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Advances in Antibody–Drug Conjugate Conjugation: Methods, Challenges, and Improvements

-- Addressing Conjugation Instability and Site-Specificity in ADC Design

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Abstract: Antibody-drug conjugates (ADCs) are targeted biopharmaceuticals that constitutes monoclonal antibody selectivity with the cytotoxic strength of small-molecule drugs. Over the past decades, a growing number of reagents and studies have been proposed, and substantial progress has been achieved in improving conjugate homogeneity, linker chemistry, and payload design. One of the typical conjugation methods, maleimide-thiol conjugation, has been proposed in the first-generation ADCs, and the problems related to retro-Michael conjugation have been tackled by the study of succinimide ring hydrolysis. Apart from that, a wide range of reagents and new linkers for disulfide bond re-bridging have been studied as well. This mini-review summarises these core strategies, critically examining their mechanistic principles, limitations, and recent innovations that collectively shape the evolution of next-generation ADC design.

Keywords: Antibody-drug Conjugates; ADCs; Antitumour Agents; Bioconjugation; Site-Specific Modification; Therapeutic Activity; Optimisation.

1. Introduction

ADCs, in recent years, have emerged as a major class of biopharmaceutical agents engineered to achieve tumour-selective chemotherapy [1]. Each ADC integrate three functional components: a monoclonal antibody (mAb) that recognises tumour-associated antigens, a chemical linker connecting the payload, and a cytotoxic drug for cell killing [2]. Due to its structure, ADCs enable the selective destruction of malignant cells while largely sparing normal tissues [3]. An effective ADC should be equipped with high stability in the systemic circulation, accumulate specifically at the tumour site, and release its active drug only within the target cells [2].

Throughout these years, biopharmaceutical drugs have developed rapidly, starting from the basic chemotherapies. Early clinical milestones were achieved with first-generation ADCs, including gemtuzumab ozogamicin and inotuzumab ozogamicin [2]. The first-generation ADCs validated the therapeutic concept of selective cytotoxic delivery. However, these initial constructs suffered from limitations, namely a low therapeutic index, rapid plasma clearance of high drug-to-antibody ratio (DAR) species, low conjugate stability, and narrow therapeutic windows [4,5]. Therefore, to address these shortcomings, second-generation ADCs have been developed with site-specificity. These ADCs with site specificity have been shown to have superior in vivo antitumour efficacy. Accordingly, site-selective modification has become a central focus in the development of next-generation ADCs for clinical use.

Apart from the progress in the homogeneity, stability of the conjugates and wider therapeutic windows, payload design has been improved as well. Among the most widely adopted are monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), which serve as potent microtubule inhibitors in clinically approved ADCs. Early conjugation strategies frequently targeted lysine residues on the antibody, yielding heterogeneous constructs with DARs typically between 2 and 8 [6].

In this review, the core types of modification strategies will be introduced and demonstrated with the typical examples, including maleimide-thiol conjugation and disulfide re-bridging. Through a critical comparison of their mechanisms, advantages, and inherent limitations, we aim to offer an integrated perspective on current strategies for constructing stable and therapeutically effective ADCs. The refinement of site-specific modification strategies continues to enhance the clinical potential of ADCs in targeted cancer therapy in the future [2].

2. Maleimide-thiol Conjugation

One of the most widely adopted methods in the second-generation ADCs is maleimide–thiol coupling. This chemistry utilises the high nucleophilicity of cysteine thiols to enable rapid and selective bond formation between the maleimide moiety and the antibody [7]. Maleimides are functionalised via the addition of various substituents on the Nitrogen atom. Adcetris®, as an example of the FDA (Food and Drug Administration)-approved ADCs, is constructed via partial reduction of the interchain disulfide bridge in an IgG1 framework, thereby generating solvent-accessible cysteine residues for subsequent maleimide functionalisation.

Despite its broad application, maleimide–thiol conjugation has been associated with notable challenges related to stability. Several studies and reviews, including that by Szijj et al. in 2018, have examined the mechanistic basis of its instability and introduced subsequent chemical strategies aimed at improving this conjugation strategy, with particular emphasis on the retro-Michael deconjugation process [7]. In 2011, Shen *et al.* observed that the microenvironment around the conjugation site heavily impacts the succinimide ring behaviour within the linker. Highly solvent-exposed sites were shown to undergo rapid loss of the maleimide–thiol linkage through exchange with plasma thiols both *in vitro* and *in vivo*, such as albumin, free cysteine, and glutathione [8].

This exchange results in the formation of covalent adducts between the released drug–maleimide derivatives and the Cys-34 residue of serum albumin during retro-Michael deconjugation in circulation [9]. This instability of the conjugates will lead to off-site toxicity and the reduction of the therapeutic efficacy, as it compromises the conjugate's pharmacological precision [7].

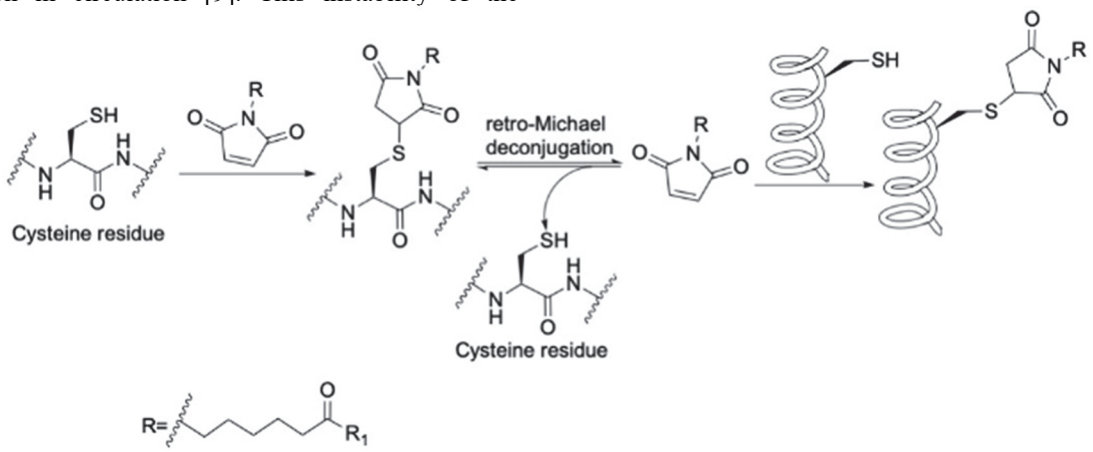

Figure 1. Maleimide-thiol conjugation with retro-Michael deconjugation before conjugating to the serum thiol

To address the issue of the off-site toxicity, numerous methods have been proposed in recent years. Shen *et al.* then proposed the model involving the post-conjugation hydrolysis.8 In this study, three thio-trastuzumab variants with conjugation sites and similar DAR value were selected to test relative therapeutic activity, *in vivo* activity and the stability of the conjugates. The conjugate located in a positively charged microenvironment exhibited rapid hydrolysis of the succinimide ring within the linker, whereas that positioned in a more neutral and solvent-exposed site experienced accelerated maleimide exchange with plasma thiols such as albumin, cysteine residues, and glutathione. This work represented the first systematic investigation to reveal the intrinsic limitations of conventional maleimide–thiol conjugation and to propose a refined model for improving its chemical stability.

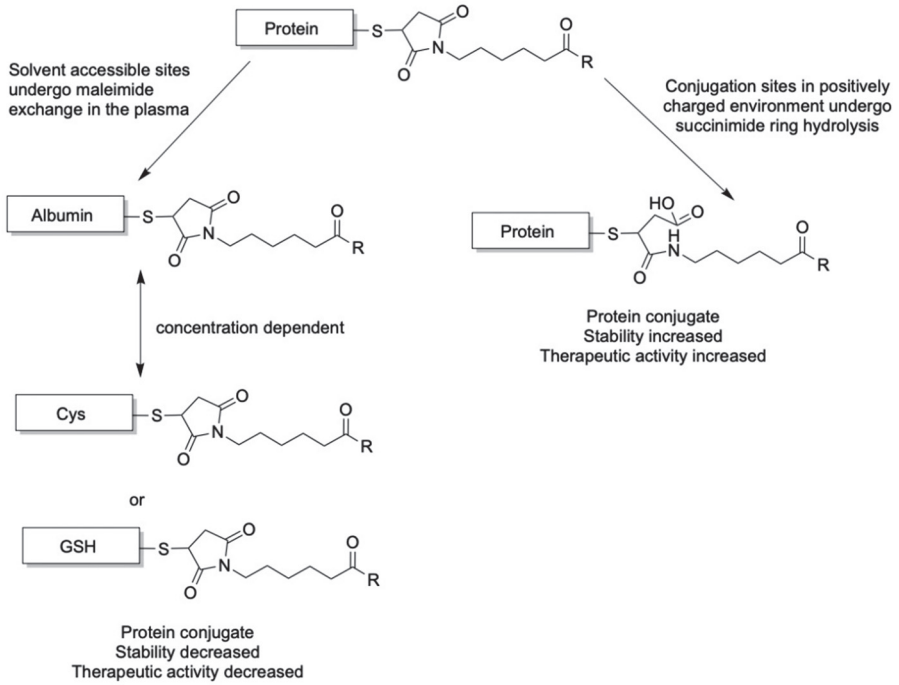

Figure 2. Reaction scheme of the competing pathways affecting maleimide–thiol conjugates. Both routes of the succinimide ring hydrolysis and the exchange of maleimide linkers with plasma thiols (albumin, cysteine, or glutathione) determine the overall stability and therapeutic efficiency of the ADCs.

After proposing this method, studies then focused on the optimisation of the maleimide–thiol linkage via controlled hydrolysis of the succinimide ring in order to suppress retro-Michael deconjugation. In 2014, Tumey *et al.* systematically examined how ring opening influences ADC performance and concluded that it does not adversely affect cytotoxic potency, antigen recognition, or thermal stability. Hence, they proposed two methods to appraise if and how the succinimide ring-opening would affect the stability after confirming to use the pH 9.2 BBS for hydrolysis conversion. One involved incubating the ADC with 500 μM glutathione at pH 7.4 and 37 °C to accelerate retro-Michael cleavage, while the other exposed the conjugate to mouse plasma to simulate in vivo conditions. Across these tests, unhydrolysed ADCs exhibited a 30–60 % loss in drug loading within six days, whereas hydrolysed counterparts retained approximately 90 % of their payload. Similarly, the ring-closed ADCs under the conditions of CO2 atmosphere at 37 °C lost the 30-45%

payload in mouse plasma. Although anti-Her2 with hydrolysed succinimide rings showed enhanced stability, pharmacokinetics and *in vivo* efficacy relative to the non-hydrolysed one, the DAR of the hydrolysed one dropped over the reaction time. Apart from that, the reason why the hydrolysed ADCs still lost a bit of payload has yet to be rationalised [10].

In the same year, Lyon and his colleagues reported a chemical strategy to mitigate the instability in alkyl-maleimide–based conjugates. Diaminopropionic acid (DPR) was introduced to the construction of the drug–linker, placing a basic amino substituent adjacent to the maleimide ring to promote intramolecular catalysis of thiosuccinimide hydrolysis under mild conditions. Such modification was hypothesised to enhance ring-opening through base-assisted catalysis [11]. However, the following studies refined this understanding. Fontaine *et al.* showed that the rate of succinimide ring opening was primarily impacted by the inductive effect, specifically, the electron-withdrawing effect. They explained that the intramolecular basic catalysis probably occurred during the reaction as well, but the improvement of the ring opening was not accounted for by such effects. The intramolecular basic catalysis, mechanistically, failed to elucidate the modification of the hydrolysis rates compared to the inductive effects [12]. Building on this insight, Christie *et al.* proposed and designed the comparison study of N-alkyl, N-phenyl, and N-(4-fluorophenyl) maleimides to quantify substituent effects on stability. The N-(4-fluorophenyl) analogue exhibited the fastest ring-opening reaction at room temperature and pH 8.6, with hydrolysis rates approximately 7-fold and fourfold greater than those of N-alkyl and N-phenyl derivatives, respectively. In this case, incorporating a fluorine atom into the phenyl ring accelerated the thiosuccinimide hydrolysis rate further. Conjugate stability was assessed by monitoring deconjugation in the aqueous buffer system containing excess thiols such as β-mercaptoethanol (BME) using mass spectrometry over 125 h. Under both freshly prepared and pre-incubated conditions (pH 8.6, 37 °C for 1 h), N-alkyl conjugates showed the highest kiss of the payload, approximately 30–35 % after 125 h. Collectively, these findings established that the extent of thiosuccinimide hydrolysis directly constrained the maximum retro-Michael deconjugation achievable in thio-maleimide-linked antibody conjugates [13].

3. Disulfide Rebridging

Another widely adopted method involves the selective re-bridging of the native disulfide bonds on the Fab. In this strategy, payloads are attached to cysteine residues, which are produced via partially reducing the interchain disulfides in IgG1, producing more homogeneous conjugates than those obtained via modification of lysine. Although early studies reported a moderate reduction in ADC half-life following disulfide disruption, the resulting products still exhibited a broad distribution of DARs of approximately 0, 2, 4, 6, and 8. Alternative methods, such as the incorporation of unnatural amino acids and the engineering of cysteine mutants via recombinant antibody technologies, have been explored as well. However, these techniques above lack the general applicability towards the ADC system, and the manufacturing cost increases due to the addition of a layer of complexity during ADC production.14 Therefore, the rebridging of the disulfide bond became another leading method, and it has drawn significant attention. Forte *et al.* provided a concise review of this strategy, summarising reagents such as bis-sulfones and other bridging chemistries [14].

Various reagents have been developed for disulfide re-bridging, among which bis-sulfones are particularly well studied. This reagent started to obtain widespread attention in 2006, when Shaunak *et al.* demonstrated that this reagent could modify antibody fragments through bis-alkylation of cysteine thiols site-specifically, reforming the original disulfide linkages. In their study, a three-carbon PEGylated bridge was yielded from the native disulfide bridge in human interferon α-2b and in a CD4-binding antibody fragment. The resulting conjugates retained native tertiary structures and biological activity while offering high yields, establishing bis-sulfone chemistry as a versatile rebridging platform [15]. However, the limitation of this mechanism was obvious as well. Mechanistically, the monosulfone reagent, as the reagent species, forms *in situ* following the elimination of p-toluene sulfonic acid. By using the TCEP reducing agent to reduce the disulfide bridge, the liberated thiols sequentially add to the enone-sulfonyl species through an addition–elimination process, ultimately generating a three-carbon bridge [14]. Consequently, two cysteines are reconnected, but two diastereomers are formed because a new stereogenic centre is introduced during the reaction [16]. Later optimisation by Godwin et al. refined this chemistry using a bis-sulfone linker bearing the cytotoxic payload MMAE for conjugation to trastuzumab (TRA). Comparative stability assays were performed using the Alexa Fluor 488-labelled bis-alkylated and maleimide-functionalised TRA conjugates in 50 % rat serum and undiluted human serum for 96 h. The SE-HPLC spectra revealed minimal changes in the bis-alkylated conjugate, whereas the maleimide analogue showed new peaks indicative of deconjugation. Both FabTRA-bisAlk-vc-MMAE and TRA-bisAlk-vc-MMAE were found to exhibited strong cytotoxicity against SK-BR-3 cells and were well tolerated without a large change in body weight [17].

In 2023, King and his colleagues introduced an alternative synthetic approach for constructing ADCs through a TetraDVP-based linker system [18]. The second generation of TetraDVP was designed to address the issue of limited conversion due to steric hindrance. This study systematically evaluated how structural variations within the linker backbone and side-chain composition affect re-bridging performance. Eight TetraDVP variants incorporating different PEG spacer lengths and side-chain configurations were synthesised. The interchain disulfide bridge was selectively reduced with ten equivalents of TCEP·HCl at 37°C before using the linkers for optimisation and bioconjugation, yielding antibody-linker conjugates (ALCs) that showed complete conversion and retained profiles of the unmodified light chain and the unmodified heavy chain. These results confirmed the steric flexibility and the high re-bridging efficiency of the TetraDVP scaffold. The new ALCs were then coupled to dibenzylcyclooctyne (DBCO)-containing payloads via strain-promoted azide–alkyne cycloaddition (SPAAC) under mild incubation conditions (37 °C for 4 h and 24 h). The conversion toward the desired ADCs was improved with the prolonged reaction time. However, although the longer azide-containing linker improved the DAR ratio compared to the shorter linker, the DAR ratio overall was still lower than 1, which might result in reduced efficacy and potency. Therefore, the efficacy of this strategy should be

further investigated before its commercial use in the future.

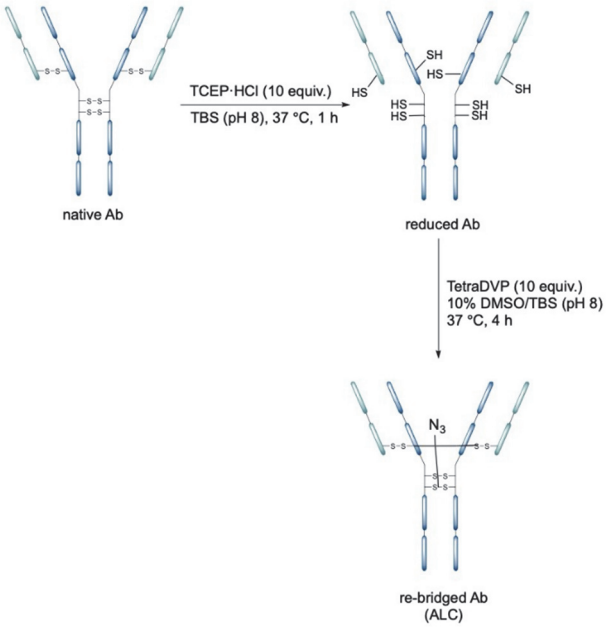

Figure 3. The reaction scheme of the disulfide bridge rebridging after the disulfide bridge has been reduced

4. Conclusion

This mini-review has summarised the development of key site-selective conjugation strategies for ADCs, highlighting both mechanistic insights and design innovations. So far, the traditional strategy of maleimide-thiol conjugation has been extensively demonstrated in the research field, as it is the most established platform for bioconjugation. Since the limitations have been evaluated, various strategies for improving the stability of the conjugates were designed and proposed in recent years, including the optimisation of succinimide ring hydrolysis with the rationalisation of the ring opening.

Disulfide re-bridging techniques have subsequently emerged as powerful alternatives, with bis-sulfone reagents in this review and their alternative reagents providing enhanced conjugate homogeneity and plasma stability. Apart from the reagents, linker engineering through the TetraDVP system has expanded the scope of re-bridging chemistries, although its relatively low DAR still limits clinical translation.

In the next generation of ADCs, future studies will likely focus on enhancing the linker stability or the precision of conjugation, which provides the direction for further refining the design of ADCs drug for more effective clinical use.

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