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Selective Reduction of Interchain Disulfides in Native Antibodies is an Underexplored Problem

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ABSTRACT

Interchain disulfide conjugation chemistry has enabled the generation of homogeneous antibody–drug conjugates (ADCs), but this is mostly restricted to drug-to-antibody ratio (DAR) 4 and DAR 8 constructs when using native antibodies. A native IgG1 has four interchain disulfide bonds: two heavy–heavy (H–H) bonds in the hinge region, and two heavy–light (H–L) bonds. Selective reduction of one pair of bonds would allow greater control over drug loading and access to dual payload ADCs without the need for complex linker design or antibody engineering, however, this pursuit has received little attention. Herein, we highlight the research in this field to elucidate the differences between these bonds and how these subtleties have been exploited to enable selective disulfide reduction.

1 | Introduction

In the last 20 years, antibody–drug conjugates (ADCs) have become established as a next-generation therapeutic to treat unmet medical needs. ADCs comprise three components: the antibody, the payload, and a linker containing a conjugation handle. Where the payload is appended to the antibody is determined by the reactivity of this handle, thus it controls site selectivity and drug-to-antibody ratio (DAR) which in turn effect the activity, pharmacokinetic profile, and propensity for antibody aggregation [1]. Therefore, developing methods that allow tuning of the number and location of payloads is crucial to produce effective treatments.

Immunoglobulin G (IgG) is the most abundant class of human antibody, accounting for 20% of plasma proteins [2] and the family is further divided into four subclasses that differ most noticeably in the character of interchain disulfide bridges that hold the two "heavy" (H) and two "light" (L) protein chains together (Figure 1A) [3]. The most abundant of these subclasses, IgG1, is utilized in the vast majority of ADC therapeutics and has four interchain bonds between the domains: two H-H bonds in the hinge, and two H–L bonds in the Fab region. The other subclasses, IgG2, IgG3, and IgG4, are characterized by having 6, 13, and 4 interchain disulfide bridges, respectively. All IgG subclasses also contain 12 intrachain disulfide bonds within the individual domains [4]. Interchain disulfide bonds have been shown to have a 100–1000 fold higher rate of reduction than intrachain bonds and there is a linear relationship between greater solvent exposure and lower bond stability [5]. This disparity between the bond types allows for the facile reduction of the four interchain disulfide bonds to eight free thiols (Figure 1B) subsequently enabling electrophilic linker-payloads to be appended. The reduction and trapping of these cysteines forms the backbone of antibody bioconjugation chemistry due to their unique reactivity and well-defined prevalence in an antibody's structure.

As IgG1 and IgG4 are the most prevalent families used in ADCs, they are the focus of this review. The four interchain disulfide bridges in IgG1/4 are usually reduced with reagents such as tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) and the eight thiols generated are then reacted either individually with

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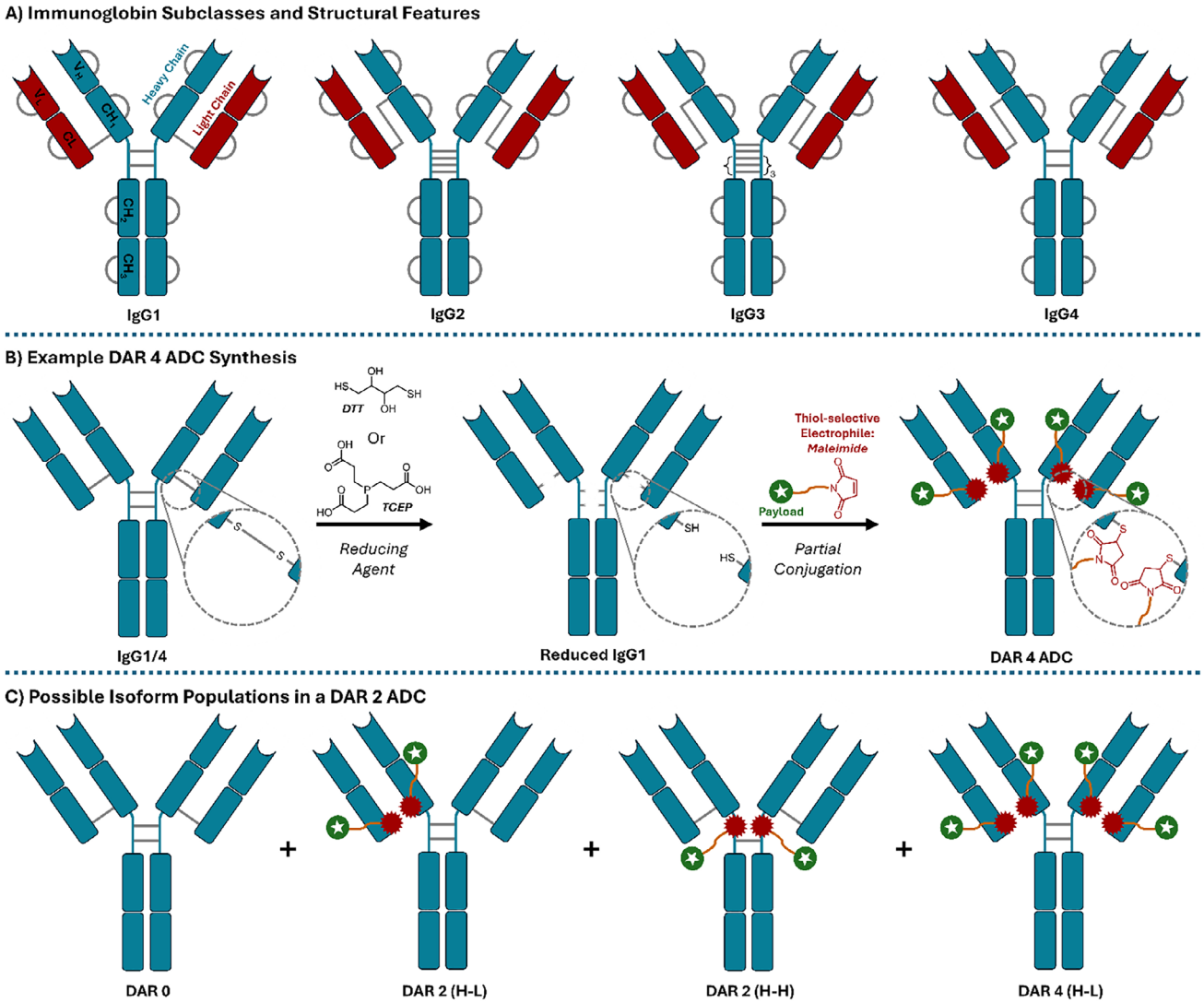


FIGURE 1 | Overview of human IgG structure and state-of-the-art in ADC synthesis. (A) IgG1–4 annotated structures showing the domains of the heavy (blue) and light (red) chains, and the relative positions of intrachain (gray; curved) and interchain (gray; angular) disulfide bridges [2, 3]. (B) Example synthesis of a DAR 4 ADC using a maleimide-based linker-payload. After reduction, eight thiols are available to react; by limiting the equivalents of electrophile a lower DAR is achievable. This example shows the DAR 4 isoform from the reaction of the H–L disulfides; isoforms with both H–H bonds or one H–L and one H–H bond reacted are also formed. (C) Example of how a distribution of isoforms and different drug-loading levels can lead to an overall DAR of 2.

mono-reactive electrophiles, such as maleimides [6], sulfonyl pyrimidines [7], or ethynylphosphonamidates [8, 9] leading to a maximum DAR of 8, or di-reactive electrophiles, such as next-generation maleimides [10], divinyl pyrimidines [11, 12], or ThioBridge [13, 14], leading to a maximum DAR of 4.

Whilst these methods allow facile and consistent access to DAR 8 and 4 constructs using native antibodies, access to other DAR values relies mostly on incomplete conjugation to the cysteine residues. Although there has been success using this method to generate DAR 2 constructs (with potent cytotoxins such as PDB (pyrrolobenzodiazepine) dimers [15, 16]), invariably there is no control over which cysteines react, thus leading to high heterogeneity with individual ADCs within a DAR 2 population containing a higher or lower number of drugs (Figure 1C). Furthermore, there is also a desire to include multiple different payloads on a single antibody construct to capitalize on the synergy between different mechanisms of action [17]. However, with classical methods on native antibodies, access to dual-payload constructs is hampered by the lack of control over site-selectivity, with the ill-defined distribution of the first conjugation compounding the issue for the second. Multi-functional linker constructs have been developed to tackle this issue [18, 19], however, the ability to achieve a dual-payload ADC directly on a native antibody would expand the toolbox of available methods and preclude the need for complex linker synthesis.

Physiologically, some antibody subclasses exhibit variable interchain disulfide bond architecture which plays an important role in the natural function of IgG2 and IgG4. Both subclasses show a tendency to swap the cysteines in their disulfide bonds to generate different isoforms; IgG2 structures can exist with 0, 1, or 2 CH_1—L bonds (Figure 2A) whilst IgG4 antibodies interconvert their H-H bonds from an inter- to intrachain format, naturally allowing

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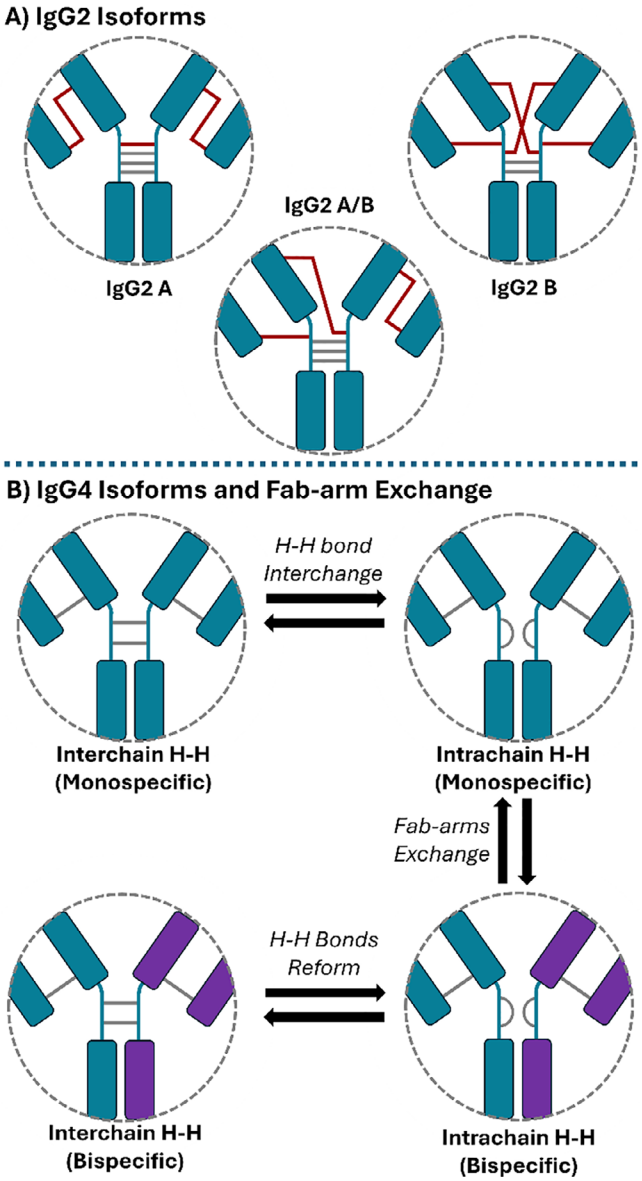


FIGURE 2 | Zoomed-in hinge regions showing natural diversity in the disulfide bond architecture. (A) IgG2 possible isoforms. As the number of CH_1–L bonds is different in each case, the bond strength and antibody binding can be affected. Variable bonds are shown in red [21]. (B) IgG4 isoforms. Inter- to intrachain H–H bond exchange results in no covalent bonds between the two half-antibodies [22]. This allows dissociation and random reassociation to different half-antibodies to generate bispecific antibodies.

bispecifics to form via Fab-arm exchange (Figure 2B) [20]. In both cases, this can affect the binding affinity and therefore potency of the antibody [21–23]. Furthermore, there are two naturally occurring types of light chains found in IgGs, κ and λ, with IgG1λ H–L bonds found to be more susceptible to reduction than IgGκ [24]. Whilst none of these cases are examples of clean selectivity, they highlight that the H–L and H–H bonds are not chemically equivalent.

This, therefore, begs the question: If efficient access to DAR 2 and dual-payload ADCs is not possible via selective conjugation to disulfides on native antibodies, is it possible to use selective disulfide reduction to limit the number of reactive thiols available for conjugation? Such a strategy ultimately relies on the subtle differences between the two H–L and two H–H disulfide bonds and whether one can be selectively reduced over the other. This review aims to highlight the work that has been undertaken to address this question and suggests where future research may focus.

2 | Studies on the Differences in Intrachain Disulfide Bonds

2.1 | Pioneering Methods for Monitoring Reduction

One of the earliest attempts at selective disulfide reduction comes from the work of Sun et al. at Seagen. They noted in prior research that drug-loading stoichiometry and conjugation site affected toxicity and efficacy [25]. Therefore, to quantify the distribution of linker-payload on a DAR 4 ADC and the effect of different reducing agents, a series of analytical methods were developed.

IgG1 DAR 4 ADCs were generated using a maleimide-based linker-payload by either partially reducing the disulfide bridges or fully reducing the bonds, then partially reoxidizing them (Figure 3) [26]. TCEP, DTT, or aminoethanethiol (AET) were used to reduce the bonds, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) was used to reoxidize them. In each case, hydrophobic interaction chromatography (HIC) was used to separate out the different DAR species. As each set of DAR species is a mixture of different disulfide architectures (e.g., a DAR 2 structure may have payloads added across an H–H or H–L bond), these were then quantified by reducing the remaining disulfide bridges, allowing H and L chain components to be separated by HPLC. Concurrently, each set of DAR species was also analysed by capillary electrophoresis in nonreducing conditions, thus retaining the native H–L and H–H bonds and allowing for species such as HL, HHL, and LHHL (full antibody) to be detected. Comparing these two datasets allowed the ratio of different disulfide architectures to be determined and therefore the effect of each partial reduction or re-oxidation set of conditions.

The researchers found that different reducing agents caused different species ratios, with DTT and TCEP leading to a 2:1 and 1:1 ratio of **4B** to **4C**, respectively [26]. More strikingly, the ratio of **2A** and **2B** was found to entirely reverse if a partial reduction or partial reoxidation strategy was used. DTT partial reduction yielded 8% **2A** compared to 77% from reoxidation with DTNB. This strongly suggests that the H–L bond is more susceptible to both reduction and oxidation. However, the fact that > 96% of DAR 6 species retain an H–L disulfide for all conditions showcases that other factors must be considered, such as that reducing one H–H bond may influence the rate of reduction for the second H–H bond; this is supported by the fact that most species across all DAR values in the study show either both H–H bonds being reduced or neither. It was also found that the spread of the number of payloads on each antibody did not match the expected values if each disulfide bond reduction had an equal rate constant. Furthermore, they showed that the ratios of species did not change over time, suggesting the process is under kinetic control.

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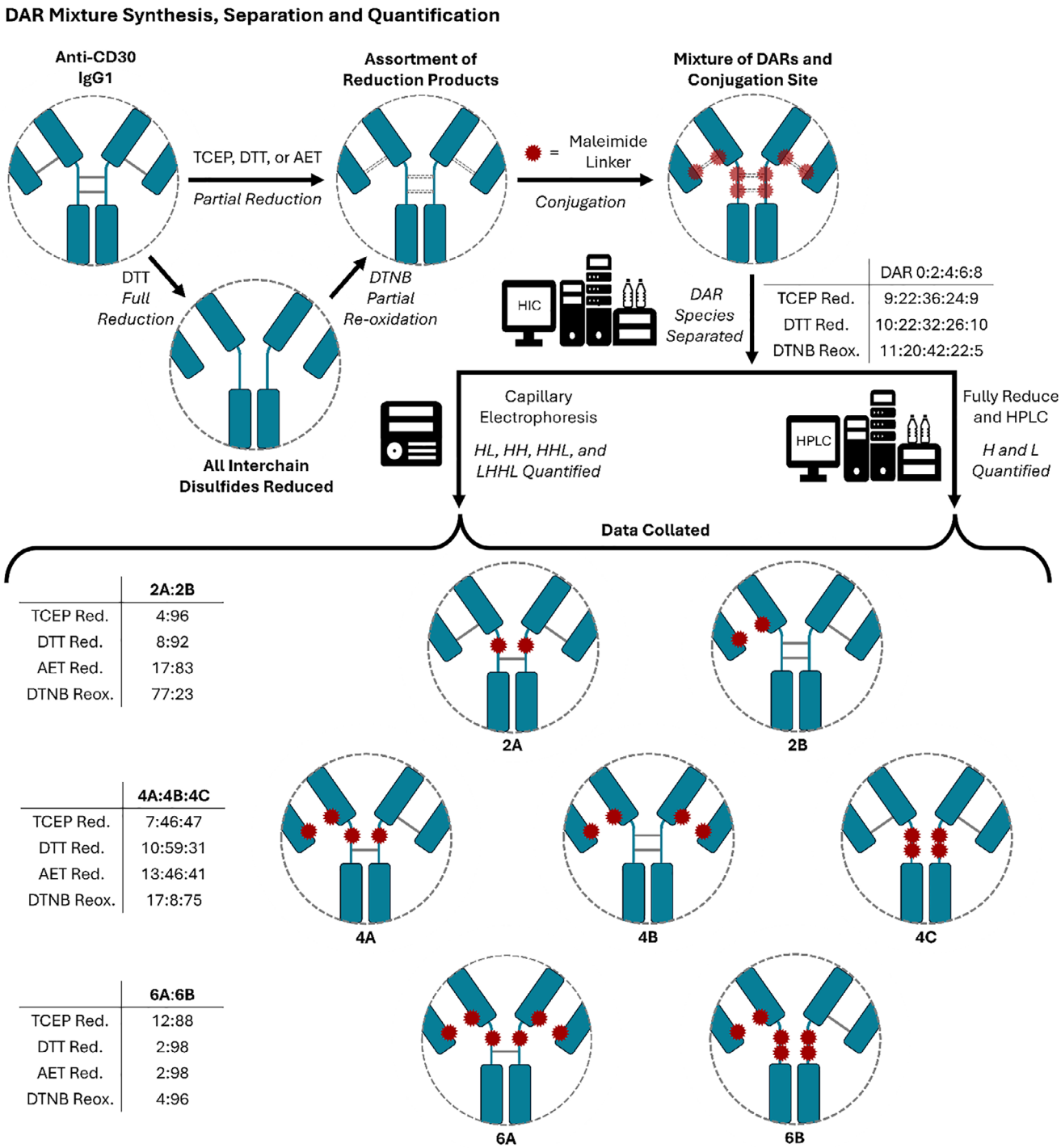


FIGURE 3 | Workflow described by Sun et al. using either partial reduction or partial reoxidation to generate a mixture of DAR species [26]. These were then separated by HIC and further studied by reducing HPLC or nonreducing capillary electrophoresis to enable the populations of each species to be determined. It shows that redox methods greatly influence which thiols are available for conjugation and that there is bias toward certain populations. AET, aminoethanethiol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; H, heavy chain; L, light chain; Red., reduction; Reox., reoxidation; TCEP = tris(2-carboxyethyl)phosphine.

Studies have also been carried out to explore the effect of analogues of TCEP as reductants [27]; unfortunately, none of these showed enhanced reduction selectivity. It is of note, however, that for specific reductants changing the pH from 7.2 to 8.0 impacted the relative ratios of H or L conjugation, albeit only by 10%. This suggests that optimising pH will also be vital to achieve selective reduction.

2.2 | Kinetic Studies

To gain greater understanding of the IgG1 disulfide bond properties, kinetic studies have been carried out, such as by Weggen et al., who developed linear regression and kinetic models to help explain and predict drug loading distribution and DAR [28]. Experimental data were collected by reducing antibodies

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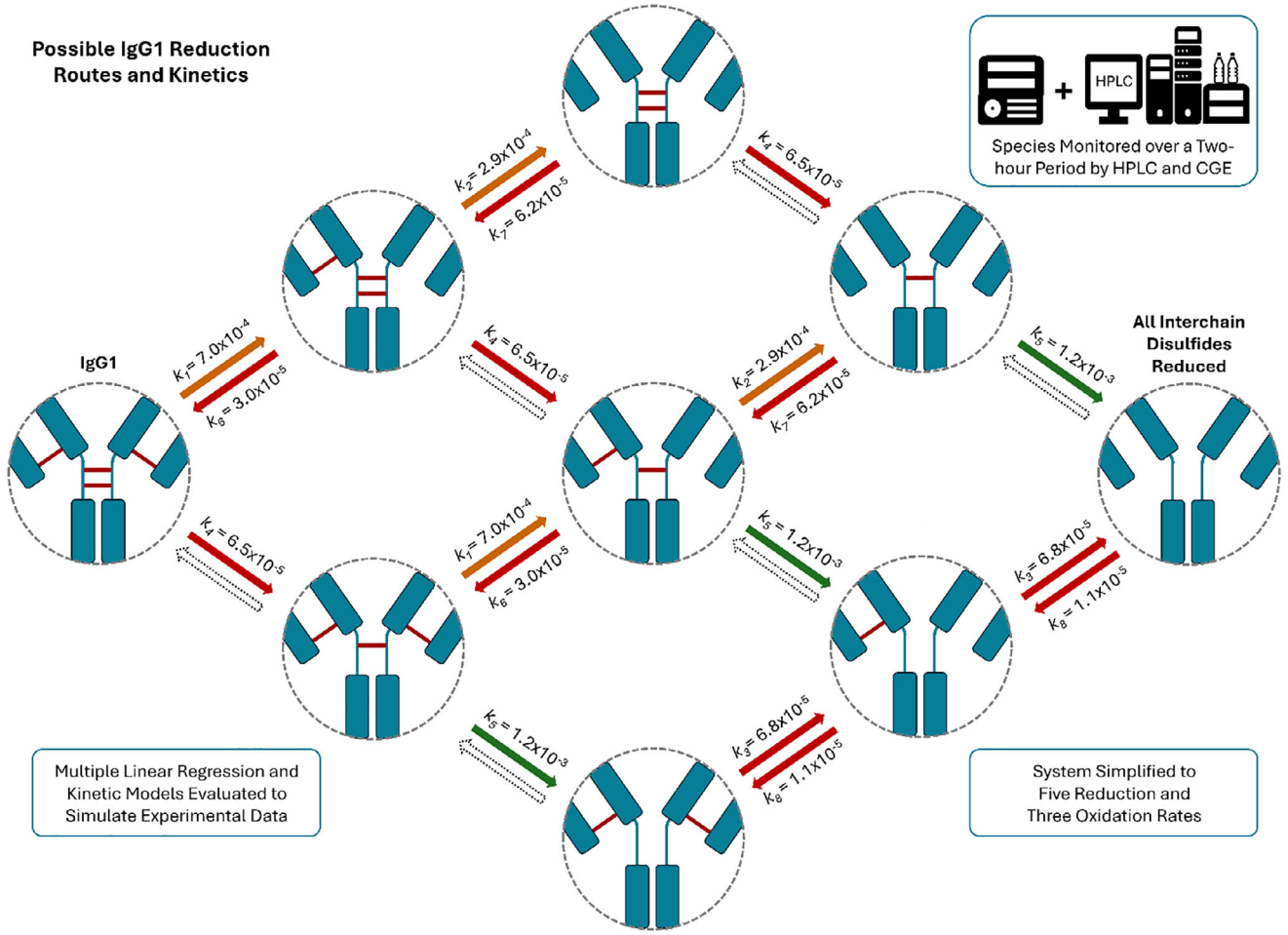


FIGURE 4 | Possible routes to reach a fully reduced antibody and rate constants (at 20°C) for each step. Figure adapted from Weggen et al. [28]. The arrows are color-coded based on the rate of the redox: fast (green), medium (orange), slow (red), and negligible (black dotted).

under various conditions (changing mAb concentration, temperature, and reducing agent concentration) then quenching with an excess of a maleimide linker. Similarly to above, capillary gel electrophoresis (CGE) and HPLC were used to study the conjugation products over a 2 hour period, and possible reduction intermediates were postulated to enable reaction routes to be established (Figure 4). By splitting the full antibody reduction into single-disulfide reduction steps, the rate of each step could be used to determine the order in which disulfide bridges were more likely to reduce, thus allowing a prediction of DAR and drug loading distribution to be made. Whilst the authors state that the rate of reduction on a bond is affected by the number of bonds of each type available and each reduction will affect the structure of the antibody, these factors were avoided to simplify the system to five reduction and three oxidation rates.

Their research corroborates that H–L bonds reduce faster than H–H in IgG1, and shows that the second H–H bond reduces 18 times faster than the first H–H bond [28]. This might be due to structural changes [4] and/or gain of entropy [29]. The experimental data also revealed that lowering the temperature increased the proportion of HH formed and decreased that of HL [28].

Interestingly, the rate of H–H bond reoxidation was found to be negligible, and the authors propose that previous evidence of reoxidation was in fact caused by exchange of the conjugation handle to other positions, thus giving the impression of H–H bond oxidation [28]. Van den Berg et al. described the phenomenon more deeply, reporting that whilst the DAR profile of an ADC sample does not change over time, the exact species do [30]. It was proposed that thiol-disulfide exchange in a partially reduced antibody allows the conjugation handle to switch between the cysteine pairs, thus changing the ratio of species over time (Figure 5). The commonly used maleimide conjugation moiety is well known to act reversibly, enabling these switches. The findings of Van den Berg et al. show a preference for an enrichment of H–H bond-based conjugation over time, at the expense of H–L conjugation, which initially dominates kinetically. The data suggested this was not caused by excess TCEP and thus is an innate process, with hinge conjugation arising as the thermodynamic product.

On first inspection, this switching appears to jeopardise the ability for selective reduction to ever allow the generation of homogeneous conjugates. However, the use of fast nonreversible conjugation strategies (such as hydrolyzed maleimides, ethynylphosphonamidates, and divinyl pyrimidines) would negate this by suppressing the establishment of an equilibrium.

Song et al. reported that IgG1 disulfide bonds are more susceptible to TCEP reduction than IgG4 bonds [31]. Furthermore, in contrast

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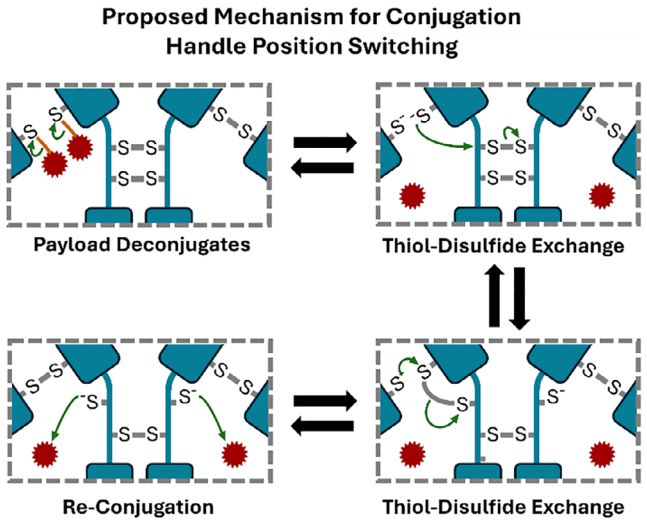


FIGURE 5 | Proposed mechanism by which a reversible conjugation handle can move between thiols in a partially reduced antibody [30]. H–H disulfide conjugation becomes enriched overtime for IgG1.

to IgG1s, it was noted that in IgG4s the H–H bonds are more susceptible than the H–L, perhaps unsurprising given the Fab-arm exchange phenomenon. This can be partially explained by solvent accessibility; however, discrepancies with molecular dynamics data have shown that other factors are at play, possibly involving peptide backbone flexibility [5]. The idea that H–L IgG1 disulfide bonds are more susceptible to TCEP reduction than in IgG4 contrasts with reports that the thermal stability of the IgG1 H–L bond is in fact higher than that in IgG4 [32]; this exemplifies the nuances in the properties of the bonds but highlights the opportunity to take advantage of these differences to develop selective reduction.

2.3 | IgG4 Fab Arm Switching

As has been previously described, IgG4 antibody H–H bonds naturally undergo inter- to intrachain thiol switching, enabling the Fab arms to dissociate and pair up with other Fabs to form bispecifics (see Figure 2B). This ability has been attributed to the amino acid sequences in the hinge (destabilizing the disulfide bonds) [20] and the CH_3 domain (weakening noncovalent interactions between the H chains) [33]. Therefore, by mutating the amino acids in these areas, it is possible to knock out or induce Fab arm switching [33–35].

Genmab utilized this concept to form human IgG1 bispecifics by mixing two antibodies with K409R and F405L mutations, respectively [36, 37]. The mutations weaken the CH_3—CH_3 interaction in the monospecifics enough for a mild reducing agent (2-mercaptoethylamine) to cause Fab arm switching. The mutations were found to be complementary, enabling a stronger CH_3—CH_3 interaction in the bispecific and thus stabilising the construct. Further kinetic studies corroborated this, showing that a greater than fivefold affinity difference between the CH_3—CH_3 interactions is required to favor the formation of the bispecific [38].

Whilst these studies have all afforded valuable insights into the nuances between the H–L and H–H disulfides and highlight that they can be tuned, the need for genetic engineering makes pursuing Fab arm switching for selective conjugation of native antibodies unproductive.

2-Mercaptoethylamine has also been used to reduce the H–H disulfides of Fab dimers [39] where the lack of an Fc region weakens the interaction between what remains of the CH_3—CH_3 domains, thus increasing reduction susceptibility. Use on full IgG leads to poor selectivity and depends heavily on the species from which the IgG was obtained [40].

3 | Methods to Exploit the Differences in Intrachain Disulfide Bonds

3.1 | Solid-Phase Reduction

H–L bonds of an IgG1 are more solvent-exposed than the H–H bonds. Therefore, if reducing agent access to the antibody was restricted, could the more solvent-exposed bonds be preferentially reduced?

Ferraz et al. described a strategy to reduce the disulfide bonds in a rabbit Fab dimer and full IgG using solid-supported mercaptohydroxypropyl ether (Figure 6) [41]. They found that all three disulfide bridges in a rabbit Fab dimer could be reduced (liberating six thiols) after 6 h of incubation at 20°C with 8 μmol of mercaptohydroxypropyl ether resin per milligram of protein. However, when used on full rabbit IgG, they found that similar conditions only liberated an average of 2.5 thiols, suggesting the larger size of full IgG was hindering access of the reducing agent to the disulfides. By increasing the temperature to 37°C and the solid-support concentration to 16 μmol/mg, all interchain thiols were liberated.

The exact species during the partial reduction were not determined; however, more recently Kirley and Norman used TCEP-agarose beads to study the reduction of a humanised IgG [42]. By trapping the reduced disulfides with a fluorescent probe, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) could be used to separate the antibody fragments, and the intensity of fluorescence measured to calculate the proportion of each fragment. They found that the H–L bonds can be preferentially reduced, with c. 85% of the conjugated fragments fully retaining their H–H bonds. It is of note that the effect of higher DAR causing the proportion of species to be overestimated is not addressed, but as this would most greatly affect species with both H–L and H–H bonds reduced (and hence more fluorescent labeling), this suggests the ratio of H–L to H–H reduction is even more favorable than reported. However, a substantial amount of unreduced full antibody remains, and the effect of other variables, such as concentration of the TCEP beads, was not disclosed; therefore, whilst the results are impressive, there is still research to be done to optimise the method.

3.2 | The Effect of Low Concentration of Reductant

Kirley and Norman have also reported research into the effect of low concentrations of TCEP to exploit the greater reduction

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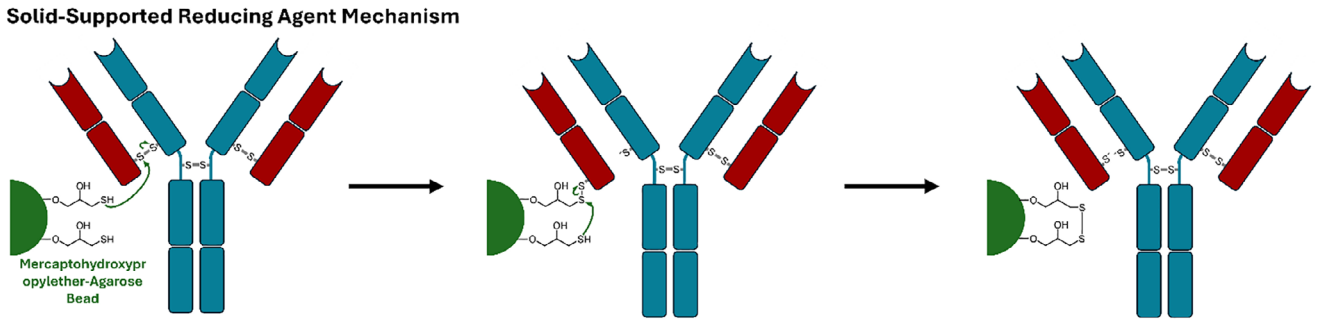


FIGURE 6 | Reduction mechanism of a rabbit IgG1 using solid-supported mercaptohydroxypropyl ether [41]. Due to the hindered nature of the reducing agent, it may be possible to favor H–L over H–H reduction.

susceptibility of the H–L bond in the presence of a cysteine-reactive aminosulfonyl linker [43]. It was found by fluorescent SDS-PAGE that reduction with 0.1 mM TCEP (3 eq.) for 20 min followed by reagent removal via SEC spin columns afforded mostly conjugated H–L reduction products (such as LC, HHL, and HH species) and that an increase in H–H products correlated with increased reaction time or TCEP concentration. However, the low TCEP concentration meant that a large proportion of nonreduced native antibody remained. As with the solid-supported TCEP work, whilst a balance is undoubtedly hard to achieve to ensure full H–L reduction whilst preventing over-reduction of the H–H bonds, it does hinder the method's utility to generate homogeneous ADCs. This could be improved by purifying out the unreacted native antibody or perhaps by carrying out repeated 20 min reaction cycles to reduce the remaining H–L disulfides. Furthermore, as the method is only demonstrated on a compact conjugator, how a full (and likely bulky) linker-payload would behave in the short reaction conditions remains to be seen.

3.3 | Metal-Ion Hinge Shielding

WuXi developed a method (so-called WuXiDAR4) that purportedly increases the selectivity of conjugation to the H–L disulfides by chelating the hinge region H–H thiols with Zn^{2+}, preventing them from reacting [49]. IgG1 antibodies are reduced with TCEP in the presence of $ZnCl_2$, followed by the addition of maleimide-based payloads (Figure 7). With the zinc purportedly preferentially chelated to the cysteines in the hinge region, the conjugator reacts with the H–L thiols. Excess cysteine is added to quench any remaining maleimide, and EDTA is used to remove the Zn^{2+}, allowing the hinge disulfides to be reoxidized with dehydroascorbic acid (DHAA). This process increased the proportion of DAR 4 species to 73%–78% compared to 35%–38% when $ZnCl_2$ wasn't used; however, to the best of our knowledge, no analysis of the composition of the DAR 4 species was carried out to determine the selectivity of H–L over H–H conjugation. The presence of 11%–14% DAR 6 and DAR 8 species illustrates that the Zn^{2+} did not successfully mask cysteines in each antibody, though it does compare favourably to the DAR profile without $ZnCl_2$ (27%–33% DAR 6 and DAR 8 species).

Although details of the species produced are deficient, perhaps further research into different chelating ions and other conditions could enable full masking of the H–H or H–L thiols.

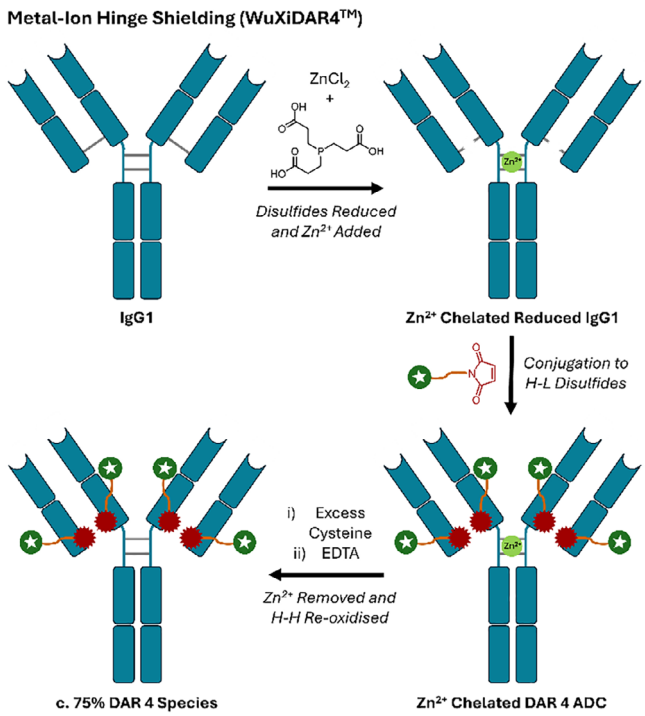


FIGURE 7 | The WuXiDAR4™ platform purportedly masks the H–H disulfides, increasing the proportion of H–L conjugation. Adapted from [49].

3.4 | Electrochemical Reduction

Morgan et al. reported the use of electrochemistry to reduce both inter- and intrachain antibody disulfides [45]. By using a flow setup, the researchers were able to reduce the antibody and directly analyse the fragments produced via LCMS (Figure 8A). They found that the number and type of disulfides reduced correlated with the voltage supplied; 0.2 V led to H–L reduction, 0.4 V to H-H reduction and LC intrachain reduction, and 0.6 V to full interchain and partial intrachain reduction. It is notable, however, that with 0.2 V the researchers report the presence of HL but no HC; this is contradictory to the idea of H–L reduction occurring first. Due to the overlap of these fragments with the full antibody in LCMS, it is not possible to conclude how much of these species are present. Regardless, the results show that, as with chemical reductants, the H–L bond reduces first, and so by changing the potential, a greater selectivity of reduction can be achieved. The authors also note that an increase in temperature

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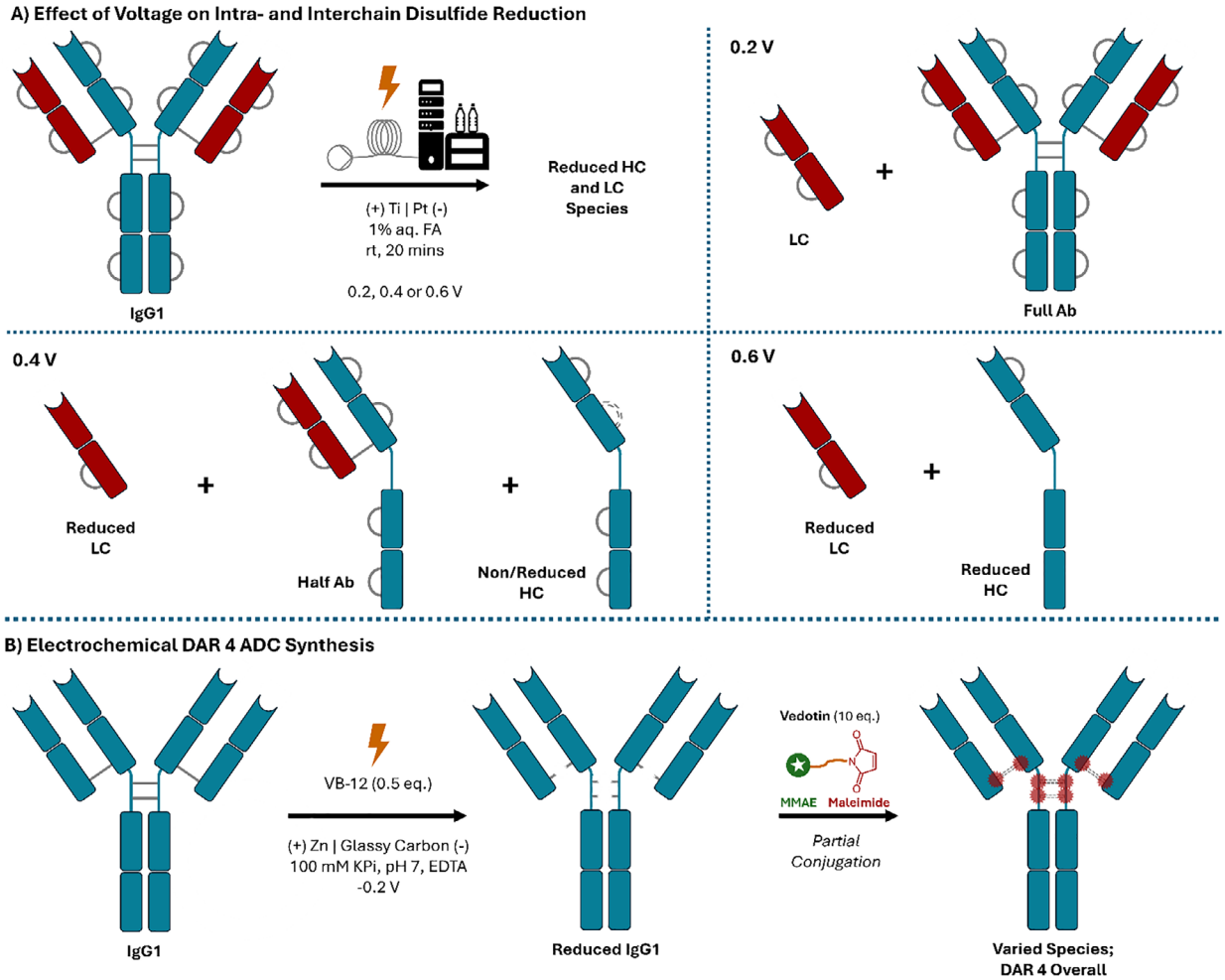


FIGURE 8 | Electrochemical reduction of disulfide bonds. (A) Varying the voltage changes the reduced species generated. Increased voltage led to increased intrachain reduction; only major species illustrated [45]. (B) Electrochemical synthesis of a DAR 4 ADC using a vitamin B 12 (VB-12) catalyst [46]. Incomplete conjugation with vedotin led to a DAR 4 ADC. FA, formic acid; KPi, potassium phosphate buffer; MMAE, monomethyl auristatin E.

and/or organic solvent proportion increased the propensity for disulfide reduction, probably due to increased denaturing. This method was developed to enable analysis of antibody fragments, and as such, no conjugation was reported.

Ruccolo et al. developed a vitamin B12-catalyzed electrochemical method to reduce disulfide bridges in a variety of peptides and proteins [46]. The protocol was found to be successful between pH 4–8 and in a range of buffers. A 97% interchain disulfide reduction was recorded for zilovertamab which, when reacted with the maleimide-based linker-payload vedotin, produced an ADC with a comparable DAR profile to standard TCEP conditions (Figure 8B). No reduction selectivity is reported, though by combining the works of Morgan et al. [45] and Ruccolo et al. [46], it may be possible to produce an attractive workflow of selective electrochemical reduction, conjugation, and in-line conjugate analysis.

4 | Summary and Outlook

Selective disulfide reduction promises to enable greater control over DAR and allow for the generation of dual payload conjugates via two sequential reduction and conjugation cycles. This would allow facile conjugation of synergistic payloads without the need for complex linker synthesis. Furthermore, the ability to form such conjugates without antibody engineering or other complex enzymic manipulation would reduce large-scale production costs. Whilst no single study has thus far achieved total selectivity of reduction, the work described in this review highlights the knowledge gained and shows the challenges and opportunities available in the pursuit of this goal.

Studies by Sun et al. [26] and Weggen et al. [28] examined the differences between the H–L and H–H bonds, showing that the conditions in which a reduction takes place affects the proportion of products formed. Kirley and Norman [42, 43] exploited these differences by either physically or stoichiometrically restricting reductant access to the disulfides in an attempt to reduce only the H–L bonds. Their work represents the most successful selective reduction approach with greater than 5:1 selectivity for H–L versus H–H conjugation, and further optimization opportunities may allow this to be increased further by changing the temperature, pH, reaction time, reducing agent loading, and resin concentration. Recent advances in the synthesis of high-density reducing agent beads may also improve IgG reduction efficiency [47].

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WuXi purportedly developed a metal chelation method to mask the four hinge cysteines, thus biasing conjugation across the H–L bond [44]. Although this method does increase the proportion of DAR 4 species formed, the lack of data on the proportion of bond types reduced and the presence of DAR 6 and DAR 8 species lessen the utility of the method at the present time. However, further optimization to increase the selectivity of the hinge masking would allow facile access to dual payload conjugates by first reacting the H–L cysteines with a linker-payload, then unmasking the hinge for a second conjugation.

Finally, the electrochemical method developed by Morgan et al. [45] has shown partial success at selective reduction, though further optimization will be required to increase the selectivity whilst ensuring less native antibody remains unreduced. By combining their work with electrochemical ADC synthesis strategies, it may be possible to utilize the precise tuning that electrochemistry enables to increase reduction selectivity. Optimization of cosolvent, temperature, potential, catalyst, and reaction time may also further improve selectivity. Another possible avenue for selectivity would be the use of photoredox chemistry, which enables precise tuning of reaction conditions and has shown promise in small peptide disulfide reduction [48]. We believe this is where the greatest future gains in the field lie.

This review showcases that although selective reduction promises the facile synthesis of truly homogeneous DAR 2 and dual-payload conjugates on native antibodies without the need for complex linker design, relatively little research has been conducted in this field. The approaches presented form a strong foundation for further study that we believe is needed to truly realize this crucial goal.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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