

Chapter 6

Generating DAR 2 Antibody-Drug Conjugates Using Bis-Divinyl Pyrimidine Reagents

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Abstract

Antibody–drug conjugates (ADCs) enable the selective delivery of drugs to specific cell types and have seen growing use as treatments for oncology targets. Homogenous and stable ADCs promise to exhibit reduced off-target effects, but achieving this goal is hampered by the lack of control of the drug-to-antibody ratio (DAR). Divinyl pyrimidine (DVP) reagents enable the re-bridging of the four native disulfide bridges in IgG1 and IgG4 antibodies to give DAR 1, 2, or 4 ADCs depending on the DVP linker architecture. This protocol describes how to conjugate and analyse bidentate DVP (BisDVP) linkers to native antibodies to achieve a controlled DAR of 2.

Keywords Antibody–drug conjugates, Antibody characterisation, Cysteine conjugation, Disulfide re-bridging, Drug-to-antibody ratio

1 Introduction

Interest in the treatment of oncology targets with antibody–drug conjugates (ADCs) continues to grow year-on-year, with more than 350 in clinical trials [1]. ADCs utilize a targeting antibody, a linker system (either cleavable or non-cleavable), and a drug [2, 3]. The antibody targets specific antigens in the body, allowing for a targeted delivery of a drug to a specific site. However, the vast majority of ADCs rely on maleimide conjugation strategies, which are known to be instable and prematurely release the payload; other systems use stochastic conjugation to the myriad lysine residues found on an antibody surface. Both of these methods lead to varied drug-to-antibody ratios (DARs), causing heterogeneity that is known to lead to poor pharmacodynamic and pharmacokinetic profiles [2, 4]. The ability to precisely control the DAR also allows tuning of the properties of an ADC such as activity and aggregation propensity [5, 6].

Gianfranco Pasut and Shawn C. Owen (eds.), *Antibody-Drug Conjugates (ADCs)*, Methods in Molecular Biology, vol. 3109,
https://doi.org/10.1007/978-1-0716-5719-5_6,
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Re-bridging the natural interchain disulfide bonds in IgG1 and IgG4 antibodies allows for a site-specific conjugation between the four pairs of cysteines. This has enabled the generation of homogenous DAR 4 conjugates using native antibodies [7–9]. Divinyl pyrimidine (DVP) linkers enable the generation of highly stable ADCs with DARs of one [10–12], two [10], and four [13–15]. Linkers that contain two DVP head groups with a single payload (thus leading to a DAR 2 ADC) are termed BisDVPs.

Herein is described the protocol for the bioconjugation of BisDVPs to antibodies and subsequent "click" chemistry to append a payload to the construct (Fig. 1). Attaching a payload after bioconjugation allows for fast derivation of a single antibody with a variety of drugs, fluorophores, and probes; it also ensures that the properties of the payload do not interfere with the reliability of the bioconjugation. Attaching the payload prior to bioconjugation is also viable, allowing for a greater diversity of chemistries to be used that would not be possible in the presence of the antibody. The bioconjugation of MonoDVP linkers to generate DAR 4 constructs has been previously discussed [16].

This chapter also includes information about the key characterization techniques used to check the purity and DAR of an ADC. Each gives different information about the ADC: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the extent of conjugation; mass spectroscopy (MS), the presence of the desired DAR 2 conjugate; hydrophobic interaction chromatography (HIC), the ratio between different DAR species; and size exclusion chromatography (SEC), the presence of aggregation.

Whilst the solution-phase synthesis of BisDVP has been published [10], we recommend synthesizing these large molecules using solid-phase synthesis (Fig. 2) techniques analogously to our TetraDVP linkers [12].

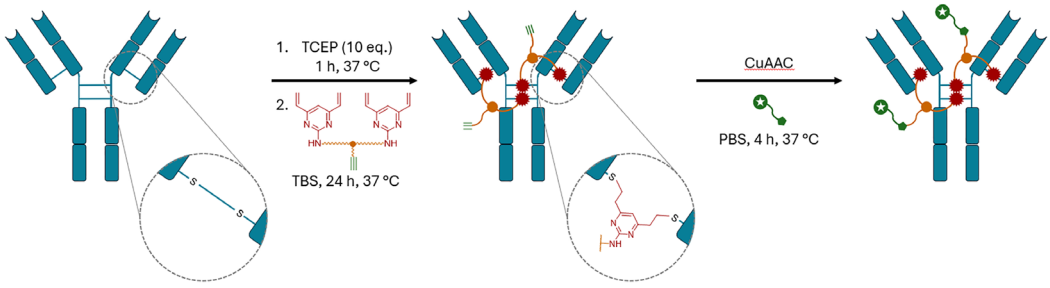


Fig. 1 Antibody schematic showing the relative locations of the interchain disulfide bridges and how BisDVP linkers conjugate to the antibody. The alkyne click handles can be modified pre- or post-bioconjugation. CuAAC = copper-catalyzed azide-alkyne cycloaddition, PBS = phosphate-buffered saline, TBS = Tris-buffered saline

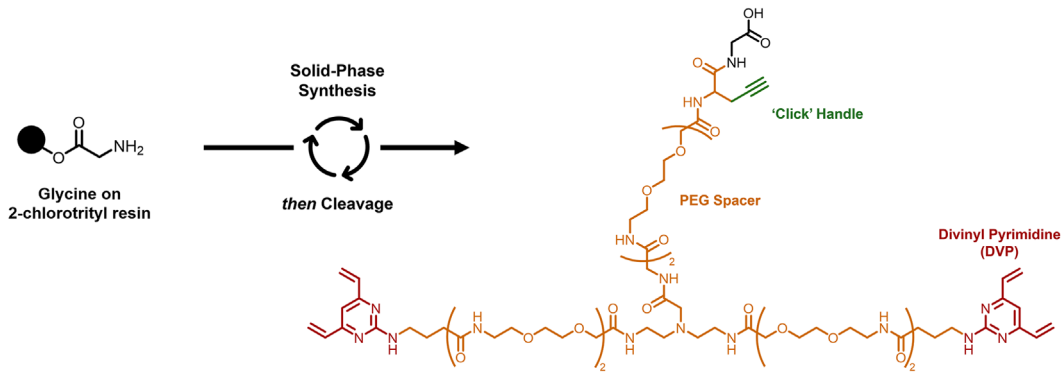


Fig. 2 Solid-phase peptide synthesis (SPPS) of a BisDVP-alkyne linker from glycine-loaded 2-chlorotrityl resin. The full linker is built up and then cleaved from the resin with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) [12]

2 Materials

All buffers are made using ultrapure water (i.e., Milli-Q) unless otherwise stated.

2.1 Antibody Preparation and Conjugation

1. IgG1 antibody (i.e., Trastuzumab; lyophilized solid) (*see* **Note 1**).
2. 500 µL Centrifuge tubes (*see* **Note 2**).
3. Tris-buffered saline (TBS) solution: 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0 with 1 M aq. NaOH.
4. Tris(2-carboxyethyl)phosphine (TCEP).
5. Thermal shaker unit (such as a Grant-bio PCMT ThermoShaker) capable of agitating a sample whilst maintaining 37 °C.
6. UV-Vis spectrometer (such as a ThermoFisher NanoDrop One) for antibody concentration determination.
7. BisDVP linker; 5 mM in DMF (*see* **Note 3**) [10, 12].
8. 0.5 mL 40k Molecular weight cutoff (MWCO) desalting columns (such as Zeba™Spin).
9. 0.5 mL 10k MWCO diafiltration columns (such as Amicon® Ultra).
10. Centrifuge capable of holding 0.5–2 mL centrifuge tubes and running at up to 14,000 × g (such as VWR Micro Star 17)
11. Phosphate-Buffer Saline (PBS) solution: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 (*see* **Note 4**).

2.2 Drug Conjugation via Click Chemistry

1. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA); 20 mM in water.
2. Copper (II) Sulfate; 5 mM in water.
3. Sodium Ascorbate; 50 mM in water.
4. Drug-Azide; 10 mM in DMSO.
5. PBS solution (as above).

2.3 SDS-PAGE Analysis

1. Heating block/sand bath capable of heating 0.5 mL vials to 90 °C.
2. 0.5 mL vials.
3. PBS solution (as above).
4. Broad range unstained protein standard, molecular weight (MW) ladder (10–200 kDa).
5. Non-reducing sample stain: 0.125 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl), 20% glycerol, 4% SDS, 0.02% bromophenol blue, adjusted to pH 6.8.
6. Reducing sample stain: 0.125 M Tris-HCl, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 1.25 mM β-mercaptoethanol, adjusted to pH 6.8.
7. Laemmli running buffer (LRB): For 1 L; 3 g 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), 14.4 g glycine, 1 g SDS.
8. 12% Acrylamide gel including 4% stacking gel (*see* **Note 5**).
9. Gel electrophoresis tank and power supply (such as Bio-Rad mini-PROTEAN™ Tetra System).
10. Coomassie brilliant blue (CBB) stain: For 500 mL, dissolve in order; 0.5 g CBB R250 dye, 50 mL EtOH, 400 mL water, then 50 mL AcOH.

2.4 Mass Spectrometry Analysis

1. PNGase F for deglycosylation.
2. Protein LC–MS system (such as Waters Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm)) with MS deconvolution software (such as Waters MaxEnt1 algorithm).
3. Mobile phase: A; 0.1% aq. formic acid. B; 95% MeCN with 5% aq. formic acid (at 0.1%).

2.5 HIC Analysis

1. HPLC (such as Agilent 1260 Infinity system) with analytical HIC column (such as Tosoh TSKgel Butyl-NPR column (3.5 cm × 4.6 mm, 2.5 μm)).
2. Mobile phase: A; 1.5 M ammonium sulfate, 25 mM Na_3PO_4, pH 7. B; 25% (v/v) isopropyl alcohol in 25 mM Na_3PO_4, pH 7.

2.6 SEC Analysis

1. HPLC (such as Agilent 1260 Infinity system) with analytical SEC column (such as Tosoh TSKgel G3000SWXL column (30 cm × 7.8 mm, 5 μm)).
2. Mobile phase: 50 mM Na_3PO_4, 100 mM NaCl, 0.2% (w/v) sodium azide, pH 7.

3 Methods

All procedures carried out at ambient temperature and in air unless otherwise stated.

3.1 Antibody Preparation and Conjugation

1. Make up an antibody solution in TBS (100 μL, 10 μM) (*see* **Note 6**). Record the UV-vis absorbance at 280 nm (using TBS for the background reading) and calculate the concentration using the extinction coefficient of the antibody (*see* **Note 7**) and the Beer–Lambert law. For trastuzumab, ε = 215,380 (*see* **Note 8**).
2. Make up a 5 mM solution of TCEP in TBS (*see* **Note 9**).
3. Add 10 eq. of TCEP (5 mM) to the antibody solution (*see* **Note 10**) and incubate on a thermal shaker (1 h, 37 °C, 400 rpm).
4. Add 5 eq. of BisDVP (5 mM) to the antibody solution (*see* **Note 10**) and incubate on a thermal shaker (24 h, 37 °C, 400 rpm).
5. Equilibrate a 0.5 mL 40k MWCO desalting column into PBS (*see* **Note 11**) according to the manufacturer's specification.
6. Pipette the antibody solution into the desalting column and centrifuge to the manufacturer's specification.
7. Transfer the filtrate into an 0.5 mL 10k diafiltration column and dilute with 300 μL PBS (*see* **Note 12**). Spin-concentrate the sample for 2 min at 14,000 × g using a centrifuge. Add a further 300 μL PBS and spin-concentrate again. Repeat this process of dilution and concentration four times. Dilute a fifth time and centrifuge for 10 min to concentrate the sample to ~30 μL.
8. Recover the re-bridged conjugate by inverting the filter section of the diafiltration column into a clean tube and centrifuging for 2 min at 4000 × *g*.
9. Record the final concentration via UV-vis (*see* **Note 13**).
10. Analyze by SDS-PAGE, MS, and SEC (see below).

For many re-bridging systems, hydrophobic interaction chromatography (HIC) is useful in determining how many linkers have become conjugated to an antibody. However, as the hydrophobicity

of a BisDVP-Alkyne linker is the same as that of a natural antibody, the addition of any number of these linkers to an antibody has no effect on the HIC spectra, and therefore, at this stage, the use of HIC is fruitless. Once a payload has been clicked to the construct, thus altering the ADC's hydrophobicity, the use of HIC once again becomes beneficial.

3.2 Drug Conjugation via Click Chemistry

1. Make up a solution of BisDVP-Antibody in PBS (100 µL; 10 µM). Record the exact concentration (as above).
2. Add THPTA (200 eq.), copper (II) sulfate (40 eq.), sodium ascorbate (300 eq.), then Drug-Azide (20 eq.). Incubate on a thermal shaker (4 h, 37 °C, 400 rpm).
3. Equilibrate two 0.5 mL 40k MWCO desalting columns into PBS (*see* **Note 11**) according to the manufacturer's specification.
4. Pipette the antibody solution into one of the desalting columns and centrifuge to the manufacturer's specification. To ensure the removal of copper salts, transfer the filtrate into the second column and centrifuge again.
5. Transfer the filtrate into an 0.5 mL 10k diafiltration column and dilute with 300 µL PBS (*see* **Note 12**). Spin-concentrate the sample for 2 min at 14,000 × g using a centrifuge. Add a further 300 µL PBS and spin-concentrate again. Repeat this process of dilution and concentration four times. Dilute a fifth time and centrifuge for 10 min to concentrate the sample to ~30 µL.
6. Recover the ADC by inverting the filter section of the diafiltration column into a clean tube and centrifuging for 2 min at 4000 × *g*.
7. Record the final concentration via UV-vis (*see* **Note 13**). Analyze via MS, HIC, and SEC (as above). Due to the relatively small size of most payloads, SDS-PAGE analysis will not look any different before and after click chemistry.

3.3 SDS-PAGE—Assessing Extent of Conversion

1. Preheat a heating block to 90 °C.
2. Make up solutions (10 µL, 1 µM; *see* **Note 14**) of the analyte and any controls (e.g., unfunctionalized antibody) in PBS buffer in 0.5 mL vials.
3. Add 10 µL sample stain (reducing or non-reducing) to all samples.
4. Aliquot a 5 µL sample of MW Ladder into a 0.5 mL vial.
5. Heat all samples to 90 °C for 5 min to denature the protein.
6. Prepare the gel electrophoresis equipment in LRB according to the manufacturer's specifications, placing the gel in the holder, and wetting the components and gaskets to ensure a good seal is made.

7. Load the individual wells of the acrylamide gel with the MW ladder followed by the analyte and control samples (*see* **Note 15**).
8. Run the gel at 200 V for 65 min.
9. Remove the gel from between the glass plates, rinse with deionized water, cutoff the staking gel, and submerge in CBB stain for at least 2 h (*see* **Note 16**).
10. Holding the gel gently down with a finger, carefully decant off the CBB stain and rinse the gel gently with water. To destain, submerge the gel in 10% aq. AcOH. Replace the aqueous acid periodically until the main face of the gel is colorless (*see* **Note 17**).
11. Remove the gel from the aq. AcOH and photograph it (*see* **Note 18**).

Running the SDS-PAGE gel with unfunctionalized antibody as a control ensures that the method has been run correctly and that comparisons can be made. An efficiently re-bridged antibody will appear as high molecular weight bands in reducing conditions as there are no remaining disulfide bonds to cleave (Fig. 3; 1 and 2). Reduced unmodified antibody will split into the constituent heavy and light chains on the gel (Fig. 3; Ab +) whilst unreduced unmodified antibody will appear as a single high MW band on the gel (Fig. 3; Ab –).

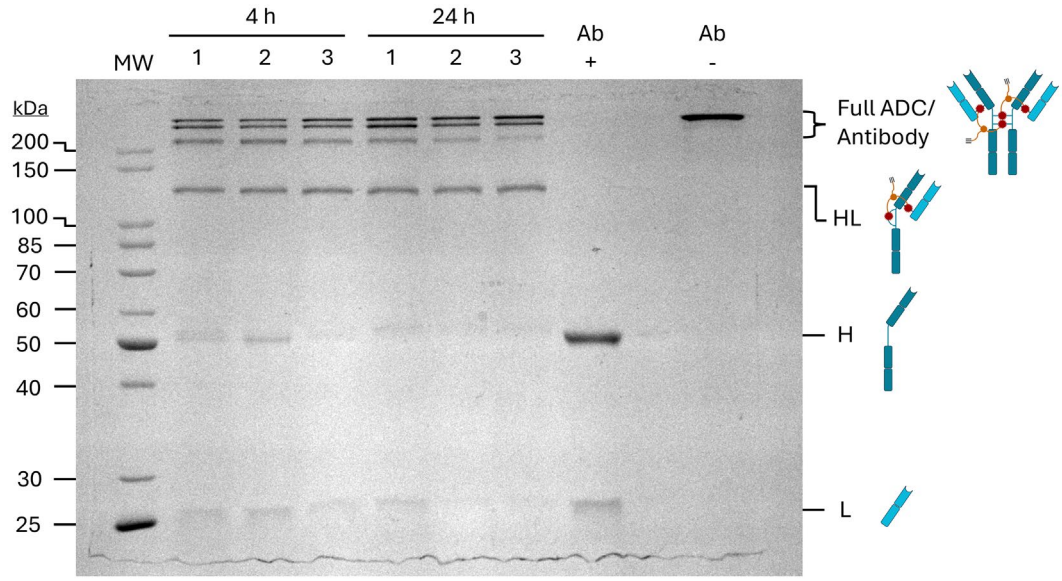


Fig. 3 SDS-PAGE gel showing the extent of conjugation of an IgG1 ADC. The ADC analytes were run in a reducing stain; therefore, bands of higher molecular weight show that the heavy and light chains have been chemically re-bridged. Three samples were analyzed after 4 and 24 h of bioconjugation; **1** = 6 eq. TCEP, 10 eq. BisDVP; **2** = 10 eq. TCEP, 5 eq. BisDVP; **3** = 10 eq. TCEP, 10 eq. BisDVP. Ab +/- = Native antibody with/without reducing stain, MW = Molecular weight ladder, H = Heavy chain, L = Light chain

The presence of re-bridged heavy-light chain (Fig. 3; HL) is causes by heavy-heavy intra-chain cysteine re-bridging competing with the inter-chain re-bridging. The second DVP head group re-bridges between the light and heavy chains. This is common for disulfide re-bridging systems and has no significant effect on ADC properties as the full ADC is held together via non-covalent interactions [7–9, 13, 14]. Due to the "bidentate" nature of BisDVP, less HL re-bridging is observed than with MonoDVP linkers.

3.4 Mass Spectrometry—Assessing Most Prevalent Antibody Species

1. Make up analytical samples (25 µL, 3–10 µM) in PBS (*see* **Note 19**).
2. Add 0.2 µL PNGase F and incubate the sample at 37 °C for 2 h to remove sugars on the antibody surface that interfere with the MS.
3. Inject 3 µL into the MS. Run the analysis at a flow rate of 0.2 mL/min with gradient: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 min, then a gradient to 95% A over 1.04 min. Capillary voltage of 2.0 kV and a cone voltage of 190 V.
4. Combine the ion count of the raw spectra and run the spectra deconvolution algorithm for *m/z* ions between 1400 and 3400, resolving for masses between 40,000 and 160,000 (Fig. 4).

3.5 HIC Analysis—Calculating Conversion and Average DAR

1. Make up analytical samples (30 µL, 3–10 µM) in PBS.
2. Inject 15 µL into HPLC system. Run with a linear gradient of 100% solvent A for 3 min, then 0–100% solvent B over 17 min at a flow rate of 0.6 mL/min.
3. Monitor UV absorbance at 280 nm, with conversion calculated by peak area compared to unmodified antibody. Coupled with MS, the DAR can be calculated via comparison of the peak positions (*see* **Note 20**) and sizes (see Fig. 5a).
4. Once all analyzes are complete, flush the HPLC system and column thoroughly with deionized water to prevent the high salt concentration buffers blocking the system.

3.6 SEC Analysis—Checking for Aggregation

1. Make up analytical samples (30 µL, 3–10 µM) in PBS.
2. Inject 6 µL into HPLC system. Run for 30 min at a flow rate of 0.5 mL/min.
3. Monitor UV absorbance at 280 nm, with aggregation calculated by peak area compared to the antibody. Aggregation appears as a peak of higher molecular weight (Fig. 5b), where a relative integration of <1.5% is considered ideal.
4. Once all analyzes are complete, flush the HPLC system and column thoroughly with deionized water to prevent the high salt concentration buffers blocking the system.

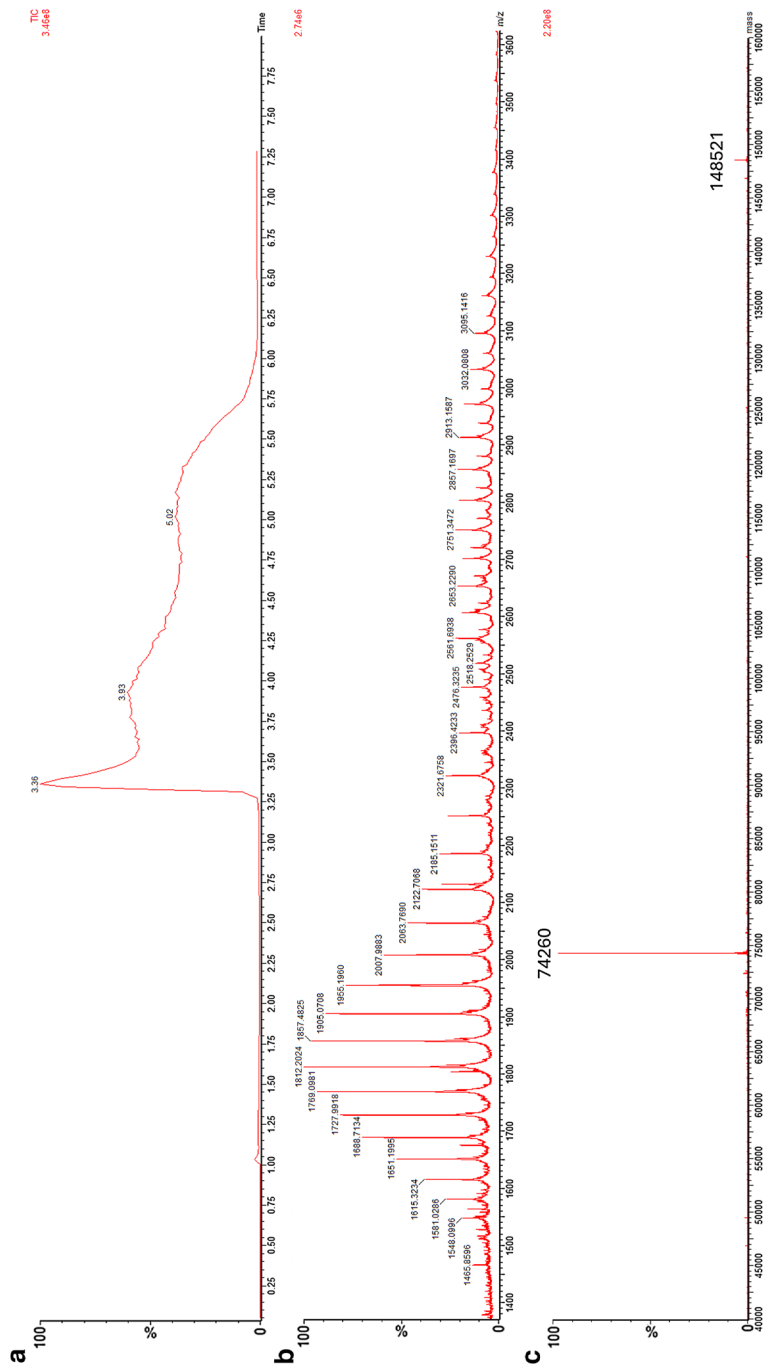


Fig. 4 (**a**), Raw total ion count, (**b**) raw *m/z* data from combining raw total ion count, (**c**) deconvoluted spectrum after running MaxEnt1 algorithm data between 1400–3600 *m/z* searching for masses between 40,000 and 160,000. Major peaks are the expected DAR 2 mass (exp. 148,513) and the half mass $[ADC+2H]^{2+}$ or half-antibody plus a single linker (exp. 74,257)

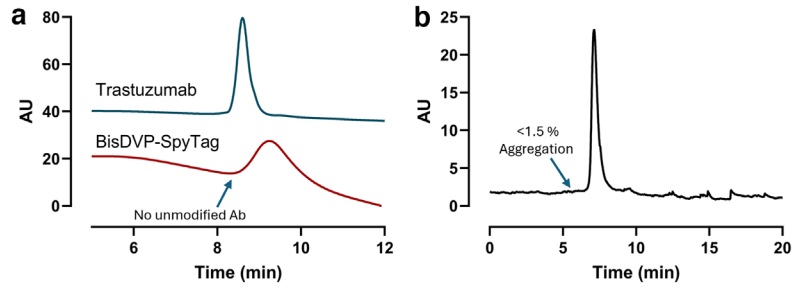


Fig. 5 (**a**), HIC spectrum showing how the addition of a BisDVP with payload affects hydrophobicity compared to native trastuzumab. (**b**) SEC spectrum showing the presence (or lack) of aggregation to the left of the antibody peak. AU = Arbitrary units

4 Notes

1. Antibodies bought in solution can be used but require buffer exchange into TBS using a 10k MWCO diafiltration column.
2. A 500 µL centrifuge tube is ideal for test reactions and reaction optimization. 2 mL Centrifuge tubes can be used for scale-up as long as the thermal shaker can accommodate their shape to provide even heating to the sample.
3. BisDVP solutions in DMF or DMSO should be stored at –4 °C (or below) and can remain viable for up to 2 years. While the BisDVPs have been successfully made in solution, we recommend synthesizing BisDVP linkers on-resin analogously to TetraDVP, described here: [12].
4. PBS can be purchased as a concentrated solution or as dissolvable pellets for simple buffer preparation.
5. SDS-PAGE gels are commercially available but can be self-cast if relevant molds are available; these should be used within 2 weeks of casting.
6. If using a lyophilised powder, about 1 mg is weighed out and diluted to 2.5 mg/ml with TBS. This gives a concentration of c. 10 µM. Antibodies acquired in solution must be buffer exchanged into TBS using an Amicon® diafiltration column via repeated dilution and concentration with TBS.
7. Concentrations between 5 µM and 20 µM are recommended. The exact concentration should be noted to allow accurate calculation of the TCEP and BisDVP reagents. Bioconjugation has been successfully carried out up to 2 mL in a single vial (20 µM). Due to size restraints on many thermal shakers, for larger scales multiple vials should be used.

8. If the amino acid sequence of the antibody is known, then the extinction coefficient can be estimated using the Expasy ProtParam online tool [17]. If the amino acid sequence is unknown, then use the extinction coefficient of a similar known antibody or trastuzumab (215,380). The differences in extinction coefficient for IgG1 antibodies tend to be less than 10%, and so inaccuracies here are not critical and will be offset by the excess of TCEP and BisDVP used.
9. TCEP oxidizes in solution over time, and so stocks must be made fresh directly before use to ensure complete disulfide reduction.
10. When adding TCEP or BisDVP to the antibody solution, gently mix the solutions by repeatedly withdrawing and readmitting the solution with the pipette. This is especially key for the BisDVP solution to prevent the DMF or DMSO settling in the buffer and creating a high local concentration, which can lead to antibody aggregation and precipitation.
11. Desalting columns help to remove excess salts (TCEP, copper, etc.), preventing further reactivity. The need for desalting can be assessed on a case-by-case basis; whilst diafiltration should remove TCEP, experience has shown that copper salts (after CuAAC) are not removed and so desalting is required. The desalting column should be equilibrated into an appropriate buffer system for any post-purification analysis or further study. PBS is commonly used as it is compatible with copper-catalyzed "click" chemistry and biological studies.
12. Repeated PBS dilution and concentration through a 10k MWCO filter affords buffer exchange (reducing DMSO and TCEP concentration) whilst allowing excess DVP linker to be filtered off, purifying the sample. The cellulose filters are delicate, so care must be taken to not pierce the membrane with the tip of a pipette. Once wetted, it is recommended not to let the membrane dry out for high sample recovery. Occasionally agitate the solution in the filter with the tip of a pipette when adding further PBS to ensure the ADC is in solution and not stuck to the cellulose.
13. Concentrations far exceeding 100 μM are not recommended due to increased aggregation propensity.
14. The following equation can be used to easily generate 10 μL solutions at 1 μM:

$$\frac{10}{x} = y$$

Where x = original sample concentration (in μM) and y = volume of sample (in μL) that needs to be diluted to 10 μL to afford

a 1 μM solution (e.g., if your sample is 5 μM then 10/5 μM = 2 μL, diluted up to 10 μL to be a 1 μM solution).

15. Aiming to pipette 10% less volume than was originally in the vial (e.g., 18 μL of a 20 μL sample) will ensure good sample loading whilst preventing bubbles forming in the pipette tip; some of the solution will have evaporated during the denaturing step. It is recommended to leave an empty well between reducing and non-reducing stain samples, as the 2-mercaptoethanol can leach between wells, causing partial reduction to other samples, thus effecting band shape.
16. The stacking gel section can be removed at any stage before imaging, but removing it early reduces the chance for parts of the stacking gel to fold over, effecting the efficiency of de/staining. Plastic pipette tip boxes are an ideal size for gel staining. A gel rocker (such as a Stuart SSM4 see-saw rocker) can be used to gently agitate the submerged gel and ensure that the gel is stained and destained evenly.
17. The gel is destained when only the protein bands are visibly blue. The protein will not be easily destained by the aqueous acid, so it is fine to leave the gel in 10% aq. AcOH for several days.
18. Due to the staining, a standard camera can be used to record the bands on the gel. Gel imagers (such as Syngene ingenious 3 with GeneSys software) are useful if fluorescent components are bioconjugated to the antibody.
19. With the full ADC, for ease, a 50 μL sample can be made up and used for HIC and SEC analyzes. The remaining material can then by deglycosylated with PNGase F for MS analysis.
20. As increasing the number of payloads conjugated to an antibody usually increases the hydrophobicity of an ADC in an additive manner, different DAR species are identifiable by HIC retention time with fairly even spacing between species as number of linkers increases. MS data is not quantitative but should show a DAR 2 species as the major product of a successful conjugation; therefore, the largest HIC peak is thus assigned "DAR 2" with any peaks around it assigned consecutively (e.g., 2 and 0).

Competing Interests

The authors have no competing interests to declare.

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