Site-specific chemical modification of antibody fragments using traceless cleavable linkers

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Antibody-drug conjugates (ADCs) are promising agents for the selective delivery of cytotoxic drugs to specific cells (for example, tumors). In this protocol, we describe two strategies for the precise modification at engineered C- or N-terminal cysteines of antibodies in IgG, diabody and small immunoprotein (SIP) formats that yield homogenous ADCs. In this protocol, cemadotin derivatives are used as model drugs, as these agents have a potent cytotoxic activity and are easy to synthesize. However, other drugs with similar functional groups could be considered. In the first approach, a cemadotin derivative containing a sulfhydryl group results in a mixed disulfide linkage. In the second approach, a cemadotin derivative containing an aldehyde group is joined via a thiazolidine linkage. The procedures outlined are robust, enabling the preparation of ADCs with a defined number of drugs per antibody in a time frame between 7 and 24 h.

INTRODUCTION

Modification of proteins by the covalent attachment of payloads has gained prominence in the development of therapeutic proteins for the treatment of cancer, diabetes, anemia and more[1], as well as in vaccine campaigns against HIV, malaria and influenza[2]. However, the accurate and consistent preparation of therapeutic modified proteins is rather difficult, owing to the use of conjugation strategies that often result in a mixture of products. These mixtures are then the basis for therapeutic studies. This presents challenges for the manufacturing, regulation and safety of therapeutic modified proteins, creating an immediate need for methods that enable specific and controllable modification of proteins.

In particular, there is a considerable interest in the area of ADCs for the targeted delivery of potent cytotoxic drugs to tumors[3,4]. This approach allows the specific delivery of potent cytotoxic drugs to tumor sites that otherwise are too toxic to be used systemically, while healthy organs can be effectively spared. Although the recent clinical successes of SGN-35 (refs. 5,6) and T-DM1 (ref. 7) clearly demonstrate the therapeutic benefit of ADCs for cancer patients, there remains a need for the development of novel classes of chemically defined ADCs.

Choice of conjugation strategy

There is a growing interest in the development of methods for the construction of homogenous therapeutic protein conjugates. However, ADCs have often been built by using conjugation approaches that typically result in heterogeneous products that contain a mixture of species with different drug-to-antibody ratios and, potentially, with different pharmacokinetic and therapeutic properties[8–10]. In the past decade, many novel site-specific, bio-orthogonal reactions that enable precise and controlled modification of proteins have been reported[11–13]. Such methods require either the substitution of an amino acid residue in the antibody molecule with a cysteine[14,15] or the introduction of a non-natural amino acid with a suitable functionality (e.g., a ketone) for drug coupling[16–18]. In the ADC field, conjugation processes often rely on more conventional modification methods. For example, interchain disulfide bonds can be reduced[19] or new cysteine residues can be inserted in the antibody molecule[14,15,20]. The resulting thiol-containing antibody can then be alkylated with drug derivatives containing linkers with suitable reactive moieties (e.g., maleimido). Linkers may contain chemical structures (e.g., protease selective amino acid sequences, disulfides and hydrazones) that can be cleaved, releasing a cytotoxic drug but leaving, at the same time, a chemical modification ('scar') on the antibody. Alternatively, acylation through lysine residues can be used to access ADCs. This strategy has been extensively characterized, and although highly reproducible protocols can be developed a range of 40 solvent-accessible lysine residues can be modified with a desired drug, potentially resulting in $>10^6$ ADC species[21].

A different approach involving traceless linkers is used in this protocol. The development and use of such site-specific modification methods where the cleavable site for drug release is the one that results from the site-specific conjugation reaction may result in cleaner conjugates and may facilitate the assessment of the structure-activity relationships of ADCs. We have used this approach to produce vascular targeting ADCs for cancer therapy[22–24].

The process of site-specific modification of antibodies is challenging, as only predetermined amino acid residues must react in a specific manner in the presence of competing residues, without compromising the antibody conjugate tumor-targeting capacity. Thus, methods should be optimized such that reactions proceed rapidly and efficiently in aqueous milieu at neutral pH, at or near room temperature (25 °C). An ideal approach would be applicable to a variety of antibodies in different recombinant formats and result in conjugates that are stable in circulation but readily and tracelessly cleaved at the tumor site.

Although our traceless conjugation method does well against these criteria, limitations may arise from the need to engineer cysteine residues that may lead to aggregation and low conjugation yields. When one follows the mixed disulfide conjugation protocol, incomplete reoxidation of antibodies that contain

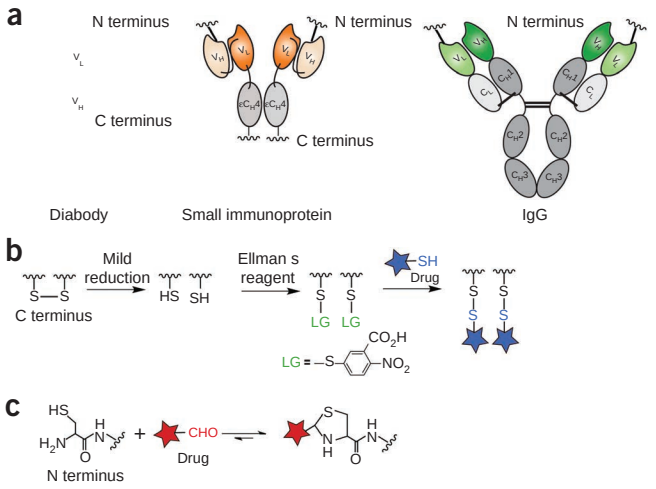

Figure 1 | Site-specific conjugation of cytotoxic payloads to antibodies using traceless cleavable linkers. (**a**) Relevant antibody formats used; wavy line represents the site of modification. (**b**) Modification at the C-terminal cysteine residues by using a disulfide strategy. (**c**) Modification at the N-terminal cysteine residues by using a thiazolidine strategy.

by antibody characteristics. It is difficult, however, to assess the contribution to therapy derived from drug release inside and outside the target cell. We[22–25] and others[26,27] have explored novel classes of ADCs that target structures in the tumor microenvironment that do not rely on internalization for drug release, such as mucin secreted by cells[21], components of the extracellular matrix and/or markers of angiogenesis. In this way, ADCs may be developed without the need to prepare individual antibodies for each specific cancer type, as vascular targeting antibodies recognize the majority of solid tumors and lymphomas. Furthermore, vascular structures are readily accessible for ADCs coming from the bloodstream, and their targeting is rapid[28]. In this protocol, we have focused on the clinical-stage F8 and L19 antibodies as model antibodies; they are specific to the alternatively spliced extra domains A and B (EDA and EDB) of fibronectin, respectively[29,30]. However, the methodologies we present are completely general and can be used for other antibodies in the same format. In analogy, we have used cemadotin derivatives as model drugs, but other drugs carrying similar functional groups could have been considered.

Smaller recombinant antibody formats (e.g., scFv, diabody, mini-antibodies, SIP, scFv-Fc) are now emerging as valid alternatives to full IgG antibodies[31]. While retaining the specificity of full antibodies, these fragments can be produced more economically and often have superior biodistribution and blood clearance properties that are favorable for use in the delivery of cytotoxic agents. For instance, we have observed that smaller formats of vascular targeting antibodies (e.g., SIP and diabody) exhibit favorable tumor to organ ratios already a few hours after i.v. administration[32]. For the construction of ADCs and choice of conjugation strategy, this is a key parameter, as the half-life of the cleavable linkage between antibody and drug should exceed the half-life of the intact conjugate. For example, the half-life of a cleavable site that may be short for ADCs based on IgG modification (e.g., 24 h) may be long for ADCs based on smaller antibody formats and/or vascular targeting antibodies. In addition, internalizing ADCs in the diabody and IgG formats linked to monomethyl auristatin E or F using a N-ethylmaleimide linker equipped with a protease-cleavable valine-citrulline site have been shown to possess similar potency *in vivo* despite 25- to 34-fold faster clearance of the diabody than IgG[33]. Together, these results encourage further development and optimization of vascular targeting ADCs used in recombinant formats.

In this protocol, we describe two novel classes of chemically defined ADCs that were built taking into account the above considerations[22–24]. In both cases, cysteines were genetically engineered at the C and N terminus to generate homogenous vascular targeting ADCs by using disulfide and thiazolidine linkages (**Box 1**). The strategies are targeted directly at C- or N-terminal cysteine residues that may be routinely engineered in any antibody format, making the developed strategies of general utility. It should be noted that the disulfide chemistry detailed here may be used in combination with the THIOMAB technology[14,15], which enables the incorporation of reactive cysteines in intact antibodies. In addition, the thiazolidine chemistry may also prove to be useful for the modification of aldehyde-tagged proteins with cysteine-bearing drugs[17]. The release process from ADCs built using the site-specific strategies reported here is exquisitely traceless, resulting in the free drug and native antibody.

Experimental design

We developed two complementary methods that enable the site-specific modification of vascular targeting antibodies in recombinant formats using traceless strategies (i.e., upon *in vivo* cleavage of the disulfide or thiazolidine bond only the native antibody and free drug will result)[22–24].

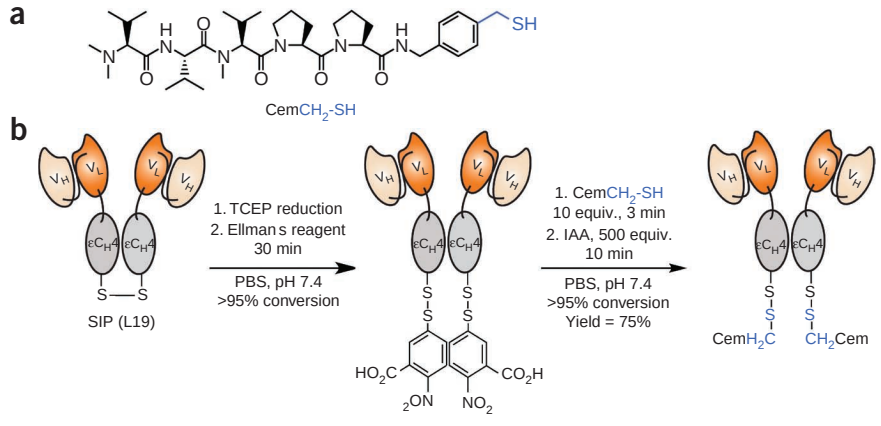

Figure 2 | Construction of a traceless chemically defined tumor-vascular targeting antibody-drug conjugate based on disulfide linkage. (**a**) $CemCH_2$-SH, a potent cytotoxic thiol derivative of cemadotin used in this protocol for the construction of traceless and homogenous ADCs. (**b**) Site-specific modification of C-terminal cysteines of SIP(L19) via mixed disulfide formation with a potent thiol drug. (Note: the same modification strategy can be used with the antibody SIP(F8); see **Supplementary Figure 2**.)

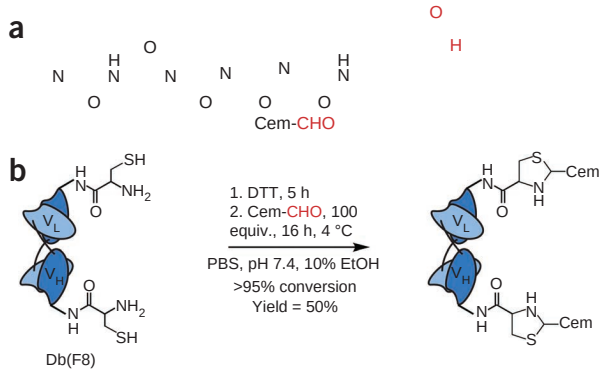

Figure 3 | Construction of a traceless chemically defined tumor-vascular targeting antibody-drug conjugate based on thiazolidine linkage. (**a**) Cem-CHO, a potent cytotoxic aldehyde derivative of cemadotin used in this protocol for the construction of traceless and homogenous ADCs. (**b**) Site-specific modification of N-terminal cysteines of Db(F8) via thiazolidine formation with a potent aldehyde drug. Similar conditions can be used to modify full IgGs (see **Fig. 1**). EtOH, ethanol.

As a tumor-targeting moiety, we used the noninternalizing human antibodies F8 and L19 that have been shown in mice and in the clinic to successfully target the EDA and EDB of fibronectin, respectively, as markers of tumor angiogenesis[28]. Unlike most ADCs developed to date that are specific for a particular cancer cell surface antigen, our vascular targeting approach offers pan-tumoral coverage, as the oncofetal fibronectin isoforms containing EDA or EDB are highly overexpressed in the neovasculature and stroma. In principle, ADCs generated using vascular targeting antibodies can be used for most types of aggressive solid tumors and in hematological malignancies[34]. The antibodies were used in the diabody and SIP formats (**Fig. 1**), as comparative evaluations demonstrated that recombinant antibody formats may possess similar or even superior biodistribution properties when compared with intact immunoglobulins in IgG format[32]. In addition, these antibody formats allow introduction of C- and N-terminal cysteines for site-specific chemical modification without compromising the tumor-targeting capacity.

As a cytotoxic moiety, we chose cemadotin, a tubulin polymerization inhibitor analog of the group of dolastatins[35,36]. This drug is a water-soluble, linear peptide that possesses in vitro cell-killing activity in the low-nanomolar range and has been studied extensively in the clinic[37]. Key in our traceless approach is the design of drugs that contain functionalities that may be used for site-specific chemical modification of engineered cysteines without the need to use linkers. We designed and synthesized analogs of cemadotin containing a sulfhydryl group (CemCH_2-SH) (**Fig. 2**) or an aldehyde group (Cem-CHO) (**Fig. 3**), respectively, which are suitable coupling partners for C- and/or N-terminal cysteines and that retained cell-killing activity in the low-nanomolar range[22–24]. The observed variability of the in vitro potency of the free thiol drug derivatives is probably due to the formation of mixed disulfides via thiol or disulfide exchange reaction with cysteine and disuflides present in the cell culture medium. Analogously to previous reports on maytansinoid drugs[38], the stable S-methyl thioether derivative of Cem-CH_2SH is as potent as the parent cemadotin drug.

First, we explored the reactivity of C-terminal cysteines in the site-specific formation of mixed disulfides[22,24]. This approach involves mild reduction of the covalent C-terminal disulfide bond using Tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP·HCl), followed by activation of the resulting two cysteine residues with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent) (**Fig. 1b**). Subsequent incubation with as little as 10 equivalents of a thiol drug, in this case ChemCH_2-SH, at room temperature in PBS (pH 7.4), with purified antibody and Ellman's reagent yielded a homogenous mixed disulfide ADC (**Fig. 2b**). This conjugation method proceeded with complete conversion (>95%) within 3 min, and it enables the preparation of ADCs with excellent overall yields. This approach is detailed in the Procedure section. The second method is based on the modification of N-terminal cysteines with aldehydes to form a thiazolidine-linked conjugate (**Fig. 1c**; ref. 23). The procedure for this approach can be found in **Box 1**. An aldehyde drug, in this case Cem-CHO, undergoes thiazolidine formation by reaction with a 1,2-aminothiol functional group introduced in a protein either by engineering of the N terminus or by the use of a suitable cross-linker (or a maleimide/thiazolidine heterobifunctional linker). The thiazolidine heterocycle is generated in a mild reducing environment (TCEP or 1,4-DTT) with an excess of aldehyde reagent in PBS at neutral pH (**Fig. 3b**). The condensation reaction proceeds with excellent conversion (>90%) and enables the preparation of ADC with good overall yields.

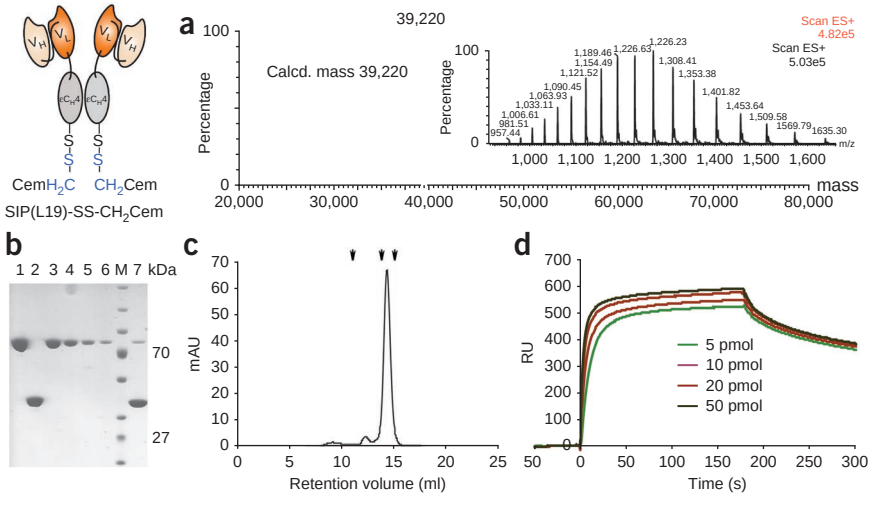

Figure 4 | Characterization of purified SIP(L19)-SS-CH_2Cem. (**a**) ESI-MS spectrum of purified SIP(L19)-SS-CH_2-Cem. (**b**) SDS-PAGE; (lane 1) SIP(L19) 0.34 mg ml $^{-1}$; (lane 2) reduced SIP(L19) 0.34 mg ml $^{-1}$; (lanes 3–6) SIP(L19) 0.17, 0.08, 0.04 and 0.02 mg ml $^{-1}$; M, molecular marker; (lane 7) purified SIP(L19)-SS-CH_2Cem conjugate 0.21 mg ml $^{-1}$. (**c**) Gel-filtration analysis of purified SIP(L19)-SS-CH_2Cem conjugate on a Superdex 200 HR 10/30; arrows indicate standard proteins (11 ml: ferritin 440 kDa; 14.1 ml: BSA 67 kDa; 15.4 ml: lactoglobulin 35 kDa). (**d**) Biacore analysis of purified SIP(L19)-SS-CH2Cem toward recombinant 7B89 fibronectin. RU, resonance units.

19| Manually collect fractions containing the modified protein. In this example, we collected 1.5-ml fractions between 12 and 24 ml of elution volume. The typical collected volume is 12 ml, and it can be used directly for the next step. (Typical recovery after this step is 75%, and the concentration is typically 0.8 mg ml $^{-1}$. In this specific example, the concentration was 0.85 mg ml $^{-1}$.)

PAUSE POINT Store the eluted fraction at 4 °C. The conjugate may be stored for a period of 10 d at 4 °C; for longer periods, store the conjugate at −80 °C.

20| Wash the column with at least 210 ml of PBS before the next purification run.

Conjugation of activated SIP(L19) to CemCH$_2$-SH, followed by IAA quenching ⌊ **TIMING 25 min**

21| Prepare a 1M stock solution of IAA (keep it in the dark).

22| Weigh CemCH$_2$-SH (10 equivalents, 2.1 mg in this example) into a 12-ml round-bottomed Falcon tube by using a microbalance.

! CAUTION The reaction conditions described are general for thiol-containing molecules, and they can be performed by using standard laboratory equipment. The coupling of cytotoxic drugs may require the use of appropriate workstations and a controlled environment, depending on the toxicity and mutagenicity of the drug under study.

CRITICAL STEP After weighing (Step 22), store CemCH$_2$-SH under argon to avoid oxidation and degradation.

? **TROUBLESHOOTING**

23| Equilibrate the Falcon tube containing CemCH$_2$-SH under argon atmosphere.

24| Transfer the purified SIP(L19)-SS-Ellman's (12 ml, 0.85 mg ml $^{-1}$ in this example) into a plastic round-bottomed Falcon tube.

25| Degas the protein with three vacuum/argon cycles. Apply vacuum by using a needle through a rubber septum and leave the protein under argon flow.

26| Transfer the degassed protein to the Falcon tube containing CemCH$_2$-SH and stir the reaction mixture for 3 min at room temperature. The protein solution should become yellowish now.

? **TROUBLESHOOTING**

27| Add 500 equivalents of the 1 M IAA solution to quench free thiol moieties that could be responsible for undesired disulfide exchange reactions with the desired product.

28| Incubate the reaction mixture for 10 min at 25 °C in the dark.

? **TROUBLESHOOTING**

29| Centrifuge the mixture for 10 min at 3,000*g* and 4 °C.

Purification of SIP(L19)-SS-CH$_2$Cem ⌊ **TIMING 60 min**

CRITICAL Purify the final conjugate SIP(L19)-SS-CH$_2$Cem by again using the ÄKTA FPLC system, this time with the HiPrep desalting column as well. The released NTB acid can be removed in this purification step.

30| Follow Steps 14–16 to prepare the system for the purification runs.

? **TROUBLESHOOTING**

31| Inject the SIP(L19)-SS-CH$_2$Cem conjugate crude mixture (12 ml in this example, with a 14 ml loop) and use the following settings for the purification run: HiPrep desalting column, flow rate: 2 ml min $^{-1}$; column pressure limit: 0.15 MPa; detection with UV detector at a wavelength of 280 nm; sample injection (empty loop with): 30 ml; fractionation: 0 ml; length of elution: 3 CVs.

32| Manually collect the fractions containing the modified protein. In this example, 1.5-ml fractions were collected between 12 ml and 24 ml of elution volume. Typical collected volume is 12 ml. (The typical recovery after this step is >90%; and the concentration is typically 0.6–0.8 mg ml $^{-1}$. In this specific example, the concentration was 0.80 mg ml $^{-1}$.)

PAUSE POINT Store the eluted fraction at 4 °C. The conjugate may be stored for a period of 10 d at 4 °C; for longer periods, store the conjugate at −80 °C.

33| Wash the column with 210 ml of PBS before the next purification run.

Concentrating the final conjugates for therapy efficacy studies ⌊ **TIMING variable**

34| To obtain the final conjugates at a high concentration, follow the instructions under Steps 9–13. As soon as the protein concentration is beyond 1 mg ml $^{-1}$, measure the absorbance of the protein at a wavelength of 280 nm with a NanoDrop. Use deionized water to initialize the instrument and use PBS as a blank.

35| Freeze the nal concentrated ADCs with liquid nitrogen and store aliquots of 1 ml at 80 °C.
PAUSE POINT The product is stable at 80 °C for months and through repetitive freeze-thaw cycles.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4, 5, **Box 1** (step 8)	Reduction is incomplete (detectable on SDS-PAGE gel; SDS-PAGE shows an upper band corresponding to the dimeric form of the Db or SIP antibodies)	TCEP or DTT might be degraded; insufficient amount of reducing agent; or insufficient reaction time	Use a new bottle of TCEP or >30 equivalents of TCEP for the reduction step; use a fresh DTT solution or >50 equivalents of DTT
10, 13, **Box 1** (steps 2, 16)	Vivaspin: more solution volume in flow-through than expected	Vivaspin membrane leaks	Measure absorbance of the flow-through at 280 nm by a UV spectrophotometer. If absorption is >0.1, prepare a new Vivaspin and use the flow-through again to concentrate the protein
14, 30, **Box 1** (step 13)	Air peaks in the elution profile of the protein	Air bubbles were injected together with the injection of the protein solution; column is at the end of its life	Reverse the flow direction and pump 100 ml of well- degassed PBS at 5 ml min ^{1} or change the system to deionized water and wash the column with 20 ml of water before you wash the column again with PBS Use a new column if washing does not help Prevent the injection of air by degassing the sample in the syringe (close the opening of the syringe, pull back the plunger and carefully tap the syringe to remove the visible air bubbles)
14	No clear separation of protein and yellow Ellman s reagent is observed (eluted protein fraction is yellowish)	Column is overloaded	Wash the column with additional 5 CV of PBS Purify the eluted protein fraction again by using exactly the same settings and collect the protein fraction
22	$CemCH_2$-SH is oxidized and/or degraded	Oxidation and/or storage at room temperature can lead to degradation	Reduce $CemCH_2$-SH with TCEP (10 mM, 1 h, 37 °C). If this fails, re-synthesize and use a new batch of $CemCH_2$-SH
26	Solution of protein does not get yellowish after adding SIP(L19)-SS-Ellman s to $CemCH_2$-SH	Incomplete reaction; $CemCH_2$-SH could be oxidized or degraded	Run LC-MS after purification: if MS shows the desired conjugate, proceed to the next step (34). If MS shows remaining SIP(L19)-SS-Ellman s reagent, add extra 10 equivalents of $CemCH_2$-SH, and check LC-MS of conjugate If the reaction did not proceed to completion, this may be an indication of $CemCH_2$-SH oxidation/degradation; run a MS and/or NMR to confirm the purity of $CemCH_2$-SH
28	SDS-PAGE shows a minor band at ~75 kDa for the reactions of SIP(L19)	This band could correspond to reoxidation of C-terminal cysteines to form a covalent dimer	Reduce reaction time between SIP(L19)-Ellman and $CemCH_2$-SH. Monitor the reaction by SDS-PAGE
Box 1 (step 9)	Excess Cem-CHO	Reasonable reaction times require a large excess of the drug aldehyde	The large excess of Cem-CHO can be recovered by simple DCM extraction of the FPLC waste: the procedure is repeated three times with a one-third volume of DCM per extraction. The combined DCM layers are subsequently dried with Na_2SO_4, filtered, evaporated and finally purified by reverse-phase HPLC, as previously described[23]

TIMING

Steps 1–5, disulfide site-specific modification of C-terminal cysteines: 3–16 h
Steps 6–8, cysteine activation using Ellman's reagent: 45 min
Steps 9–20, purification of the SIP(L19)-SS-Ellman's reagent: 90–120 min
Steps 21–29, conjugation of activated SIP(L19) to CemCH_2-SH followed by IAA quenching: 25 min
Steps 30–33, purification of SIP(L19)-SS-CH_2Cem: 60 min
Steps 34 and 35, concentrating the final conjugates for therapy efficacy studies: variable
Box 1, steps 1–6, prepare protein solution for conjugation reactions: 30–90 min
Box 1, steps 7 and 8, N-terminal cysteine reduction: 5 h
Box 1, steps 9–12, conjugation of the reduced Cys-Db(F8) to Cem-CHO via thiazolidine condensation: 16 h
Box 1, steps 13–15, purification of Cem-thz-Db(F8): 1 h per FPLC run
Box 1, step 16, concentrating the final conjugates for therapy efficacy studies: variable

ANTICIPATED RESULTS

The purity of the FPLC-purified ADCs can be confirmed by LC-MS, SDS-PAGE and size-exclusion chromatography, whereas immunoreactivity can be studied using surface plasmon resonance (Biacore). These data are shown in **Figures 4** and **5** and **Supplementary Figure 1**. After site-specific conjugation of the drugs to the antibodies, >95% conversion should be observed and ADCs should be detected in a homogenous form by using LC-MS and SDS-PAGE. LC-MS can be used to monitor the progression of the reaction (see below conjugation analysis by LC-MS and **Supplementary Fig. 2**). Typical yields of 50–75% are obtained, from modification to purification and final concentration of ADCs for *in vivo* evaluation, when using the site-specific strategies for the modification of antibody fragments reported in this protocol. In addition, after modification, the ADCs should remain immunoreactive.

Characterization of SIP(L19)-SS-CH_2Cem

A single peak after deconvolution of the multiply charged ions spectrum should be detected by LC-MS analysis of SIP(L19)-SS-CH_2Cem at 39,220 Da; SDS-PAGE analysis should give a single major band at ~39 kDa; in some cases, SDS-PAGE shows a very minor band at ~75 kDa (see TROUBLESHOOTING); a peak eluting at a retention volume of 14.9 ml corresponding to the noncovalent homodimeric form of SIP(L19)-SS-CH_2Cem should be detected by gel filtration; and finally, SIP(L19)-SS-CH_2Cem should remain immunoreactive toward recombinant 7B89 fibronectin, as observed by direct comparison of the sensorgram profiles of modified and nonmodified antibody fragments.

Characterization of Db(F8)-thz-Cem

A single peak after deconvolution of the multiply charged ion spectrum should be detected by LC-MS analysis of Db(F8)-thz-Cem at 25,598 Da; SDS-PAGE analysis should give a single major band at ~26 kDa; a peak eluting at a retention volume of 15.1 ml corresponding to the noncovalent homodimeric form of Db(F8)-thz-Cem should be detected by gel filtration; and finally, Db(F8)-thz-Cem should remain immunoreactive toward recombinant 11A12 fibronectin, as observed by direct comparison of the sensorgram profiles of modified and nonmodified antibody fragments.

Conjugation analysis by LC-MS

A typical analysis of a conjugation reaction by LC-MS is shown in **Supplementary Figure 1**. The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the starting material and product of the reaction of SIP(F8)-Ellman's with CemCH_2-SH. Identical analyses can be carried out for all the conjugation reactions described in this protocol.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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