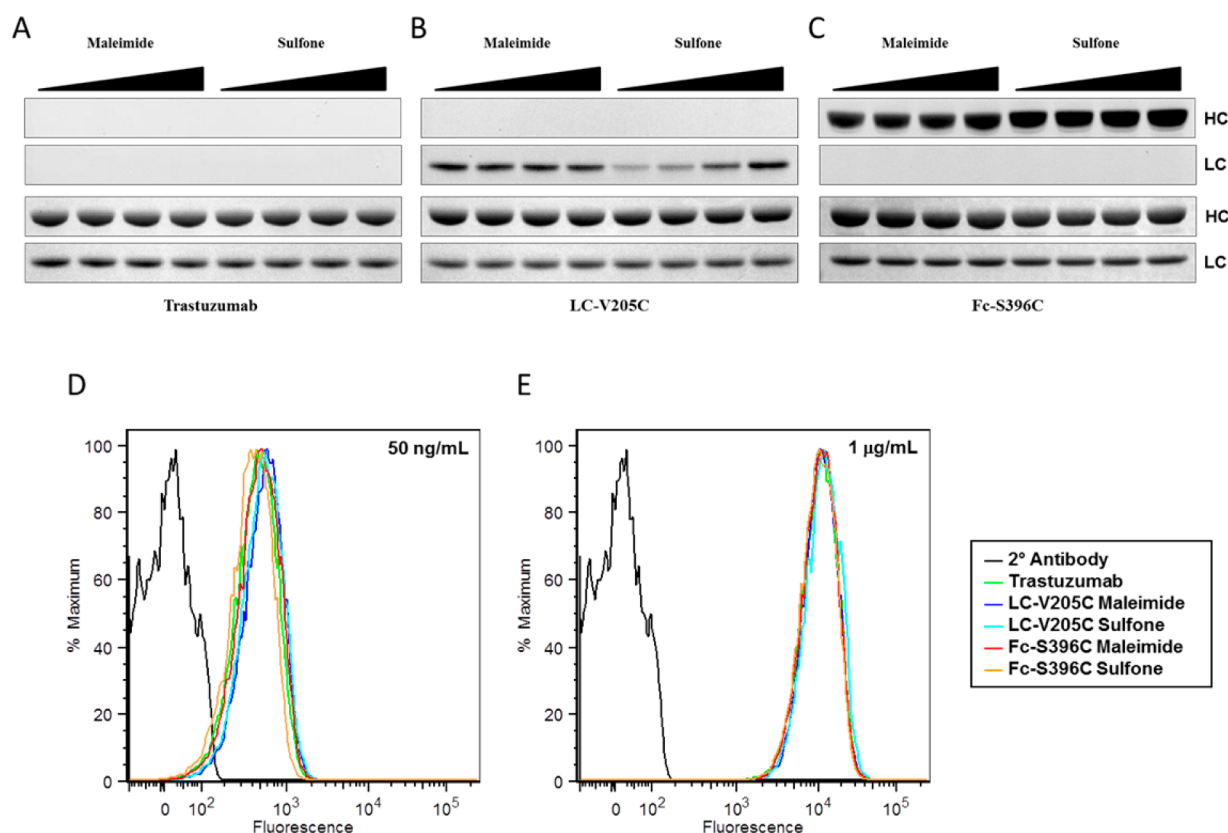

Figure 2. Site-specific conjugation of THIOMABs using maleimide or phenyloxadiazole sulfone fluorescent compounds. (A−C) SDS-PAGE analysis of labeled conjugates. Reactions progressed for 1, 2, 4, and 8 h at room temperature. Fluorescent (top) and Coomassie stained (bottom) gels are shown. Heavy chains (HC) and light chains (LC) are indicated. (D−E) Trastuzumab and THIOMAB conjugate binding to HER2 on BT-474 cells by flow cytometry.

extensively formed until 4 or 8 h (Figure 2B). However, we observed that increasing the reaction temperature from room temperature to 37 °C for 1 to 2 h increased labeling with the sulfone compound (data not shown). Both the maleimide and sulfone compounds labeled Fc-S396C within 1 h (Figure 2C). The sulfone linker was thus determined to be capable of producing site-specifically conjugated THIOMABs (SI Table S1).

HER2 antigen recognition on BT-474 cells was examined by flow cytometry to ensure high-affinity binding by the THIOMAB sulfone conjugates. Subsaturating (50 ng/mL) and saturating (1 μg/mL) concentrations of antibody were used for this analysis (SI Figure S2). Each conjugate bound HER2 with affinity similar to that of trastuzumab (Figure 2D,E), and comparable binding was also seen by ELISA (SI Figure S3). Hence, no impairment in HER2 binding was observed due to conjugation site (LC-V205C and Fc-S396C) or linker chemistry (maleimide and sulfone). These data are consistent with previous results that showed trastuzumab and trastuzumab THIOMABs bound MCF-7 cells and inhibited SK-BR-3 cell proliferation with similar affinities and potencies, respectively.[16] In addition, THIOMABs were shown to retain internalization properties,[16] suggesting that antibody–sulfone conjugates should also be suitable for ADC applications.

We next evaluated THIOMAB conjugate stability in human plasma. Maleimide labeling of LC-V205C led to improved conjugate stability in comparison to Fc-S396C (Figure 3).[16] In particular, the LC-V205C maleimide conjugate maintained ~80% of fluorescent label after incubation at 37 °C for 72 h (Figure 3A). In contrast, ~20% of the Fc-S396C conjugate remained intact after incubation for 72 h (Figure 3B). Notably, thioether exchange with albumin was evident at least 24 h after conjugation. Sulfone conjugate stability was less dependent on the conjugation site. While similar plasma stability was observed between LC-V205C (Figure 3A) and Fc-S396C (Figure 3B) after 24 h with the phenyloxadiazole sulfone fluorescein compound, we found that approximately two-thirds of the Fc-S396C sulfone conjugate was intact compared to the LC-V205C sulfone conjugate by 72 h. Additionally, thioether exchange with albumin was not observed. It is likely that the heteroaromatic structure of the sulfone linker as well as the methyl sulfone acting as leaving group contributes to this enhanced stability, especially considering that deprotonation at the α carbon in the succinimide ring of the maleimide conjugate greatly increases instability of the thioether bond.

Incorporation of the highly nucleophilic Sec residue through genetic engineering also allows site-specific conjugation of antibodies (SELENOMABs), further providing alternative strategies for producing ADCs.[25−27] Co-translational Sec insertion at the C-terminus of anti-ROR1 R11-scFv-Fc enabled conjugation at pH 5.2 with either maleimide or sulfone compounds without conjugation to hinge Cys residues (Figure 4A).[27] Interestingly, the increased reactivity of Sec compared to Cys greatly enhanced labeling with the fluorescent benzothiazole and phenyltetrazole sulfone compounds to similar levels as maleimide and phenyloxadiazole sulfone (Figure 4A and SI Figure S1). R11-scFv-Fc-Sec conjugation was well tolerated as conjugates maintained binding to ROR1 expressed on HBL-2 cells (Figure 4B). SELENOMAB conjugate stability was evaluated over 72 h in human plasma revealing the strong propensity of maleimide conjugates to rapidly undergo exchange with albumin, with the majority of maleimide conjugate being transferred within 4 h (SI Figure S4). However,
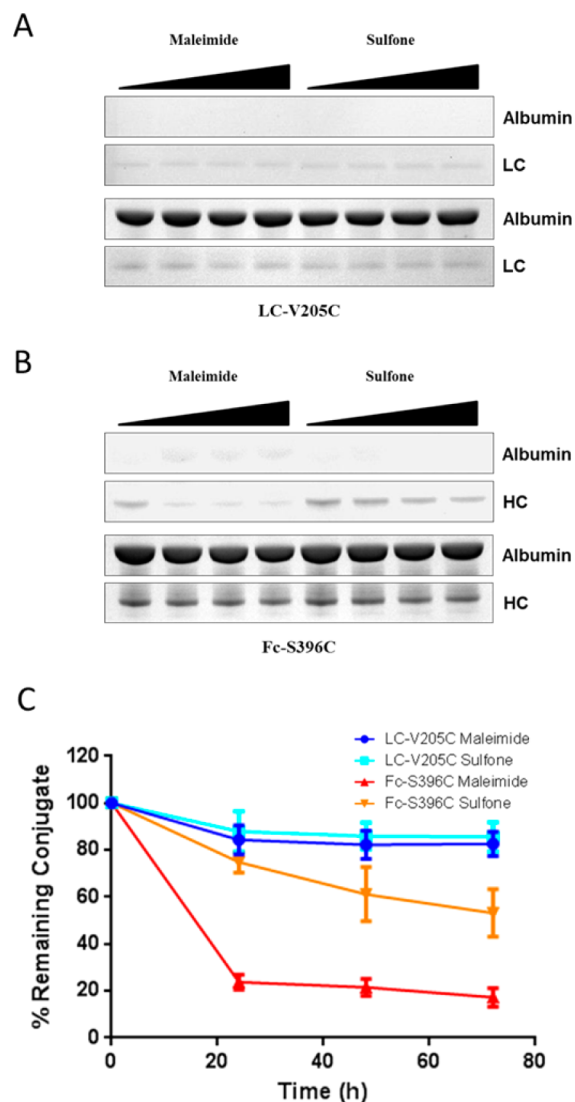

Figure 3. Antibody conjugate stability in human plasma at 0, 24, 48, and 72 h for maleimide and phenyloxadiazole sulfone conjugates. (A–B) Fluorescent (top) and Coomassie stained (bottom) SDS-PAGE gels are shown for LC-V205C and Fc-S396C conjugates. Albumin and the respective fluorescently labeled antibody chains are denoted. (C) Conjugate stability in human plasma.

exchange between the sulfone conjugate and albumin was not evident, and sulfone conjugate stability extended out to 24 h.

The enhanced stability of the sulfone conjugates at Fc-S396C and R11-scFv-Fc-Sec encouraged us to explore HC-A114C, which was reported to have intermediate maleimide conjugate stability.[16] HC-A114C, much like LC-V205C, resides within a partially buried cleft. However, unlike LC-V205C, HC-A114C does not feature a local basic environment that promotes succinimide ring hydrolysis to prevent retro-Michael elimination of conjugate. Conjugation of HC-A114C with both maleimide and sulfone linkers went to completion within 1 h (SI Figure S5 and Table S1), with HER2 antigen recognition being maintained (SI Figure S3). Conjugate stability was then assessed over a 1 month period in human plasma at 37 °C. The HC-A114C maleimide conjugate retained slightly greater than half its label (Figure 5). However, sulfone conjugation led to a substantial improvement in HC-A114C stability, as conjugate was retained for 1 month with ~90% remaining (Figure 5).
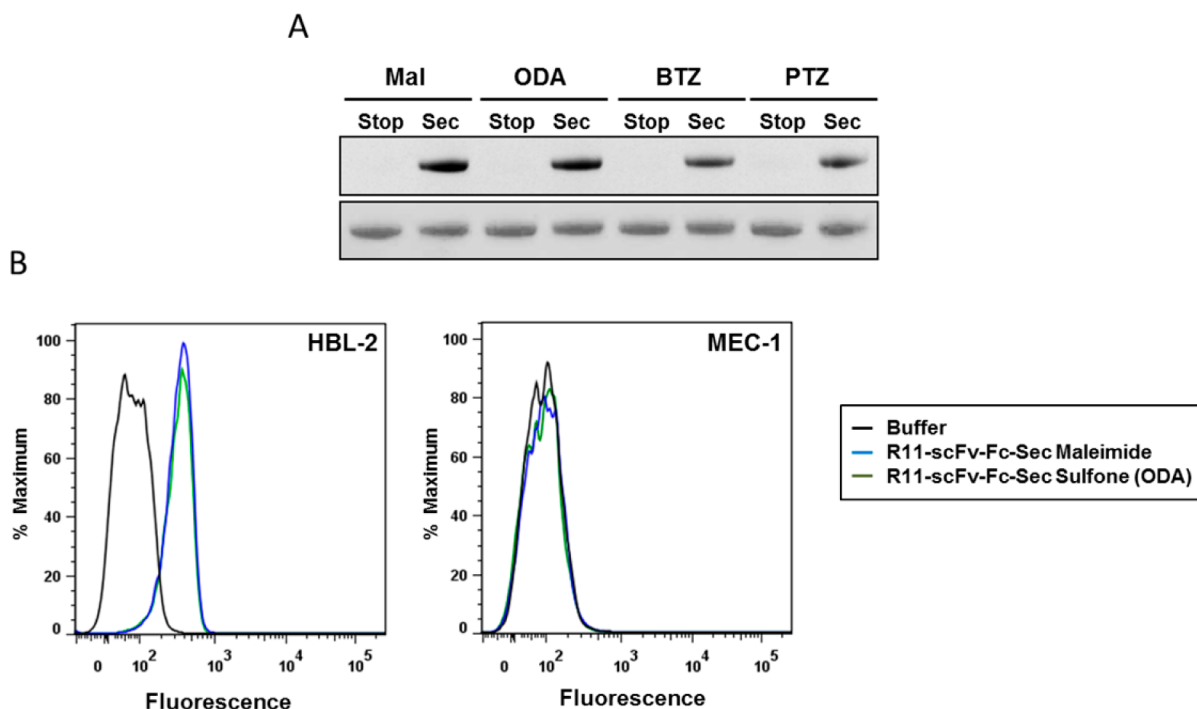
Figure 4. Site-specific conjugation of antibody via Sec using maleimide or sulfone fluorescein compounds. (A) SDS-PAGE analysis of labeled conjugates. Fluorescent (top) and Coomassie stained (bottom) gels are shown. Antibody R11-scFv-Fc-stop (Stop) and R11-scFv-Fc-Sec (Sec) are indicated. (B) Binding of fluorescent R11-scFv-Fc-Sec conjugates to ROR1-positive cell line HBL-2 but not ROR1-negative cell line MEC-1 by flow cytometry.
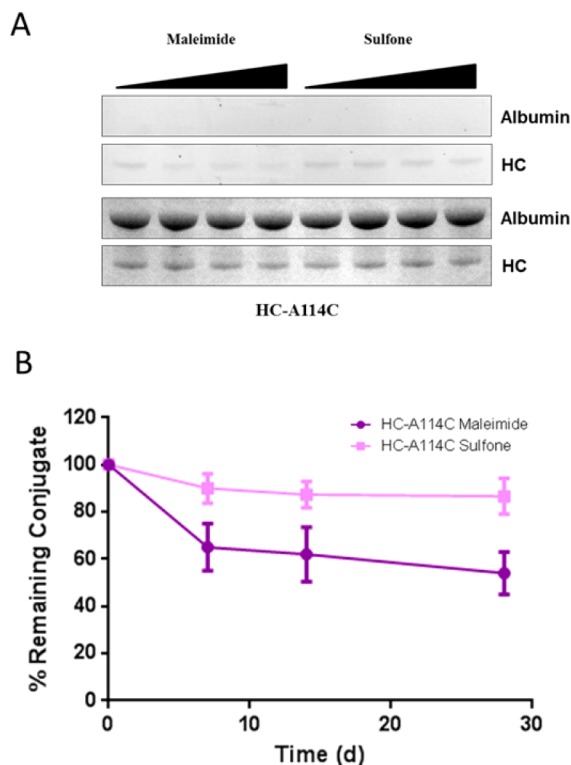

Figure 5. HC-A114C maleimide and phenyloxadiazole sulfone conjugate stability in human plasma. (A) SDS-PAGE of labeled conjugates at 0, 7, 14, and 28 d. Fluorescent (top) and Coomassie stained (bottom) gels are shown with albumin and the heavy chain indicated. (B) Stability of HC-A114C conjugates in human plasma.

These results indicate that the phenyloxadiazole sulfone linker enables conjugation at THIOMAB sites with intermediate maleimide stability. Furthermore, such sites can be used for engineering bispecific antibody constructs to optimize binding for multiple targets simultaneously.[28−32]

Finally, the ability to increase antibody valency enables expanded recognition of tumor antigens. We previously accomplished this task by chemically programming the monoclonal antibody 38C2 with a bifunctional linker that combined targeting of the $\alpha\nu\beta3/\alpha\nu\beta5$ integrins with the luteinizing hormone releasing hormone receptor as well as by trastuzumab conjugation through tyrosine-selective modification.[33−35] Given the observation that the sulfone linker labeled LC-V205C and Fc-S396C at different rates, we speculated that kinetically controlled dual labeling of a LC-V205C/Fc-S396C THIOMAB could be performed. To test this hypothesis, LC-V205C/Fc-S396C THIOMAB was labeled in two steps. First, the Fc-S396C site was conjugated with the sulfone linker for 1 h, and second, the LC-V205C site was labeled with either the maleimide or sulfone linkers for 1 or 8 h, respectively (SI Figure S6). Treatment of LC-V205C/Fc-S396C with the maleimide compound led to labeling at both sites, indicating heterogeneous conjugation. Conjugation with sulfone compound first, however, induced biased labeling for Fc-S396C and enabled subsequent labeling of LC-V205C with either maleimide or sulfone compounds, without impairing HER2 binding (SI Figure S3). Such kinetically controlled dual labeling should allow conjugation of additional targeting moieties or cytotoxic payloads.

■ CONCLUSIONS

We have improved antibody conjugate stability by employing a sulfone linker for site-specific modification rather than

conventional maleimide chemistry. Labeling of engineered Cys and Sec residues in anti-HER2 THIOMABs and an anti-ROR1 SELENOMAB, respectively, was achieved using a phenyloxadiazole sulfone fluorescein compound.[21] Neither chemoselective conjugation nor antigen binding was impaired with the sulfone linker. Studies utilizing cytotoxic payloads will be essential for validating the use of sulfone linkers in preparing ADCs. Conjugation of cytotoxic agents increases the local hydrophobic character at the site(s) of labeling which can lead to aggregation, particularly for ADCs with high drug-to-antibody ratios.[36,37] However, ADCs site-specifically labeled at engineered cysteine residues have shown much improved physical stability.[13]

The stability of antibody conjugates was also evaluated in human plasma. In general, LC-V205C conjugates were more stable compared to Fc-S396C conjugates. However, the Fc-S396C sulfone conjugate was much more stable than the corresponding maleimide conjugate. We suspect that further enhancements in stability are possible via conjugation at sites with intermediate conjugate stability, such as HC-A114C. Moreover, the kinetics of sulfone labeling can be exploited to generate conjugates with expanded valency, which may prove to be valuable in gaining selective tumor targeting, increased internalization, or more potent cytotoxicity. These results indicate that sulfone linker chemistry provides a new synthetic route for developing antibody conjugates with improved serum stability.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Experimental procedures; comparison of THIOMAB labeling using phenyloxadiazole, benzothiazole, and phenyltetrazole fluorescein-linked compounds; flow cytometry histogram of the dose-dependence for HER2 antigen binding by trastuzumab on BT-474 cells; trastuzumab and THIOMAB conjugate binding to HER2-Fc by ELISA; R11-scFv-Fc-Sec conjugate stability in human plasma; time course for HC-A114C labeling with maleimide or sulfone fluorescent compound; kinetically controlled dual labeling of LC-V205C/Fc-S396C THIOMAB; dye-to-antibody ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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