Review

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# The Future of Antibody Drug Conjugation by Comparing Various Methods of Site-Specific Conjugation

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**The field of oncology is continuously seeking to find effective treatment therapies with limited side effects. Antibody-drug conjugates (ADCs) are a promising class of cancer therapies that have been shown to be effective with limited side effects. Although promising, these therapies experience shortcomings, such as the stability and reproducibility of current conjugation methods. Historically, ADCs have been produced by stochastic conjugation methods; however, new methods of site-specific conjugation have evolved to mitigate current ADC shortcomings. In this article, we highlight the success of ADCs as well as some of their challenges. We also highlight the shortcomings of stochastic conjugation and explore the various site-specific conjugation methods and their advantages over stochastic conjugation.**

**Keywords:** antibody-drug conjugates; ADC; conjugation; stochastic conjugation; site-specific conjugation

## Introduction

Cancer is the second leading cause of death in the United States with 1,958,310 new cases and 609,820 deaths expected for the year 2023 [1]. The conventional or traditional treatment options for cancers include surgery, chemotherapy, and radiation therapy [2]. Chemotherapy and radiation tend to have negative side effects due to their nondiscriminatory killing of cells regardless of whether they are cancerous [3]. Increasing numbers of innovative and targeted anticancer therapies have been clinically approved due to a better understanding of the molecular nature of cancers [4]. Targeted therapies are defined by the National Cancer Institute as any type of treatment that uses drugs or other substances to target specific molecules that cancer cells need to survive [5]. Targeted therapies are primarily small-molecule drugs or monoclonal antibody therapies. Examples include Neratinib, a small-molecule drug that targets the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor (HER) family, and Trastuzumab, a monoclonal antibody that targets human epidermal growth factor receptor 2 (HER2) [6,7].

Another classification of targeted therapy is antibody-drug conjugates (ADCs) [8]. ADCs are a rapidly developing therapeutic and diagnostic option in oncology; 14 ADCs have been clinically approved worldwide, and 12 of those have been approved in the United States as of December 2022 [8,9]. They are composed of an antibody specific for a particular antigen conjugated to a linker and a payload. In terms of treating tumors, ADCs have the advantage over conventional cancer therapeutics because they reduce the exposure of the drug to noncancerous tissues and organs, thus decreasing the side effects and increasing the efficacy and therapeutic index of the drug [10,11]. ADCs are also categorized under the family of immunoconjugates, which include immunotoxins, immunocytokines, and radioimmunoconjugates. Immunoconjugates all have similar structural properties due to the antibody delivery system, but they differ in the type of payload used [12,13].

The first approved ADC was gemtuzumab ozogamicin, which consists of an anti-CD33 antibody conjugated to an acid-cleavable linker and an ozogamicin payload. Gemtuzumab ozogamicin is used to treat acute myeloid leukemia [14,15]. The success of gemtuzumab ozogamicin and other ADCs has opened a new avenue for cancer therapies, but current clinical barriers still prevent ADCs from being more widely accepted. Some of these challenges include unique target antigen selection, chemical instability of the linkers, extravasation of the ADC, and immunoreactivity associated with the components of ADCs [10,16,17].

An ADC works by binding to a specific antigen that is overexpressed on the surface of cancer cells. Typical antigen targets are membrane-bound proteins and receptors [16]. The challenge is in finding a uniquely specific antigen since some of these antigens are also expressed in normal tissues [16]. For example, EGFR, a target in head and neck squamous cell carcinomas (HNSCC), is overexpressed in these cancers, but EGFR is also expressed in normal epithe-

lial tissue [18]. Another example is HER2, a target in breast cancers. HER2 is overexpressed in various solid tumors including breast cancers, gastric cancers, and lung cancers, but it is also expressed in normal epithelial cells and cells in the nervous system [19]. In both of these examples, although the antigen is not uniquely specific to the cancer cells, ADCs work on the principle of the overexpression or amplification of these antigens. In the case of HER2, there are as many as 50 copies of the Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*) gene in breast cancer with a fold increase of up to 40 to 100 in the HER2 protein, resulting in the expression of 2 million receptors on the cell surface of the tumor [20]. Ado-trastuzumab emtansine, an ADC approved in 2013 for the treatment of breast cancer, works on the basis of targeting overexpressed HER2 in breast cancer [21,22].

The linkers used in ADC development also present a challenge because of their potential to be unstable and release the cytotoxic payload prior to tumor penetration and accumulation. There are two types of linkers used, namely cleavable and non-cleavable linkers [23,24]. Cleavable linkers are made up of chemically and enzymatically cleavable linkers. Under the right conditions, covalent bonds break, releasing the payload from the antibody [25]. Non-cleavable linkers rely on lysosomal degradation. They include thioether linkers, maleimidocaproyl linkers, and others [25]. Ideally, linkers should be stable in circulation and should be able to release the payload once the ADC has been internalized into the tumor, but unfortunately, this is not always the case. For example, the ADC gemtuzumab ozogamicin was shortly withdrawn in 2010 because its linker was unstable, which caused a premature release of the drug's payload. Gemtuzumab ozogamicin was reapproved in 2017 after a redesign that included a more stable linker [23,24].

The extravasation of the ADCs becomes a challenge when targeting solid tumors. In treating blood cancers, the only step required for the ADC to reach its target is the distribution of the injected ADC throughout the circulatory system [16]. In solid tumors, the ADC must first extravasate from the circulating blood into the extracellular matrix before it can be transported through the extracellular matrix to reach its target [16]. The lower barrier of targeting hematological cancers is evidenced by 7 of the 12 ADCs approved by the U.S. Food and Drug Administration (FDA) are approved for hematological tumors (Table 1) [8]. Vascularity of solid tumors also impacts the effectiveness of ADCs. The effectiveness of ADCs increases in highly vascularized tumors and decreases in less vascularized tumors. Vascularity is a hallmark of most cancers because tumors require increased nutrients and oxygen and therefore undergo angiogenesis to produce new vasculature to provide the necessary nutrients and oxygen [26]. Cancer vasculature tends to be aberrant and leaky, thus allowing macromolecules, including ADCs, to be transported out of the circulation and into the tumor [16,26]. In highly vascularized solid tumors like glioblastoma, hemangioendothelioma, angiosarcoma, synovial sarcoma, and pancreatic neuroendocrine carcinomas, ADCs may have a greater effect due to the leakiness of the vasculature, whereas in less vascularized solid tumors like pancreatic ductal adenocarcinomas, they may have less of an effect [27–29].

Although ADCs still have barriers to overcome for widespread acceptance, the success of gemtuzumab ozogamicin and other FDA-approved ADCs has facilitated an increase in research interest to develop more effective and more stable ADCs. One way to improve the stability and predictability of ADCs involves conjugation techniques.

## Conjugation Techniques

### *Stochastic Conjugation Techniques*

There are two main methods of conjugating an antibody: stochastic and site-specific. Stochastic conjugation, also known as random conjugation, is the more common of the two methods. This method relies on chemical means such as thiol alkylation of native cysteine residues and amine acylation of lysine residues [30]. Both methods have the advantage of using native residues without any modification and can be performed under mild reactions [31]. Most clinically approved ADCs were produced using these methods, including gemtuzumab ozogamicin, which is produced by amine acylation on lysine residues, and Brentuximab vedotin, which is produced by thiol alkylation on native cysteine residues [32]. Although these methods have been used successfully in generating clinically approved and available ADCs, they still have certain flaws. ADCs produced stochastically form heterogeneous mixtures with a wide range of drug-to-antibody ratios and have poor reproducibility (Fig. 1) [30,33]. These methods can also potentially affect the biological function by interfering sterically with the antigen recognition domain and prohibiting the binding of the antibody to the antigen [30]. These disadvantages stem from the fact that the conjugations are random and can occur at any position in the antibody where there are lysine or cysteine residues. For example, if the conjugation occurs at or close to the complementarity-determining region (CDR) of the antibody, the specificity and binding of the antibody to the antigen will be inhibited [30].

### *Site-Specific Conjugation Techniques*

Site-specific conjugation tends to solve the shortcomings of stochastic conjugation methods because it produces more homogeneous mixtures with a less varied range of drug-to-antibody ratios [30], but oftentimes it requires further engineering of the antibody. Site-specific conjugation refers to conjugation at a specific site or location on the antibody. This method can be carried out by chemical or en-

**Table 1. Summary of antibody-drug conjugates (ADCs) currently approved in the United States as of December 2021.**

| ADC | Trade name | Company | Target | Year of approval | Approved indication |
|---|---|---|---|---|---|
| Gemtuzumab ozogamicin | Mylotarg® | Pfizer | CD33 | 2000 | Acute myeloid leukemia |
| Brentuximab vedotin | Adcetris® | Seagen | CD30 | 2011 | Hodgkin leukemia; systemic anaplastic large-cell lymphoma |
| Ado-trastuzumab emtansine | Kadcyla® | Roche | HER2 | 2013 | HER2-positive breast cancer |
| Inotuzumab ozogamicin | Bespona® | Pfizer | CD22 | 2017 | B-cell acute lymphocytic leukemia |
| Polatuzumab vedotin | Polivy® | Roche | CD79B | 2019 | Diffuse large B-cell lymphoma |
| Moxetumomab pasudotox | Lumoxiti® | AstraZeneca | CD22 | 2018 | Hairy cell leukemia |
| Enfortumab vedotin | Padcev® | Seagen | Nectin-4 | 2019 | Urothelial cancer |
| Fam-trastuzumab deruxtecan | Enhertu® | Daiichi Sankyo | HER2 | 2019 | HER2-postive breast cancer |
| Sacituzumab govitecan | Trodelvy® | Immunomedics | Trop-2 | 2020 | Breast cancer |
| Belantamab mafodotin | Blenrep® | GlaxoSmithKline (GSK) | BCMA | 2020 | Multiple myeloma |
| Loncastuximab tesirine | Zynlonta® | ADC Therapeutics | CD19 | 2021 | B-cell lymphoma |
| Tisotumab vedotin | Tivdak® | Genmab/Seagen | Tissue factor | 2021 | Cervical cancer |

HER2, human epidermal growth factor receptor 2; Nectin-4, nectin cell adhesion molecule 4; Trop-2, trophoblast antigen 2; BCMA, B-cell maturation antigen.

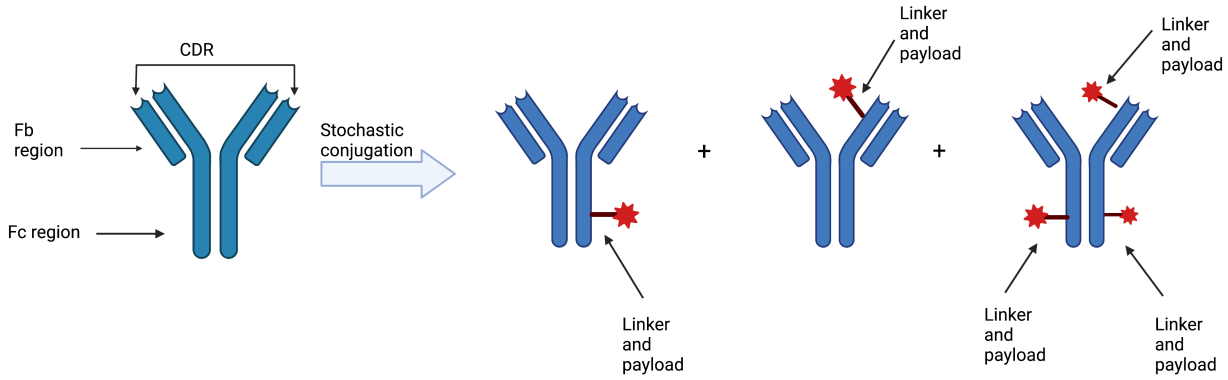


**Fig. 1. Stochastic conjugation of an antibody.** A native antibody conjugated stochastically producing multiple conjugates with one being conjugated at the complementarity-determining region (CDR) and another with multiple drugs conjugated to the antibody. Fb, constant region; Fc, fragment crystallizable region. The figure is an original image created with https://www.biorender.com/.

zymatic means, which further divide it into four methods based on what is used as the site [17,30]. This article highlights the four methods based on what is used as the site for conjugation: specific amino acids, unnatural amino acids, glycans, and short peptide tags [31,34,35].

## Site-Specific Conjugation Using Specific Amino Acids

In this method, native or engineered amino acids are chosen as the conjugation site. These amino acids typically include cysteines and glutamines [34]. In this method, the cysteine used could either be engineered or be native to the antibody, whereas the glutamine used is native to the antibody. This method requires either engineering amino acids, reducing and oxidizing reagents when using cysteine, or microbial transglutaminase when using glutamine [34].

One of the first site-specific ADC methods developed, THIOMAB™, is based on using specific amino acids. THIOMAB™antibodies refer to antibodies bearing engineered cysteine residues [36]. THIOMAB™conjugation approach is based on the introduction of unpaired cysteine residue by engineering into a specific position of an antibody to allow site-specific conjugation; ADCs developed by this method are typically called THIOMAB™antibody-drug conjugates (TDCs) [36,37]. Junutula *et al.* [38] developed an HER2-targeting ADC using the method and showed that it had an improved therapeutic index to its target. In an attempt to improve this method, Zhou *et al.* [37] recently investigated the feasibility of having multiple engineered unpaired cysteines in the CH2 and CH3 in an antibody to generate ADCs and showed that the conju-

**Table 2. Summary of advantages of site-specific conjugation methods and stochastic conjugation methods.**

| ADC characteristics | Site-specific | Stochastic |
|---|---|---|
| Reproducibility | very high | lower |
| Specific DAR | precise | random |
| Stability in blood | higher | lower |
| Chemistry | knowledge based | ubiquitously common |
| Reaction conditions | harsher | milder |
| Cost to generate | higher | lower |
| Potential immunogenicity | higher | lower |

gates formed had a drug-to-antibody ratio (DAR) above 3.4. DAR refers to the total number of drug molecules that are attached to a single antibody, with the most optimal DAR ranging from 2 to 4.

### Site-Specific Conjugation Using Unnatural Amino Acids

In this method, unnatural amino acids are incorporated into the antibody as sites for conjugation [34,39]. ADCs conjugated through this method were determined *in vivo* to be more efficacious than those conjugated using an unpaired cysteine-mediated site-specific method [34]. This method requires both antibody and cell line engineering [8,34]. Axup *et al.* [39] used this method to generate two ADCs using trastuzumab, an anti-HER2 antibody as the antibody; a non-cleavable linker; and auristatin F as the cytotoxic payload. The first ADC was generated by incorporating *p*-acetylphenylalanine (pAcPhe) into the fragment antigen-binding (Fab) fragment of the antibody [39]. This process was completed by coexpressing an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthase (tRNA/aaRs) pair with a truncated form of the immunoglobulin heavy constant gamma 1 (*IGHG1*) gene that generates an IgG1 Fab that binds and included TAG codons at specific residues [39]. The second ADC was generated by substituting an amber codon at the heavy chain residue A121 of the full-length *IGHG1* gene and incorporating pAcPhe into the genome of the antibody and expressing the antibody in Chinese hamster ovary cells (CHO-K1) [39]. The genes of the light and mutant heavy chains were then incorporated into the CHO cell line [39]. Both conjugates were seen to be selective for HER2 and potent against breast cancer xenograft models [39]. Although there have been successes in using this method, the immunogenicity of ADCs conjugated using this method in humans is not known, and it is suggested that the unnatural amino acid may induce immunogenicity [8,34].

### Site-Specific Conjugation Using Glycans

In this method, antibodies are conjugated using the N297 glycans in the CH2 domain of the antibody. This method works on the basis that glycosylation at the N297 position is conserved for all IgG antibodies [33,40]. The main advantages of this method are that *N*-glycans are conserved across all IgG subtypes; they are distant from the CDR and are disparate from the antibodies' polypeptide backbone [33]. This method can be accomplished by using unique reagents and enzymes [34,41,42]. There are several commercially available kits that can be used to produce conjugates with this method. For instance, GlycoConnect from SynAffix were used in the development of ADCT-601, an ADC targeting AXL Receptor Tyrosine kinase (AXL)-expressing cancers [43]. Another example is the SiteClick™ antibody labeling kit from Thermo Fisher, which was used by Kristensen *et al.* [44] in comparing trastuzumab 89Zr conjugates. The caveat with these kits is that they come with specific linkers and payloads, and those are the only ones that can be used, which can be a problem if those linkers and payloads are not suitable. If that is the case, chemical modification of the N297 glycan and the linker of choice can still be done with some knowledge of chemistry, as was done by Bejot *et al.* [45]. Shivatare *et al.* [42] were also able to use *in vitro* glycoengineering with enzymes to create site-specific ADCs by inserting an azido-fucose tag at the fragment crystallizable region (Fc)-glycan; these ADCs had potent cytotoxicity, were specific for their target, and were internalized rapidly.

### Site-Specific Conjugation Using Short Peptide Tags

In this method, specific short peptide tags containing four to six amino acid residues are used as the conjugation sites [34]. There are two main ways peptide tags can be used for site-specific conjugation: recognition sites for enzymes or chelators. Chelators are cage-like chemicals that are commonly used to capture metallic radioisotopes and can be conjugated to antibodies [46]. Peptide tags used as recognition sites can either be done with modifications by enzymes such as Transglutaminase (TGase), Sortase (SrtA) or fusion proteins that are genetically engineered to have both the antibody and enzyme together, such as in SNAP-tag and inteins [46]. Peptide tags can also be used as chelators as in the case of $(His)_6$ Tag and $(Gly)_x$Cys Tag [46]. Kampmeier *et al.* [47] used the $(His)_6$ Tag as a chelator in designing a $^{99m}$Tc-labelled diabody of mAb J591. Berndorff *et al.* [48] used a $(Gly)_3$Cys Tag in designing a $^{99m}$Tc-Labeled human recombinant Anti-Extra-domain B (ED-B) fibronectin antibody fragments. The biggest drawbacks with this method are the scalability and the potential immunogenicity [34].

Each of these site-specific methods has been shown to produce more homogeneous conjugates with a greater therapeutic index and greater stability in blood plasma [30,34,44,49]. With these advantages, site-specific conjugation appears to be the future of ADC. This forecast can be seen with the approval of Polatuzumab vedotin, a site-specially conjugated ADC that was approved by the FDA in 2019, and the increase in the number of ADCs entering clinical trials that are created using site-specific conjugation methods [8,30,50–52]. Numerous site-specific conjugated ADCs are currently in various phases of clinical trials

with examples including HDP 101 and SYD985, which are currently in Phase 1/2 and Phase 3, respectively [53,54]. Although there have been a few site-specific conjugated ADCs that have been withdrawn from clinical trials, most notably SGN-CD70A, which was discontinued after Phase 1, the majority of ADCs in clinical trials are conjugated by site-specific methods [51,55].

## Conclusions

As discussed above, there are many methods of generating site-specific ADCs including specific amino acids, unnatural amino acids, glycans, and short peptide tags [31,34,35]. The various site-specific conjugation methods each have their respective advantages and disadvantages (Table 2); therefore, the choice of which method to use will depend on an assessment of the respective advantages. In summary, ADCs are a great therapeutic option in cancer therapy. There are currently two main approaches to conjugation, and site-specific conjugation has superior chemical properties of uniformity and stability. However, the drawbacks of limited versatility in linkers and payloads, the potential for immunogenicity, and the extensive steps of bioengineering resulting in greater costs prevent a more universal acceptance of the site-specific conjugation methods. Site-specific conjugation seeks to address the problems associated with stochastic conjugation, and the advantage of reproducible DAR is paramount in obtaining FDA approval for clinical dosing. Many selected targets provide a narrow therapeutic window before ADCs can become toxic, thus maintaining a tight control of how much drug is administered is essential. Although there are other issues associated with all ADCs that still need to be addressed, such as extravasation and linker stability, ADCs continue to be a promising therapeutic option in cancer therapy.

## Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

## Author Contributions

Conceptualization: JO, SM and JB. Funding acquisition: JB. Supervision: JB. Visualization: JO. Writing—original draft preparation, review and editing: JO, SM and JB. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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