Application of Next Generation Maleimides (NGMs) to Site-Selective Antibody Conjugation

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Abstract

Site-selective antibody conjugation is widely recognised as a key strategy for the optimum construction of antibody-drug conjugates (ADCs). Achieving such bioconjugation directly onto native antibodies would represent the ideal solution, as it would afford greatly improved homogeneity whilst avoiding the need for genetic engineering, and even allow the repurposing of existing antibodies 'off-the shelf'. Here we describe a protocol for the use of Next Generation Maleimides (NGMs) for the selective modification of the 4 interchain disulfide bonds present in a typical IgG1 antibody format. These reagents retain the efficiency of classical maleimides whilst serving to re-bridge each reduced disulfide bond, affording one attachment per disulfide. The approach is simple, uses readily available reagents, and generates robustly stable conjugates which are ideal for *in vitro* or *in vivo* applications. In addition to use in the construction of ADCs these reagents can also be used to develop antibody-conjugates for imaging, bispecifics, and broadly for use across biology and medicine.

Keywords

Site-selective antibody conjugation, antibody drug conjugates (ADCs), disulfide bridging, Next Generation Maleimides (NGMs)

1. Introduction

Antibody conjugates represent one of the most diversely exploited class of bioconjugates. They form the basis of immunoassays such as ELISAs, radioimmunoconjugates for imaging and therapy, and antibody-drug conjugates (ADCs) for the targeted delivery of cytotoxins. Approaches to antibody conjugation have been dominated by non-selective methods, most commonly targeting lysine residues which cover the surface of antibodies. On a typical antibody there are >80 lysine residues, and it has been estimated for ADCs that a desired average loading of 3-4 drugs per antibody leads to around 10^6 molecular species present in the resulting heterogeneous conjugates [1, 2]. Each component in such a mixture will have a different pharmacological profile, and thus this represents a far from optimal outcome [2, 3]. Alternatively cysteine residues can be targeted as sites for attachment. There are four solvent accessible disulfide bonds in the major therapeutically relevant antibody isotype (IgG1), and these can be readily reduced in preference to the buried intrachain disulfides to generate 8 cysteines. In practice partial reduction is usually carried out, with subsequent conjugation affording products with drug-to-antibody ratios (DARs) of 0, 2, 4, 6 and 8 as the major components [4]. Such conjugates still represent heterogeneous mixtures. Notably the presence of unmodified antibody can inhibit the activity of the ADC, and the higher loaded species have been identified as having reduced stability [5, 6], and poorer outcomes *in vivo* due to their accelerated clearance [4, 7]. The use of classical maleimides as the favoured approach for efficient cysteine conjugation has also been widely demonstrated to suffer a further limitation, as the conjugates are unstable over several days *in vivo*, due to their ability to undergo retro conjugate additions [8, 9].

Increased homogeneity in conjugates has been shown to offer the prospect of improved therapeutic windows [2, 7, 10, 11], and thus site-selectivity is considered a key component in the design of future ADCs [12, 13]. Strategies being explored include the use of genetic engineering to incorporate cysteine mutants, non-natural amino-acids or enzymatic recognition sequences as handles for controlled drug loading [10, 12-14]. Site-selective methods which avoid this requirement for engineering can offer

the further advantage of being directly applicable to native antibodies. To this end disulfide bridging is a leading strategy, in which the interchain disulfide bonds are targeted with reagents which reconnect the two cysteine residues [13, 15-19]. This approach targets antibody conjugates with a controlled loading of one drug per disulfide bond, and thus a DAR of ~4 in IgG1s.

We have recently described the use of next generation maleimides (NGMs) [20-22], which are able to achieve efficient disulfide bridging and thus represent an ideal platform for site-selective antibody conjugation (**Fig. 1**) [23-27]. These reagents retain the rapid kinetics of maleimide conjugation (the conjugation step occurs in < 1 min), and are 'locked' as robustly stable conjugates following quantitative hydrolysis after just 2 hours in the conjugation buffer. Here we summarise a step-by-step guide to their use.

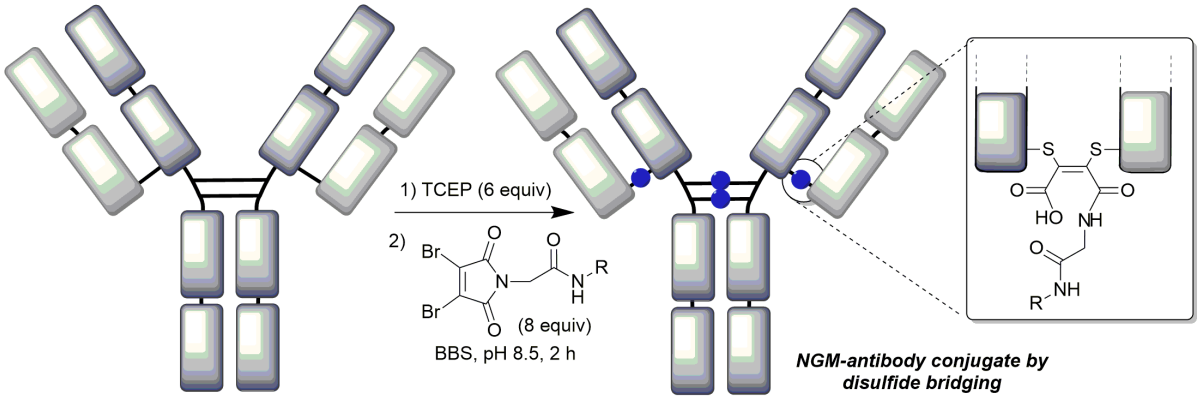

Fig. 1 Antibody conjugation by disulfide bridging using next generation maleimides

2. Materials

The optimum NGM reagents we suggest for antibody bioconjugation by disulfide bridging are dibromomaleimides (DBM) with a C-2 linker (DBM-C_2 reagent, **Fig. 2**) [28]. These reagents are readily synthesised in two steps, starting from commercially available dibromomaleic acid (e.g. Sigma-Aldrich). The synthesis involves initial condensation with glycine to generate DBM-C_2-acid, which is

then coupled with the functional amine of choice (e.g. drug, imaging agent etc.). Details of this synthesis are described elsewhere [28], and are not the focus of this protocol chapter.

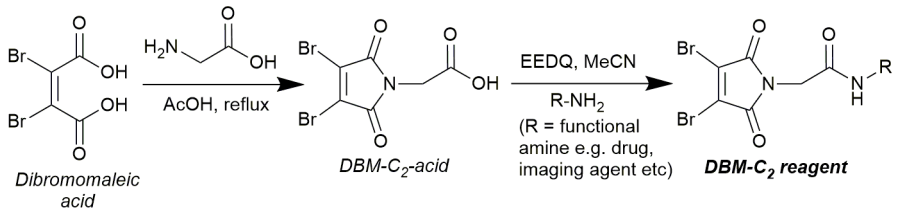

Fig. 2 Synthesis of the DBM-C_2 reagent

2.1 Antibody reduction and conjugation.

1. Prepare buffer solutions with double-deionised water. Filter-sterilise (through 0.22 µm membrane) and degas them, prior to use (*see* **Note 1**).
2. Borate buffered saline (BBS) solution: 50 mM Sodium Borate, 50 mM NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA), at pH 8.5.
3. IgG1 antibody (e.g. lyophilized).
4. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl).
5. DBM-C_2 reagent [28].
6. *N*,*N*-Dimethylformamide, anhydrous (DMF).
7. Vivaspin® 500 polyethersulfone (PES) membrane concentrators with a molecular weight cut-off (MWCO) of 10 kDa for ultrafiltration.
8. Centrifuge operating at 14,000 rcf at 20 °C (e.g. Eppendorf 5415R fixed angle rotor centrifuge).
9. Thermomixer for temperature and agitation controlled experiments.
10. UV spectrophotometer to determine the antibody concentration (e.g. Thermoscientific Nanodrop 2000C operating in A280 mode). Use ε_{280} = 215,380 M^{-1} cm^{-1} for trastuzumab mAb.

2.2 SDS-PAGE analysis of the conjugates

1. 12% acrylamide gels, with a 4% stacking gel.
2. Mini-Protean® Electrophoresis system.

3. Broad-range MW marker (10-250 kDa, BioLabs) to estimate protein molecular weights.
4. Loading buffer stock solution (5x): 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg R-250 dye. *See* **Note 2**.
5. 10 mM dithiothreitol (DTT) solution in water, *see* **Note 3**.
6. SDS-PAGE running buffer (composition for 10x buffer): 30 g Tris base, 144 g glycine, 10 g SDS in 1 L double-deionised water. Store the buffer at room temperature and dilute to 1 x with double-deionised water before use.
7. Staining solution: coomassie blue (0.05% w/v) solution (1 L), contains 49.95% water, 40% methanol and 10% acetic acid (AcOH).
8. Destaining solution (1 L, % v/v): 10% methanol, 10% AcOH and 80% water.

2.3 LCMS analysis of the conjugates

1. 50 mM ammonium acetate buffer, pH 6.9.
2. HPLC-grade acetonitrile, water and formic acid solvents.
3. 15,000 units·mL^{-1} PNGase F in 20 mM Tris-HCl, 50mM NaCl, 5 mM EDTA, pH 7.5 (New England BioLabs Inc.).
4. Tris buffer: 20 mM Tris-HCl, 50mM NaCl, 5 mM EDTA, pH 7.5
5. 0.22 µm centrifuge tube filters (Corning® Costar® Spin-X®).
6. Agilent 6510 QTOF LC-MS system (Agilent, UK) with MassHunter software (version B.07.00) or alternative high accuracy LC-MS with deconvolution software.
7. PLRP-S column, 1000 Å, 8 µm, 150 mm x 2.1 mm.
8. Mobile phase of A (5% MeCN in aqueous 0.1% formic acid) and B (95% MeCN, 5% water, 0.1% formic acid).

3. Methods

The following procedure has been optimised for trastuzumab (an IgG1 antibody). For other antibodies the equivalents of reducing agent will have to be tested to determine the amount which is required

for full reduction. For different antibody isotypes, which contain higher numbers of interchain disulfide bonds, the equivalents of the DBM-C2 reagent would also require increasing.

3.1 Antibody reduction and conjugation.

1. Dissolve the lyophilized antibody in double-deionised water to a final concentration of 10 mg/mL. Buffer exchange it into BBS, *via* ultrafiltration (*see* **Note 4**). Determine the concentration and adjust it to 22.9 µM (3.32 mg/mL, *see* **Note 5**) with BBS (*see* **Note 6**).
2. Dissolve TCEP.HCl in BBS to make a 10 mM (2.87 mg/mL) solution (*see* **Note 7**).
3. Dissolve the DBM-C2 reagent in dry DMF to make a 10 mM solution (*see* **Note 8**).
4. To the antibody solution (22.9 µM, 100 µL) add TCEP (10 mM, 1.4 µL, 6 equiv.) and warm at 37 °C for 2 h, under mild agitation.
5. Add the DBM-C_2 reagent (10 mM, 1.8 µL, 8 equiv) to the reduced antibody solution and incubate at 20 °C for 5 min, under mild agitation.
6. Carry out ultrafiltration (10 kDa MWCO) with BBS to remove any remaining reagent (*see* **Note 9**).
7. Retain these conjugates in BBS buffer (22.9 µM) at 25 °C for 2 h to ensure complete hydrolysis of the maleimides generating robustly stable conjugates.

3.2 SDS-PAGE analysis of the conjugates

1. Take a 3 µL sample (~35 µM mAb) and dilute in 10 µL of water. Add 2 µL of the loading buffer and heat at 65 °C for 5 min to denature the sample.
2. For the reducing conditions take a 3 µL sample (~35 µM in total mAb) and dilute in 10 µL of water, then add 2 µL of the loading buffer (5x) along with 1 µL of the DTT solution (10 mM), vortex (10 sec), and heat at 65 °C for 5 min.
3. Centrifuge the samples for 5 s and load (5 µL of each sample) onto the gel, along with the marker. Also include samples for the unmodified antibody and the reduced antibody.
4. Run the gel at constant current (30-35 mA) for 40 min in 1× SDS running buffer.

5. Following gel electrophoresis, separate the two plates with the use of a cassette opening lever. Carefully remove the gel and transfer it to a container with staining solution (enough to cover the gel). Incubate at room temperature for ca. 45 min (*see* **Note 10**). Discard the staining solution and rinse the gel twice with double-deionised water. Add destaining solution and incubate at RT, until a clear background is obtained (*see* **Note 11**).
6. The lanes generated in such a gel are shown in **Fig. 3**. The key observations are: i) that the reduced antibody (lane 2) confirms that the TCEP procedure reduces all the interchain disulfide bonds, to afford bands for the heavy chain (~50 kDa) and light chain (~25 kDa); ii) the DBM-C_2 reagent is efficient in bridging the cysteine residues generated (lane 3), reconnecting the heavy and light chains; iii) the conjugate is resistant to reducing conditions (lane 3+), confirming the installation of robustly stable maleamic acid bridges. NOTE: two dominant isomeric forms are observed in the conjugate, due to competing formation of inter- and intra- heavy chain bridges [28]. The denaturing conditions of SDS-PAGE separates these species, which are otherwise inseparable by native analytical or purification methods.

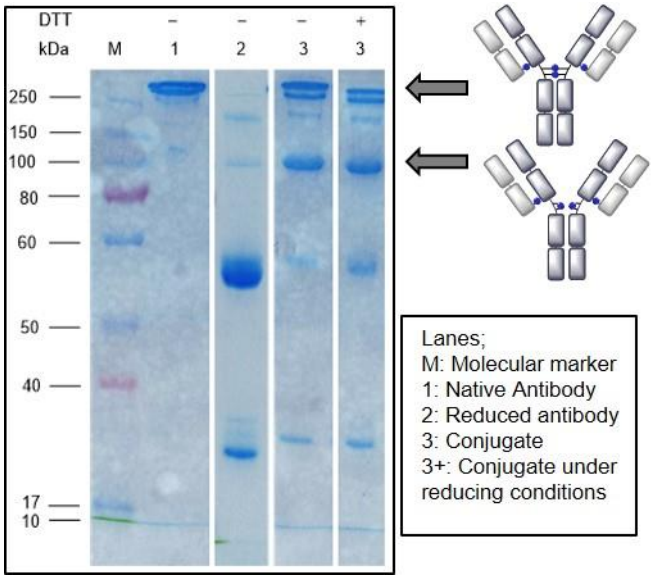

Fig. 3. SDS-PAGE analysis of DBM-C_2 reagent antibody conjugation.

3.3 LC-MS analysis of the conjugates

1. Take a 20 µL aliquot of the antibody conjugate and exchange it into ammonium acetate buffer *via* ultrafiltration (10 kDa MWCO). Determine the concentration by UV/Vis absorbance and adjust it to 0.7 µM with ammonium acetate buffer.
2. Add PNGase F (1 µL) to the antibody conjugate and incubate at 37 °C for 2 h, to effect deglycosylation.
3. Filter the resultant solution though a 0.22 µm centrifuge tube filter to remove any residual particles, prior to LC-MS analysis.
4. 10 µL of the resultant solution is injected onto the PLRP column, which is maintained at 60 °C. The flow rate used was 300 µL/min and the gradient as follows: 15% B for the first 2 min followed by increase to 32% B over 1 min, remained at 32% B for 1 min. Mobile phase B then increased to 50% over 10 min, with further increase of B to 95% over 4 min and maintained at 95% B for 2 min. The mobile phase B was changed to 15% B for the final 3 min.
5. Acquire mass spectra in positive electrospray ionization (ESI) mode using the m/z range 800−5,000.
6. Convert the raw data to zero charge mass spectra using a maximum entropy deconvolution algorithm.

The LC-MS data for trastuzumab, reduced trastuzumab, and a trastuzumab DBM-C_2 conjugate are shown in **Fig. 4**, to highlight what is expected. In each case the total ion count (TIC), raw data and deconvoluted spectrum are shown. The following key observations are made; i) the PNGase F step is important to remove heterogeneity due to glycosylation, leading to well resolved spectra; ii) the reduced antibody is observed as separated heavy and light chains due to the denaturing conditions of the analytical method; iii) the antibody DBM-C_2 conjugate is observed as two dominant species, i.e. the full antibody conjugate with four maleamic acid bridges and the half-antibody with two maleamic acid bridges. The latter species is derived from the presence of the intra heavy chain bridge, consistent with the SDS-PAGE. Under the highly acidic LC-MS conditions (pH 2-3), maleamic acid conjugates are

sometimes observed to undergo a small amount of fragmentation that leads to loss of the payload, retaining a maleic acid bridge [28].

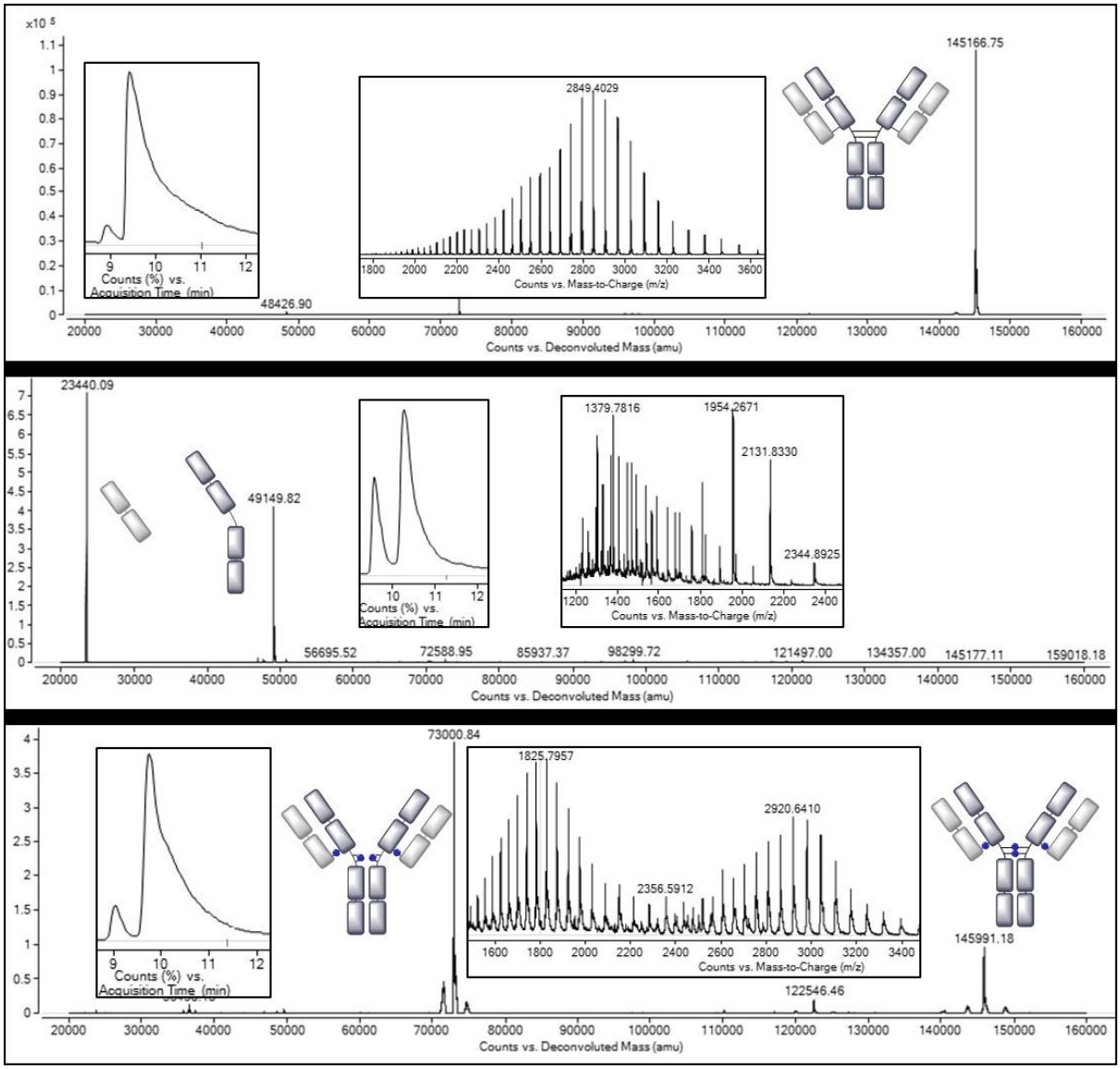

Fig. 4 LC-MS data on **top** – trastuzumab, **middle** – reduced trastuzumab**, bottom** – trastuzumab DBM-C_2 conjugate (for this particular example the R group attached to the DBM C_2 amide was the alkyne: -CH_2CCH) [28].

4. Notes.

1. The term ‘degassing’ refers to the process of removing O_2 from a solution by bubbling argon through it for 10 mins.
2. SDS-PAGE loading buffers can be stored in aliquots at -20 ^oC for up to 12 months.
3. DTT solution should be prepared fresh each time.
4. If the antibody is already obtained in solution, buffer exchange this directly into BBS buffer.
5. This exact concentration is not critical. The equivalents of TCEP and DBM-C_2 relative to the antibody must be maintained as stated, the volumes can be adjusted depending on the chosen concentration and volumes of the initial antibody solution. We suggest that the concentration should be in the range 20-40 μM.
6. Antibody aliquots can be flash-frozen and stored at -80 ^oC.
7. The TCEP solution in BBS should be prepared fresh prior to use.
8. DBM-C_2 solution should be prepared fresh each time.
9. Ultrafiltration at this stage is not essential, and can instead be carried out after the subsequent hydrolysis step for convenience.
10. Staining can be accelerated by microwaving the gel at full power for 1 min, let it cool to room temperature and repeat for 1 min.
11. Destaining can be accelerated by microwaving the gel at full power for 1 min.

Figure legends;

Fig. 1 Antibody conjugation by disulfide bridging using next generation maleimides

Fig. 2 Synthesis of the DBM-C_2 reagent

Fig. 3. SDS-PAGE analysis of DBM-C2 reagent antibody conjugation.

Fig. 4 LC-MS data on **top** – trastuzumab, **middle** – reduced trastuzumab**, bottom** – trastuzumab DBM-C_2 conjugate (for this particular example the R group attached to the DBM C_2 amide was the alkyne: -CH_2CCH) [28].

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