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Glyco-Engineered ADCs Homogeneous Conjugation for Next-Generation Cancer Therapy

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Abstract. Antibody-drug conjugates (ADCs) represent a rapidly advancing class of targeted anticancer therapeutics. This review focuses on glycan-engineered site-specific conjugation strategies, a promising approach to construct homogeneous Antibody-drug conjugates with improved therapeutic indices. The paper examines the impact of various glyco-engineered conjugation strategies on key Antibody-drug conjugates performance parameters, including targeting capacity, bystander effects, drug-to-antibody ratio, and immunogenicity. Glyco-engineering significantly enhances Antibody-drug conjugates homogeneity, stability, and therapeutic index while mitigating immunogenic risks. By systematically evaluating cutting-edge techniques such as enzymatic remodeling, metabolic engineering, and one-pot strategies, the study highlights their potential to streamline processes, increase drug-to-antibody ratio, and optimize efficacy. This review provides a comprehensive and critical perspective on glyco-engineered conjugation technologies, facilitating the rational design and clinical translation of next-generation Antibody-drug conjugates.

Keywords: Antibody-drug Conjugates (ADCs), Glycan Engineering, Site-specific Conjugation, Drug-to-Antibody Ratio (DAR)

1. Introduction

In 1967, the concept of Antibody–drug conjugates (ADCs) was first introduced and the radioimmunotherapy was disclosed [1]. ADCs have since emerged as a leading approach in oncology by delivering targeted chemotherapy. By 2025, nineteen ADCs have gained global approval, with more than 200 candidates in clinical trials, driving a market exceeding USD 10 billion.

ADCs consist of three parts: an antibody, a cytotoxic payload and a linker. The conjugation method, which governs the sites, number, and quality of linker attachment, is critical for ADC performance. These methods fall into two broad classes. The first is stochastic conjugation, which exploits either lysine residues or cysteines generated by reducing inter-chain disulfides. First-generation ADCs relied on random lysine coupling: easy to perform but yielding highly heterogeneous drug-to-antibody ratios (DARs). Second-generation ADCs switched to cysteine-based coupling, improving DAR uniformity yet disrupting the structural integrity of the antibody. Building on these lessons, third-generation ADCs have adopted the second class, site-specific conjugation,

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greatly enhancing product homogeneity. Among these technologies, glycan-engineered site-specific conjugation offers particularly compelling advantages.

This review examines glyco-engineered ADC conjugation strategies and their advantage in overcoming traditional limitations through enhanced homogeneity. By evaluating enzymatic remodeling, metabolic engineering and other advanced approaches, it provides critical insights for designing safer, more efficacious next-generation ADCs, paving the way for their clinical translation in targeted cancer therapy.

2. Application of glycoengineering in conjugation

2.1. The basis of conjugation via glycan engineering

The N297 glycan on the IgG Fc region is a highly conserved complex-type N-glycan. Its core heptasaccharide consist of four N-acetylglucosamines (GlcNAc) and three mannose residues. Glycosylation at this site maintains the global quaternary structure of Fc, thereby providing an ideal locus for payload conjugation. After enzymatic remodeling of the native glycan to expose a unique chemical handle (e.g., azide or alkyne), a bio-orthogonal click reaction installs the linker–payload module at this precisely defined, structurally remote location—affording stoichiometrically controlled, homogenous ADCs without genetic antibody engineering or disruption of antigen-binding integrity.

2.2. Conjugation chemistry

2.2.1. Periodate oxidation method

The principle involves the use of sodium periodate ($NaIO_4$) to oxidize the vicinal diols of the Fc glycan. The aldehyde groups generated by periodate oxidation represent the first widely used bioorthogonal functional groups, capable of undergoing reductive amination and chemical conjugation with small-molecule hydrazides and aminooxy compounds. Compared with other monosaccharides, sialic acid can be oxidized under relatively mild conditions. In addition, the vicinal diol structure within the glycerol side chain of sialic acid is more flexible and less sterically hindered than those found in other monosaccharides. Zhou et al. employed β1,4-galactosyltransferase (GalT) and α2,6-sialyltransferase (SialT) to install α2,6-linked sialic acid at the termini of the native Fc-glycans, followed by oxidation with a low concentration of $NaIO_4$ (1 mM) to generate aldehyde (–CHO) groups for subsequent conjugation. This approach is limited by an uncontrollable DAR; the resulting aldehyde-hydrazone linkage is reversible and unstable. Moreover, the aldehydes formed on the antibody can react with amino groups on the antibody itself, leading to aggregation.

2.2.2. Metabolic oligosaccharide engineering

The principle involves incorporating azide-modified sugars into glycosylation sites via cellular metabolism, followed by payload conjugation using click chemistry [2]. Screening of fucose analogue libraries identified 6-thiofucose peracetate as a highly efficient metabolic precursor. This compound is intracellularly converted to 6-thiofucose and incorporated into antibody glycans. The resulting modified antibody can be reduced to expose terminal thiol groups on glycans, enabling site-specific conjugation via a maleimide-vc-PAB-MMAE linker.

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This method does not require genetic engineering, which means it is applicable to wild-type IgG. Since the glycosylation site is distant from the antigen-binding region, antibody binding affinity remains unaffected. Additionally, the incorporation of exogenous azide sugars enables tracking of the binding process. Tian et al. introduced the element selenium (Se) into monosaccharide analogs to synthesize “SeMOE probes”. These probes can be metabolized by cells and incorporated into glycans. By measuring the selenium content using ICP-MS, quantitative analysis of glycans is achieved. This enables label-free, high-sensitivity, and multimodal detection [3].

2.2.3. GlycoConnect™ technology

The GlycoConnect™ technology, developed by Synaffix, is a non-genetic, site-specific, and stable ADC construction method applicable to native antibodies. Its core principle involves three steps: first, the heterogeneous N-glycans at the Fc region N297 site of the antibody are trimmed using an endoglycosidase (e.g., Endo S2), leaving the core GlcNAc intact. Next, a mutant galactosyltransferase (GalT-Y289L) is employed to transfer an azide-modified GalNAz sugar moiety onto the GlcNAc. Finally, a bicyclononyne (BCN)-linked payload is conjugated to the antibody via a SPAAC reaction. ADCs produced using GlycoConnect™ also benefit from the simultaneous loss of CD16/CD32 (Fc-γ receptor III and II) binding and a marked drop in CD64 (Fc-γ receptor I) binding to below 30%, minimizing the unwanted Fc-γ-receptor-mediated uptake that can occur in healthy tissues [4].

However, the method is constrained by low galactosyltransferase (GalT or GalNAcT) catalytic efficiency, necessitating high enzyme loads and prolonged incubation; limited DAR to 2 due to the specificity for azide- or keto-modified sugar derivatives; and costly multi-step purification imposed by the enzymatic cascade. To address these limitations, Wijdeven et al. employed anionic surfactants such as sodium deoxycholate. This approach enhanced the GalT system by enabling the use of native TnGalNAcT for efficient 6-azidoGalNAc transfer and facilitated a gram-scale synthesis of UDP-6-azidoGalNAc [4].

2.2.4. One-pot strategy

Researchers have sought to develop a one-step, high-efficiency, and structurally diversified strategy for synthesizing glycosite-specific antibody–drug conjugates (gsADCs) that obviates antibody sequence engineering and bioorthogonal reactions. A foundation for this approach was laid by Zhang et al., who screened the transglycosylation activity of various ENGases and found that wild-type Endo-S2 exhibited the highest activity. This enzyme efficiently transferred the functionalized disaccharides without hydrolyzing the products. Leveraging the dual activities of Endo-S2—deglycosylation and transglycosylation, they achieved Fc glycan remodeling of antibodies in a single reaction step, eliminating the need for intermediate purification and enabling direct access to functionalized antibodies [5].

Based on above findings, Shi et al. directly conjugated the payload to LacNAc, enabling single-step installation of the drug at the N297 site using a LacNAc-drug substrate. This strategy bypasses the need for α-fucosidase and bioorthogonal handles (e.g., azide or DBCO), allowing direct synthesis of gsADCs from native IgG with a streamlined process, reduced DAR heterogeneity, high yield, and maintained in vivo efficacy [6]. Zhang et al. further evaluated non-natural disaccharide oxazolines—Manβ1,4GlcNAc, Glcβ1,4GlcNAc, and Galβ1,4GlcNAc,as substrates for Endo-S2, conjugating MMAE via a Val-Cit linker.

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These azide-modified oxazolines were efficiently recognized by Endo-S2, demonstrating the enzyme's broad substrate tolerance. The resulting ADCs showed high homogeneity, targeted cytotoxicity, and potent in vitro activity [7].

Table 1. Comparison of glyco-engineered ADC conjugation methods

Conjugation Method	Principle	Advantages	Limitations
Periodate Oxidation	Oxidizes vicinal diols on glycans to generate aldehydes group for conjugation.	Simple operation Early widespread use.	Uncontrollable DAR Unstable linkage; prone to aggregation.
Metabolic Oligosaccharide Engineering	Incorporates non-natural azido-sugars via metabolism for click chemistry.	No genetic engineering; Applicable to wild-type IgG Preserves antigen binding.	Metabolic efficiency cell-dependent complex precursor synthesis.
GlycoConnect™ Technology	Enzymatic trimming followed by GalT-Y289L transfer of GalNAz for site SPAAC conjugation.	Non-genetic and site-specific, stable Reduced FcγR binding.	Low catalytic efficiency DAR limited to 2; multi-step purification increases cost
One-Pot Strategy	Single enzyme (e.g., Endo-S2) mediates deglycosylation + transglycosylation.	No intermediate purification Streamlined process High DAR homogeneity.	High specificity and stability requirements for enzymes.

Note. The summarized information is derived from the following references: Metabolic Oligosaccharide Engineering [2-3], GlycoConnect™ Technology [4], One-Pot Strategy [5-7].

According to their chronological development, there are four main methods for conjugation via glycoengineering. Table 1 summarizes the principles, advantages, and limitations of these four methods. Each of these methods will be introduced below, accompanied by relevant cases.

3. Key attributes of the resulting ADCs

3.1. Impact on DAR

Native IgG antibodies carry only one conserved N-glycosylation site at Asn297 in the Fc region, which results in a maximum of two glycans per antibody, one on each heavy chain. If one payload is attached per glycan, the theoretical DAR cannot exceed two. However, higher DAR values are generally associated with enhanced cytotoxic potency and a reduced risk of drug resistance. Hyongi Chon et al. adopted a bi-antennary complex-type glycan whose two antennae are chemically equivalent and can be modified simultaneously under identical conditions. By introducing azide groups at both termini, they created two conjugation handles per glycan. Attaching two such dual-azide glycans to the antibody and then performing conjugation affords a homogeneous ADC with a DAR of 4 [8]. Tang et al. combined glycan remodeling with affinity-directed traceless conjugation to boost the DAR. This method exploits the high-affinity interaction between an Fc-binding peptide (FcBP) and the antibody Fc region; a thioester mediates traceless acyl transfer, site-specifically installing the payload at Lys248. Both conjugations proceed simultaneously in one pot, accommodate single-or dual-payload combinations, and produce dual-site-specific ADCs with a DAR of 4 [9].

Despite the limitation of a low DAR, the conserved N297 glycosylation site in the Fc region of the antibody serves as the sole reactive handle. Through enzymatic remodeling, a single chemical

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tag is introduced onto the glycan, followed by a bio-orthogonal reaction to attach the payload, ensuring the production of ADCs with a highly homogeneous DAR.

3.2. Impact on immunogenicity

DAR heterogeneity is a major driver of ADC aggregation. Molecules with different DAR values differ sharply in physicochemical properties, especially hydrophobicity and isoelectric point. This molecular heterogeneity directly lowers the colloidal stability of the ADC, making it far more prone to aggregation and degradation. Once aggregated, the ADC becomes more immunogenic and is cleared faster. In contrast, glyco-engineered conjugation yields ADCs with low, highly uniform DAR values, greatly reducing the likelihood of immune recognition.

Furthermore, glyco-engineering enhances homogeneity and thereby lowers immunogenicity by reducing the number of immune-recognition motifs. Wolf et al. systematically elucidated the pivotal role of antibody Fc glycosylation in immunogenicity. Their work showed that high-mannose glycans enhance dendritic-cell (DC) uptake and antigen presentation, markedly increasing T-cell activation and anti-drug antibody (ADA) responses, whereas hypersialylation or aglycosylation reduces DC recognition and immune reactivity. The study demonstrated that improving glycoform homogeneity and eliminating heterogeneous glycan epitopes can effectively attenuate immune recognition and thereby lower the immunogenicity of ADCs. For example, glyco-engineering can trim the glycans to a single core structure or introduce a uniform glycan epitope. This approach not only increases glycoform uniformity but also allows tight control of high-mannose and sialylation levels, minimizing the immunogenic potential of ADCs [10].

4. Glycoengineering-mediated control of ADC function

4.1. Targeting capacity

Glycoengineering of antibodies enhances antibody-dependent cellular cytotoxicity (ADCC), thereby improving the targeting capability of ADCs. Claudia Ferrara et al. determined the crystal structures of glycosylated FcγRIIIa in complex with afucosylated and fucosylated Fc fragments. These structures reveal that core fucose on the Fc glycan exerts direct steric hindrance against carbohydrate-mediated interactions with FcγRIIIa. This provides a molecular mechanism explaining the increased receptor affinity of IgG lacking core fucose. This implies that afucosylation reduces the steric bulk of the glycan, strengthens carbohydrate–carbohydrate contacts, and stabilizes FcγRIIIa binding, thereby enhancing ADCC. Yen-Pang Hsu et al. further demonstrated that galactosylated and sialylated ADCs exhibit a modest increase in binding affinity to FcγRIIIa. Moreover, ADC glycoforms exert a more pronounced effect on FcγRIIIa binding: galactosylation increases the biolayer interferometry (BLI) response by 40%, whereas sialylation causes a sharp 55% decrease. These findings underscore glycoengineering as a feasible and meaningful strategy for enhancing the targeting capacity of ADCs [11].

4.2. Bystander effect

The bystander effect of ADCs refers to the diffusion of cytotoxic payloads from antigen-positive tumor cells to adjacent antigen-negative cells, thereby amplifying antitumor efficacy. This mechanism enables ADCs to remain effective in tumors with heterogeneous antigen expression. The process involves payload release, either prior to internalization or following endocytosis and

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lysosomal degradation, after which the non-polar payload crosses membranes to kill neighboring antigen-negative cells.

This effect offers a key advantage in overcoming tumor heterogeneity and expanding therapeutic coverage, but it also carries the risk of off-target toxicity to healthy tissues. Recent studies demonstrate that Fc-domain modifications concurrently influence both ADC distribution and immune-mediated bystander effects [12]. Optimization strategies, including the use of membrane-permeable payloads such as DXd via linker-payload engineering, enhance the bystander effect, while Fc glycoengineering balances immune effector functions with safety profiles [12].

5. Future directions

Enhancing the DAR of glycosylation conjugates is a critical challenge. Hybrid models that combine mechanistic and data-driven approaches may predict novel optimal sites or glycoform combinations for antibody engineering. This can facilitate the development of bioorthogonal handles to produce dual-loaded ADCs, which are promising for combating drug resistance. Integrating these approaches with complementary methods and employing multiple click chemistry reactions can increase drug loading. After developing novel ADCs, process optimization at large-scale production is also crucial. Digital twins can be employed for virtual scale-up and parameter sensitivity analysis, achieving “one experiment-multi-level scale-up”. Continued innovation in this field will expand the therapeutic potential of ADCs in oncology and beyond.

6. Conclusion

This review systematically outlines the technical principles and advances in glyco-engineered conjugation strategies. Glycan-engineered site-specific conjugation has emerged as a robust platform for generating homogeneous ADCs with enhanced stability, efficacy, and safety profiles. Current methodologies, including enzymatic remodeling and bioorthogonal chemistry, enable precise control over conjugation sites and significantly improve product homogeneity. These approaches effectively address limitations associated with traditional stochastic conjugation methods by reducing structural heterogeneity and minimizing undesirable immunogenicity. The optimization of enzymes, substrates, and reaction conditions has further enhanced conjugation efficiency and product quality, establishing glycan engineering as a reliable strategy for ADC development.

The study also has several limitations. The discussion focuses more on mechanistic insights than on clinical translation, including scalable manufacturing, regulatory issues, and cost-effectiveness. Furthermore, the comparisons between methods are qualitative and primarily based on existing literature The analysis primarily focuses on IgG-based platforms, with limited exploration of their applicability to emerging formats such as bispecific antibodies. Future efforts should focus on developing quantitative, multi-scale models that balance manufacturing scalability and cost-effectiveness with other factors.

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