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Field Guide to Challenges and Opportunities in Antibody-Drug Conjugates for Chemists

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Abstract

Antibody-drug conjugates have attracted a great amount of attention as a therapeutic strategy for diseases where targeting specific tissues and cells are critical components, such as in cancer therapy. Although promising, the number of approved ADC drugs is relatively limited. This emanates from the challenges associated with generating the conjugates and the complexities associated with the stability requirements for these conjugates during circulation and after reaching the target. Here, we provide a comprehensive overview of the design challenges facing the ADC field. These challenges also provide several unique research and development opportunities, which are also highlighted throughout the review.

INTRODUCTION

Cancer is the second leading cause of death in the United States. This year, an estimated 589,430 patients in the United States will die from this disease.[1] Typically, when a patient's tumor is localized to a particular tissue, surgery and radiotherapy are used; however, chemotherapy is required when the cancer has metastasized. The basis of chemotherapy is that cytotoxic agents would kill rapidly dividing abnormal cancer cells preferentially over the normal healthy cells by targeting the cell cycle. However, the observed systemic toxicity and cell killing of normal highly proliferating cells including those in the gastrointestinal tract and bone marrow have been strong indicators of the need for tumor selectivity. In order for chemotherapy to be a successful treatment, remission requires more than 99% of tumor cell kill, and even higher for eradication.[2] In order to increase the efficiency of chemotherapeutics, the therapeutic window needs improvement. This can be achieved by lowering the minimum effective dose (MED) through increased potency, or raising the maximum tolerated dose (MTD) through improved the tumor selectivity.[3] *In vitro* high potency drugs alone have often not improved clinical activity due to low MTDs. Better targeting approaches may be able to address the therapeutic index through preferential

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cancer cell uptake allowing for higher potency drug load or higher dosage that are specifically targeted to the tissue type.

In the late 19th century Paul Ehrlich proposed a lock-and-key model in selective targeting of a disease organism with a toxin.[4] Today one of the most pursued strategies in the pharmaceutical industry for cancer therapy is an off-shoot of that model, the targeted approach of antibody drug conjugates (ADCs). This prodrug strategy links cytotoxic payloads to monoclonal antibodies (mAbs) with the aim to selectively bind to tumor-specific antigens to decrease systemic toxicity and improve cell kill efficiency.[5] Several mAbs have been approved for therapeutics for different indications, including cancer. Monoclonal antibodies can bind to antigens uniquely expressed or overexpressed on various human cancer cell types.[6] These markers are typically involved in the processes of cell growth or progression, and are either surface proteins, glycoproteins, or carbohydrates.[3] Certain mAbs have been approved for cancer therapy that can bind to cancer-specific antigens and induce an immunological response,[7] however the therapeutic benefit is often insufficient and resistance to the mAb can occur. Alternatively, monoclonal antibodies can be used as targeting missiles for therapeutics through modification with cytotoxic drugs, with greater opportunity for cell kill.

ADCs are comprised of the monoclonal antibody and the toxic payload, which are conjugated *via* a linker (Scheme 1). Following selective cell uptake, chemical or enzymatic cleavage of the linker releases the drug from the mAb vehicle to cause cytotoxic activity. Each structural component of ADCs have a variety of requirements, as the sophisticated prodrug is exposed to a variety of environments including the blood stream, tumor tissue, and subcellular compartments. Challenges arise in optimizing these various and interdependent parameters and have led to a variety of approaches to generating ADCs. Even those that are far into clinical trials or approved by the US Food and Drug Administration (FDA) are diverse in design, structure, and function (Table 1). Regardless, the following requirements must be met in an ADC: *Circulation:* the ADC must be stable in circulation, and thus be structural and chemically as stealthy as the naked mAb. Humanized antibodies have half-lives up to three weeks, and ADCs should likewise have long circulation times, and not accumulate off-target. Further, the linker should be stable in serum so that the drug is not systemically released causing off-target toxicity. *Antigen Binding:* the immunoaffinity of the mAb should not be compromised by modification with a drug. This requires that the antigen-binding region is not modified during drug attachment. Substantial change in the hydrophobicity of the ADC construct, compared to the mAb, due to the attachment of hydrophobic drug molecules can also affect the antigen-antibody binding. *Internalization:* Following antigen binding, the ADC must be internalized through receptor-mediated endocytosis, limiting ADC development for only mAbs that are delivered intracellularly. The ADC is thus exposed to the mildly acidic endosome, then often the more acidic and catabolic environment of the lysosome or reducing conditions of the cytosol. *Payload Release:* The drug should be released from the ADC in an active form in some way due to this subcellular processing. This requires the drug has a functional handle for modification to the stimuli-sensitive linker and, after exposure to appropriate chemical or enzymatic stimuli for release, the drug or drug catabolite has sufficient cytotoxicity for cell kill. *Payload Action:* The cytotoxic effect should be achieved even with low amounts of internalized drug,

requiring drug potency in the picomolar range. Early ADCs generally had lower potency than the parent free drug due to changes in route of ADC cellular processing compared to freely diffusing hydrophobic drug. For example, free dox orubicin has an effective cytotoxic concentration (EC_{50}) around 10^{-7} M,[8] but BR96-doxorubicin ADC was about eight times less potent.[9] Highly potent cytotoxins in the picomolar range such as microtubulin binding dolastatin and maytansinoids or DNA damaging calicheamicin, which had previously been abandoned in classical chemotherapeutics due to high toxicity or nonspecificity, have become more attractive candidates for ADCs. Conjugation to the mAb imparts the target specificity for the payload, and further their high potency is important considering that only a small percentage of the administered ADC will reach the target.[10, 11] Further considering that mAbs can also be taken up by healthy cells, nonspecific pinocytosis, and the Fc receptors,[12] these potent drugs are ideally more toxic to cancerous cells.

While there are a variety of excellent published ADC reviews,[3, 10, 13-20] our goal is to present a comprehensive review from a chemical perspective, with focus on the synthetic methodology and structural components to achieve desired design elements of antibody drug conjugates, and the resultant biological implications on circulation stability, binding specificity, payload release, and drug action. Here we summarize the various strategies pursued in ADC development such that principles gained can serve as a guide for future designs.

CONJUGATION METHODS

The conjugation method of toxic payload to mAb determines (*i*) drug load stoichiometry, (*ii*) species homogeneity, (*iii*) mAb structural stability and (*iv*) binding capacity. The drug load stoichiometry is the drug-to-antibody ratio (DAR) in the ADC that can greatly affect the potency of the construct. Species homogeneity, *i.e* site of conjugation and DAR variations in the ADCs, can impact the predictability of the pharmacokinetic profiles. The mAb structural features can be impacted by both the extent of conjugated drug load, such as increased hydrophobicity, and also the chemical route of modification, such as sulfhyryl reduction, so that the ADC can be recognized as a damaged biomacromolecule or as foreign in the body, which can impact circulation half-life. The binding capacity of the antibody, which is essential for targeting, is retained if the binding region of the antibody is unaltered from drug attachment. In this section, we will briefly introduce the structure of an antibody, and discuss the various methods employed to conjugate toxic payloads to mAbs that are a) random and b) site-specific.

Antibody Structure

Immunoglobulin G (IgG) antibody is composed of two identical heavy (H) and two identical light (L) chain polypeptides that are associated with each other through four interchain disulfide bonds. The H-chain consists of one variable and three constant domains whereas L-chain has one variable and one constant domain. Each of these domains is the same size (110 amino acid long) and contains one intrachain disulfide bond. The *N*-terminal variable domains of H- and L- chains form the antigen binding site and give the antibody the ability to bind to two molecules of the same antigens simultaneously (Figure 1).

The antibody contains two antigen binding sites and one effector domain. The antigen binding domain is called the Fab (antigen binding fragment) and has the ability to bind antigen independent of the rest of the molecule. The Fc (crystallizable fragment) carries out the effector function by interacting with Fc receptors of several cells of the immune system. Antibodies also contain two carbohydrate chains on the Fc region (Figure 1), which are important for Fc receptor recognition. The Fab and Fc regions are connected by a hinge region, which gives this 150 kDa molecule the requisite structural flexibility. Antibodies contain lysine and cysteine residues that have been used as handles for modification. Lysine residues are randomly distributed through the antibody (shown in green in Figure 1) and found in numbers of 80-90 per antibody. The number of cysteines differs for each four subclasses of IgG. IgG1 is most commonly used as a therapeutic agent and it has a total of 32 cysteine residues forming disulfide bridges within the macromolecule. 24 of these form intrachain disulfide bonds and are in general considered to be stronger than the other 8 cysteines that form interchain disulfide bonds and two of these are located at the hinge region of the antibody (shown in red in Figure 1).[21]

Random Conjugation

Cysteine conjugation methods use mild reducing agent such as DTT to give an antibody mixture with one to four reduced interchain sulfhydryls, quantified typically with Ellman's reagent, providing two to eight cysteine thiols per antibody available for modification.[21, 22] A typical method for drug conjugation through cysteine residues is using maleimide linkers.[9, 23, 24] However, the resultant succinimide thioether can undergo retro-Michael addition reactions in the presence of thiols in solution, such that the drug can be removed from the antibody during circulation through reaction with free thiol on albumin (Figure 2A).[25-26] The succinimide thioether can also undergo hydrolysis, which retains the drug attachment, however minimally increases the structural heterogeneity of the conjugate. Other thiol-specific bioorthogonal reactions have been developed that are more stable to hydrolysis and competing thiols than maleimides and are opportune reactions for mAb modification (Figure 2B).[18] These include reaction with 1) monobromomaleimide,[27] 2) vinyl sulfones[28] and 3) allenamides,[29] or 4) somewhat conversely modification to dehydroalanine followed by Michael addition reaction with thiol.[30] Other thiol-selective reactions include 5) light initiated thiol-ene,[31] 6) perfluoroaromatic molecules[32] and 7) sulfone reagents that are Julia-Kocienski-like[33]. Using these thiol modification reactions in ADC synthesis may improve the species homogeneity. However, because these methods still require interchain disulfide reduction, there is potential for mAb structural instability with increasing drug antibody ratio.

A recent report may provide a viable avenue for antibody modification through bridged conjugation through interchain disulfides that can potentially improve both ADC species homogeneity and stability.[34] A bis(sulfone) reagent alkylates both thiols of a reduced mAb disulfide bond to regenerate a covalent three carbon bridge to impart structural stability (Figure 3). The MMAE drug was conjugated to a *p*-aminobenzyl ether valine citrulline linker with an ethylene glycol spacer to impart water solubility and bis-alkylating moiety for conjugation to mAb trastuzumab (TRA). Conjugation of four drugs per antibody was achieved by first mAb disulfide reduction using DTT then addition of bis-alkylating reagent.

SDS-PAGE of TRA fragment antigen-binding (Fab_{TRA}) and bis-alkylated Fab drug conjugate (Fab_{TRA}-bisAlk-vc-MMAE) under reducing conditions showed that Fab_{TRA}-bisAlk-vc-MMAE did not result in dissociation of heavy and light chain, suggesting that the bis-alkylating agent is forming an interchain bridge. The stability in serum was evaluated by forming TRA Alexa Fluor 488 conjugates using both the bis-alkylating agent and maleimide linker. The bis-alkyl TRA conjugate was shown to be stable in human and rat serum for incubation after 96 hours, whereas the maleimide conjugate showed reduction of TRA-maleimide species and new peaks were observed in the SE-HPLC chromatograms corresponding to free dye, TRA-albumin adduct, light chain-dye conjugate. These results supported that thiol conjugation using maleimide reagents can lead to retro-Michael addition reactions with albumin and mAb structural instability, however that a thiol bridging reagent can circumvent these concerns. Further, DARs of ADCs were monitored with hydrophobic interaction chromatography (HIC) over time incubated with HAS for TRA-bisAlk-vc-MMAE and TRA-maleimide-vc-MMAE. While the DAR of TRA-maleimide-vc-MMAE decreased over 120 hrs, the DAR of TRA-bisAlk-vc-MMAE remained relatively constant.

For specific ADCs, has been shown that a DAR of 2-4 through cysteine residue conjugation yields the best therapeutic effect.[23, 35] MMAE anti-CD30 mAb cAC10 (brentuximab) conjugates with two, four, or eight drugs per antibody retained antigen binding, but had increasing potency with increased drug load.[23] However, eight MMAE per antibody was cleared from circulation faster than ADCs loaded with two or four MMAEs. It was concluded that the therapeutic index was highest for 2-4 drugs per antibody. This result could also be attributed to decreased mAb structural stability through cleavage of interchain disulfide bonds for MMAE conjugation. Further, for this study the linker was conjugated to the mAb thiols using a maleimide group. It is possible that higher drug loads using less structurally deterring conjugation methods may achieve improved potency with higher extent of conjugation.

Drug conjugates can be formed by acylation of abundant native lysine residues on the mAb surface using activated esters; predominantly *N*-hydroxysuccinimide (NHS) esters. The challenge with this conjugation method is synthetically achieving a low DAR and drug load distribution, without leaving a large fraction of the mAb unmodified. The random distribution of 80-90 lysines on the antibody results in a heterogeneous mixture of ADCs, in both stoichiometry and conjugation site. The relationship between DAR and species distribution when conjugation through lysines had not been as well understood as cysteines due to the purification difficulty, so recently, a statistical modeling of ADC Trastuzumab Emtansine (*Kadcyla*) was investigated.[36] *Kadcyla* has an average DAR of 3.5, determined by UV spectrometry, and contains 0-8 drugs per antibody, determined by mass spectroscopy. A series of ADCs with DARs ranging from 2.58-4.00 were characterized by LC-MS, and obtained numerical analysis showed a predictive relationship between DAR and species distribution according to the Poisson equation, which is consistent with lysine-linked conjugates of smaller data sets.[37, 38] Thus they demonstrated that UV spectroscopy could be used as a simple method to secondarily assess distribution from DAR for *Kadcyla*. However, this result is not general to all lysine-linked ADCs, as ADC *Mylotarg* was shown to not follow a Poisson distribution and the results could be specific to conjugation method.[39]

To further investigate the relationship between DAR and pharmacokinetics for lysine-linked ADC Kadcyla, two pharmacokinetic modeling approaches were developed to quantify the rates of DMI deconjugation from the mAb in relation to DAR.[40] The ADC and total mAb plasma concentrations were obtained and DARs were quantified. NONMEM 7.2 software was used to obtain a mechanistic model fit to mAb and DAR concentrations simultaneously using LC-MS data, and a reduced model fit to mAb and ADC concentrations simultaneously based on ELISA measurements. The mechanistic model indicated the DAR of 1-7 did not alter the mAb disposition as they followed the same clearance pathways and distributional volumes. The model also showed that deconjugation rates of DAR species were similar in vitro and in vivo. The pharmacokinetic modeling showed that ADC species with 3 or more DMI per mAb deconjugated faster. Also, the rate of clearance for the ADC was two times faster than the rate of total mAb clearance, as attributed to deconjugation of the DM1. Interestingly, they observed that the ADCs of a particular DAR had a similar PK profile whether or not they were present as such in the injected does, or generated due to deconjugation in circulation. The reduced model fit was able to show an average ADC deconjugation rate, which may be used to investigate linker stability but not in relation to DAR.

Generation of ADCs through lysine conjugation is heavily pursued due to the advantages of a biologically robust amide bond that attaches the linker and the limited antibody processing steps. As such, a variety of lysine reactive moieties have been developed are typically bifunctional and if conjugated to mAb directly provide thiolation (Figure 4). Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) contains a maleimide group which can react with thiols to generate a thioether link. *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) is a conjugation molecule for which the 2-pyridyldithiol group can react with thiol groups to generate a disulfide bond.[41] 4-succinimidyloxycarbonyl-α-methyl-α-[2-pyridyldithio]toluene (SMPT) is similar to SPDP, except that the methyl group makes the disulfide bond hindered, resulting in more stable conjugations.[42] The disadvantage of these two molecules is that they need additional reducing agent to create the free thiol groups for conjugation. The often lose part of their activity during this kind of modification due to the breaking of native disulfide bonds. To solve this problem, *N*-succinimidyl S-acetylthioacetate (SATA)[43, 44], *N*-Succinimidyl S-Acetylthiopropionate (SATP)[45] and S-acetylmercaptsuccinic anhydride (SAMSA)[46] have been developed. Deprotection of the acetyl group is triggered by addition of excess hydroxylamine, which does not impart mAb structural instability.

SITE SPECIFIC CONJUGATION

N-terminal Conjugation

One method for site-specific antibody modification is a transamination reaction to generate a ketone group at the N-terminus of an antibody.[47] Incubation of anti-HER2 human IgG1 (herceptin) with *N*-methylpyridinium-4-carboxaldehyde benzenesulfonate salt generates carbonyl groups on the N-terminal glutamate residues of the heavy chain. Because two heavy chain N-termini for Herceptin are glutamate residues, this would lead to two sites for bioorthogonal chemistry. Mutation of two other N-termini residues of herceptin light chains

to glutamate could thus lead to a four sites for bioorthogonal chemistry. As such a DAR up to 4 is potentially achievable with this N-terminal modification technique. The generated ketones or aldehydes can be reacted with oxyamines to generate an oxime linkage. Flow cytometry with AlexaFluor oxamine-Herceptin conjugate suggested that N-terminal oxime formation with small molecules did not impact antigen-binding capacity.

ThioMAb

Genetic recombinant methods offer advantages of improved precision in mAb conjugation site and stoichiometric control of drug load.[35, 48] Because of the inherently low amounts of free thiol groups in the recombinant antibodies, the recently established genetically engineered cysteine containing mAbs, or ThioMAbs have attracted considerable interest.[49, 50] A first approach to creating site specific ADCs was achieved by engineered cysteine residues in the heavy chain of Fab domain of an IgG antibody by substituting Ala-114 (Kabat numbering**)**, so that the antibody would have two cysteines available for modification. These cysteine substitutions did not interfere with the antigen binding capacity. Engineered antibodies with two cysteines per antibody at various positions were reacted with maleimido-MMAE (monomethyl auristatin E). The resulting ADCs had thiol-maleimide instability in plasma depending on the conjugation site. Engineered mAb with cysteine in a less solvent accessible location with positively charged environment resulted in maleimide hydrolysis and not thiol exchange, thereby retaining the drug conjugate.[26] These results support that not only stoichiometry but conjugation location can lead to dramatic differences in overall ADC efficacy. [49, 50]

Cysteine-engineered ThioMAb antibodies were used in several studies to conjugate cytotoxic drugs. These studies demonstrated that while conventional ADC prepared by partial sulfhydryl reduction had an average DAR of around 3 with a species distrubution of 0 to 8 drugs, while engineered ThioMAb drug conjugates (TDCs) with two engineered cysteines had DARs ranging from 1.6-1.8 with a species distribution of 0 to 2 drugs.[48, 51, 52] The obtained DAR values reflects an approximate conjugation efficiency of 80-90%. TDC showed less toxicity and lower risk of liver damage when compared against the ADC based on the cytotoxic drug dosage (μg/m^2).[48, 51] Overall, the results showed improved or similar therapeutic index in xenograft models of ThioMAb drug conjugates of Anti-MUC16 (antibody against an ovarian cancer antigen)[48], Anti-Her2 (commonly called Trastuzuman, FDA approved antibody for the breast cancer therapy)[51] and Anti-STEAP1 (antibody against prostate cancer antigen)[52]. *In vivo* studies in rats and also in cynomolgus monkeys with anti-MUC16 antibody showed an increase in serum half-life of TDC compared to ADC.[48] The clearance rate of anti-STEAP1 ADC was also found to be 2.7 times faster than anti-STEAP1 TDC when the ELISA method was used to detect anti-STEAP1 antibody loaded with drug (MMAE); however, the total anti-STEAP1 antibody found in the serum was similar in both cases. The result might indicate that MMAE deconjugation is faster in the case of ADC compared to TDC.[52] However, it is difficult to draw robust conclusions from this due to the polydisperse nature of ADCs.

Conventional ADC methods use interchain disulfide reduction for conjugation, which destabilizes the antibody and can lead to aggregation. This phenomenon was observed with

aggregation of anti-CD70 antibody when modified with hydrophobic pyrrolobenzodiazepine using a maleimide-linker.[53] In order to rescue the ADC from aggregation, a cysteine-engineered anti-CD70 was used. Substitution of Ser-239 to cysteine on the heavy chain of anti-CD70 followed by drug attachment decreased the aggregation level to 1.6% from 13% observed for the non-engineered ADC with the same average DAR by size-exclusion chromatography. Further, the overall conjugation efficiency increased from 49% for partially reduced mAb to nearly 95% for engineered mAb, leading to the conclusion that cysteine-engineered mAb was a more viable modification route than reduction of interchain sulfhydryl.

A drawback of site-specific engineered mAbs is the requirement of extra steps in antibody cell culture generation and the complexity of the overall process. Further, ThioMAb antibodies are expressed in CHO (Chinese hamster ovary) cells and in cells, engineered cysteine residue was found to be conjugated to a glutathione or a cysteine.[50] Thus, the system still requires a reduction and oxidation process like the conventional ADC methods, which can potentially lead to interchain sulfhydryl cleavage (Figure 5).

Unnatural amino acids

In 2012, the first unnatural amino acid was incorporated into an antibody with the synthesis of an ADC encoded with an unnatural amino acid, *p*-acetylphenyalanine (pAcPhe).[54] In order to produce this antibody, a stable CHO line expressing an orthogonal *E.coli* $tRNA^{Tyr}$/aaRS (amino acyl tRNA sythetase) pair was generated. Ala-121 residue of the heavy chain of antibody is substituted to an amber codon for pAcPhe incorporation. The ketone group of pAcPhe was reacted with alkoxyamine derivatized drug compound and resulted in the formation of a stable, noncleavable oxime bond with 95% coupling efficiency. Blood clearance rate of ADC was the same as the recombinant antibody, but had the ability to suppress the tumor growth at 5 mg/kg dose.

Glycoengineering

One available strategy for site-specific conjugation is the utilization of the oligosaccharides present in the Fc region of the mAb. Antibodies typically have restricted glycosylation site distal from the antigen-binding site (represented in Figure 6A), providing an opportunity to specifically conjugate molecules onto this site. Two approaches have been used to modify the oligosaccharide structures on antibodies to achieve homogeneous ADC products that include chemical modification and chemoenzymatic strategies.

Chemical modification—In early approaches to modify oligosaccharides, radioactive tags were immobilized onto monoclonal antibodies through chemical modification using oxidized oligosaccharide moieties.[55] Briefly, the oligosaccharide on antibodies was first oxidized by sodium periodate and then reacted with amine-containing radioactive tags to give an imine linkage. Further reduction of imine using sodium cyanoborohydride affords uncleavable radioactive labeled antibody. For comparison a modified mAb was prepared using a conventional lysine conjugation method and showed reduced binding affinity due to species heterogeneity, however antibodies modified via oligosaccharides retained homogeneous antigen binding and high affinity. More efficient accumulation at the target

tumor of the antibody conjugates modified via oligosaccharide compared to modified via inspired further chemical modification through the glycosylation sites to prepare antibody conjugates.

An ADC of homogeneous hydrazide-linked IgG conjugates targeting tumor neovasculature has been efficiently prepared using oxidized fucose as a handle.[56] As shown in Figure 7, a fucose residue attached to the GlcNAc unit (Asn297) can be selectively oxidized by sodium periodate. LC-MS indicates that only a single aldehyde group that is readily available for drug conjugation has been achieved due to the over-oxidation of one of the two aldehyde groups to the carboxylic acid. Their different hydrazide-derivatives were successfully conjugated to the antibody via a labile hydrazone linker. Quantitative conjugation of hydrazine derivatives can be achieved after the optimization of reaction conditions. These IgG conjugates were found to retain comparable binding affinity to unmodified IgG. The conjugates exhibit a half-life of approximately 18 hours at physiological pH and temperature.

Hydrazines and alkoxyamines have been intensively utilized for aldehyde or ketone functionalized protein conjugation and thus have been of interest in modified mAbs for generation of ADCs.[57] Though these types of conjugation can be simply operated with bioorthogonality and good yield, the resulting C=N bonds even in oximes, which are identified as hydrolytically stable, can undergo hydrolysis via an acid-catalyzed mechanism under dilute conditions. To avoid the susceptibility of C=N bonds, conjugation chemistries targeting on formation of stable C-C bonds with aldehyde and ketone containing protein have been developed. Among them, the Pictet-Spengler ligation is the most developed one used to prepare hydrolytically stable conjugates. The proposed mechanism of Pictet-Spengler reaction, a cyclization reaction by which 3-(2-aminoethyl)indole undergoes a Mannich-type ring formation with an aldehyde, is shown in Figure 8. The typical 3-(2-aminoethyl)indole based Pictet-Spengler reaction requires strong Brønsted acid as a catalyst greatly impairing its protein-compatibility. Though the reaction can occur in protein-compatible conditions the reaction is too slow to achieve a good yield.[58] Kinetic studies indicate that the formation of iminium ion intermediate is partially rate-limiting in the Pictet-Spengler reaction.[59] By focusing on enhancing the iminium ion formation, a new Pictet-Spengler ligation with accelerated reaction rate has been developed (Figure 9).[60] The increasing rate of iminium ion formation was contributed by replacing the aliphatic amine of tryptamine with aminooxy moiety. The rate of intermediate formation was further increased by moving the aminooxy group to the 2-position of indole and engaging the more nucleophilic 3-position in the reaction. The oxacarboline product, resulting from the intramolecular C-C bond formation of the iminium intermediate, is reasonably proposed to be hydrolytically stable. This method was successfully utilized for site-specific conjugation of monoclonal antibody, a-HER2 human IgG.[60]

One of the minor limitations of the Pictet-Spengler ligation based on aminoxy-functionalized indoles is the acidic operating conditions, where the optimal pH of the reaction is less than 5.0. In order to move the pH to a preferable neutral condition readily for bioconjugation, a similar methodology, Hydrazino-Pictet-Spengler Ligation (HPSL) was developed from the same group.[61] In this method, a new indole molecule in which the

aminoxy moiety was replaced by hydrazine was synthesized as shown in Figure 10. The molecule was found to react with benzyloxyacetaldehyde more than three times faster than its aminoxy counterpart in near neutral pH. The hydrazine replacement captures both the speed and bioorthogonality of hydrazine conjugation in neutral condition and stability of C-C bond.

Chemoenzymatic modification: A significant concern in the chemical oxidation of oligosaccharide moieties with sodium periodate to generate aldehydes is that methionine residues are also susceptible to oxidative modification, and oxidation of Met-252 and Met-428 residues affects the Fc receptor binding of antibody and shortens serum half-life.[62, 63] In order to avoid this fate, an alternative approach was taken by generating more reactive glycan groups on the antibody though enzymatic reactions.

Sialic acid is used as a selective target for oxidation reagents, since it is prone to oxidation even in low concentration of sodium periodate. However, only 1% of natural antibodies contains terminal sialic acid in their native structure.[64] Native glycan groups of anti-Her antibody are introduced to sialic acid units *in vitro* through enzymatic addition by using α-2,6-sialyltransferase.[63] Sialylated anti-Her antibody contained average 2.2 sialic acids per antibody (Figure 6C). Sialic acid addition is then followed by oxidation with sodium periodate, and methionine oxidation is monitored by LC-MS analysis. The results showed that by lowering the sodium periodate concentration to 1 mM major stability changes in the antibody can be avoided, and the Fc receptor binding ability was decreased by 10% compared to a 40% decrease observed above 4 mM. The conventional oxidation methods of glycan on antibody required ~10 mM sodium periodate, which might explain why ADCs generated in these conditions were not successful *in vivo*.

Another approach is the engineered incorporation of unnatural sugar units onto the glycans. A fucose group is incorporated into anti-CD70 and anti-CD30 antibodies by culturing the CHO cells in the presence of engineered fucose at 1 mM concentration.[65] The fucose with free thiol groups (6-thiofucose) was incorporated into antibody with 70% efficiency; however, purified antibody contained cysteines attached to the free thiol on the engineered fucose group. This required a partial reduction then re-oxidation step of the antibody before drug conjugation. Conjugation of the thiol to a drug compound by thio-maledimide chemistry yielded 1.3 drug molecules per antibody with the advantage of decreased heterogeneity (Figure 6B).

A unique approach for site-specific conjugation has been demonstrated using a transglutaminase enzyme derived from *Streptoverticillium mobaraensa*.[66-68] The bacterial transglutaminase (mTG) catalyzes the formation of an isopeptide bond between a primary amine and an acyl group of glutamine side chain. However, mTG does not modify glutamine residues of the native antibody. Two approaches are generated to use mTG based site-specific conjugation; (*i*) Engineering glutamine-tag on antibodies[66], and (*ii*) Deglycosylation of antibodies (Figure 6 D)[67, 68]. In the first approach, a glutamine-tag (LLQG) is incorporated into the surface exposed domains of antibody.[66] The conjugate yield was homogenous with a DAR ranging from 1.7-2. The latter, Gln-295 residue in the heavy chain is hindered by the glycan group on the Asp-297, so deglycosylation of mAb with N-

glycosidase F (PNGase F) exposes the Gln-295 residue for mTG catalyzes.[67, 68] However, this approach is not feasible for applications that require long serum half-life, since deglycosylated mAb will be depleted quickly in serum.

Linker Chemistry

The linker that conjugates the toxic payload to the antibody determines the mode of drug release and therefore can impact the selectivity of release following target cell uptake compared to release in serum, which impacts systemic toxicity and overall efficacy. A variety of stimuli sensitive linkers have been designed according to the unique microenvironment of various subcellular compartments, where the active form of the drug is expected to cleave from the mAb following receptor-mediated endocytosis of the ADC. Further, the chemical and structural nature of the linker that remains attached to the cytotoxic payload following cleavage can potentially alter potency and cellular processing.

pH Sensitive Linkers

Early approaches to generating stimuli-sensitive drug release for ADCs used acid cleavable hydrazone linkers that are ideally stable in serum conditions (pH 7.3-7.5), but should be cleaved at low pH, such as the acidic compartments of the late endosome (pH 5.0-6.5) and lysosome (pH 4.5-5.0) to generate drug release. Among the first clinically evaluated ADC candidates was a series of ADCs generated using murine KS1/4 antibody that were modified with (*i*) methotrexate linked via an amide bond (KS1/4-methotrexate)[69], (*ii*) desacetylvinblastine linked via an esterase sensitive hemisuccinate link (KSI/44-DAVLB), and (*iii*) desacetylvinblastine linked via a pH sensitive hydrazone bond (KSI/4-DAVLBHYD)[70, 71] (Figure 11). *In vitro*, target-selective potency was not well demonstrated, indicating linker cleavage could occur prior to cell internalization. For the hydrazone-linked vinblastine ADC, the *in vivo* antitumor activity was higher than free vinblastine, and for KS1/4-methotrexate 15% of the injected dose per gram of tumor was localized in the tumor. The ADCs were discontinued after Phase I evaluation due to immune response and lack of therapeutic benefit. For the majority of patients, the human anti-mouse antibody (HAMA) was observed in circulation, which could lead to faster clearance of the foreign murine antibody. Overall, any conclusions on the impact of the linker on ADC success would be difficult due to the elicited immune response of the murine antibody.

Similarly, chimeric mAb BR96 was modified with doxorubicin using a pH-sensitive cysteine thiol-reactive (6-maleimidocapropyl)hydrazone linker for the treatment of metastatic breast cancer, and following preclinical success, the BR96-Dox ADC was advanced to Phase II clinical trials (Figure 11).[8] High doses (more than 100 mg/kg) of the ADC modified with 8 doxorubicin molecules per BR96 were required to achieve an effect *in vivo*, which is attributed to the low potency of the payload.[9] The half-life of BR96 alone is days to weeks, where as the half-life of the ADC was about 43 hours. As discussed previously, a high DAR achieved with reduced interchain sulfhydryls can impact antibody stability and increase rate of clearance from circulation. Drug cleavage was also found to occur in serum conditions, suggesting the hydrazone bond was not ideal. However, ADC delivery platform did impact biodistribution as the toxicity of BR96-dox was different compared to free doxorubicin. This

ADC did not achieve significant therapeutic benefit, and an immune response was observed for about half of the patients.

The ADC *Mylotarg*, (gemtuzumab ozogamicin), developed by Pfizer, was prepared by the conjugation of calicheamicin antibiotic (EC_{50} of about 10^{-9} – 10^{-10} M) to the anti-CD33 antibody gemtuzumab for the treatment of CD33 expressing acute myeloid leukemia (AML).[39, 72] Calicheamicin was conjugated to gemtuzumab using a bifunctional linker 4-(4-acetylphenoxy)butanoic acid, where the acid moiety is reacted with an mAb lysine to form an amide bond, and the acetyl moiety is reacted with *N*-acetyl-γ-calicheamicin dimethyl hydrazide to form an acyl hydrazone linkage (Figure 12). The resultant ADC had considerable species heterogeneity, with about 50% of gemtuzumab non-modified, and the other 50% modified with 3-4 calicheamicin drug. The hydrolysis of the hydrazone bond over 24 hrs at 37 °C was 97% at pH 4.5 and 6% at pH 7.4. The rate of hydrazone hydrolysis was shown to be affected by substituents on the adjacent aromatic group.[71] The linker also contains a disulfide moiety that is sterically hindered with an adjacent gem-dimethyl group and expected to cleave following hydrazone hydrolysis to release the active drug. *In vivo* stability assessments in mice showed that half of the calicheamicin was released within 48 hours.[73] Significant liver toxicity was also observed, suggesting that calicheamicin was also being released from the ADC in plasma conditions. An earlier ADC design (Figure 12) included no hydrazone linkage and lacked potency, suggesting the disulfide alone did not cause sufficient drug release *in vitro*.[74] However, it's worth noting that for the same linker-calicheamicin ADC designs for a murine mAb CTM01 showed equal to higher activity *in vivo* and *in vitro* for the ADC not containing the pH sensitive hydrazone.[75]

Mylotarg was approved by the FDA in 2000, however required a post phase III study as the approval was accelerated based on a response rate of around 30%.[76] In this phase III study hepatotoxicity was observed in a significant amount of patients. While less common, adverse hypersensitivity reactions were observed that include reaction at the infusion site, pulmonary events, and anaphylaxis, which could be fatal. A follow up trial of the clinical benefits of *Mylotarg* were not demonstrated. However, additional phase III studies showed that while there were some cytogenetics risks, response in AML patients also occurred.[77, 78] In 2010, Pfizer voluntarily removed *Mylotarg* from the market.

Redox-Sensitive Linkers

Disulfide containing linkers have been extensively studied due to the increased concentration of glutathione (GSH) in the cytosol (~1-10 mM)[79] compared to the extracellular milieu (~5 μM)[80]. Concentrations of GSH are even higher in tumor cells, as their hypoxic state leads to enhanced activity of reductive enzymes. To reduce the extent of payload release that occurs at these low extracellular GSH concentrations, a series of disulfide linked maytansinoid ADCs were prepared for which the carbon adjacent to the disulfide were substituted to various extents with methyl groups.[81, 82] Thiol-modified maytansinoids were prepared for conjugation to the modified antibody. A NHS bearing heterobifunctional linker was reacted to lysine groups of the humanized C242 antibody leaving a 2-thiopyridine group for reaction with the thiol-modified maytansinoid, resulting in a series of disulfide containing GSH sensitive ADCs (Figure 13). A non-disulfide bearing ADC control was also prepared using

heterobifunctional SMCC linker, containing and NHS ester for lysine conjugation and a maleimido group for reaction with thiol-modified maytansinoid (Figure 13). The resultant ADCs contained 3-4 maytansinoid molecules per antibody. Incubation of conjugates with DTT showed the less-hindered disulfide ADCs released maytansinoids faster than more-hindered ADCs.[81] The *in vitro* toxicity was tested against huC242 targeting CanAG antigen positive COLO 205 cells, and all conjugates were potent, including the GSH non-cleavable linker. This suggests that for this ADC statistical proteolytic digestion of the antibody in the endosome and lysosome may be a sufficient mechanism of drug release.[24, 83]

The addition of huC242-maytansinoid conjugates with various linkers to antigen-positive COLO 205 cells elucidated the importance of the catabolite species generated from drug cleavage.[84] The linkers tested included cleavable disulfide (SPP and SPDB) and noncleavable thioether (SMCC), for which the latter generated a lysine bearing species as the sole catabolite indicating full antibody digestion, while cleavable linkers showed additional metabolites such as a more hydrophobic thioether maytansinoids. The hydrophobic uncharged catabolites were shown to efflux to bystander cells greater than hydrophilic charged catabolites such as lysine-maytansinoid of the non-cleavable linker. These results illustrate the importance of linker design on cellular processing and potential for efflux for bystander cell killing.

For these maytansinoid-humanized C242 antibody ADCs, *in vivo* pharmacokinetic profiles showed that circulation half-life was increased with greater disulfide steric hindrance, but the extent of disulfide hindrance did not affect the tolerability in mice as demonstrated with similar MTDs regardless of hindrance. However the noncleavable linker was best tolerated with MTDs 2-3 times higher than disulfide linkers.[85] *In vivo* efficacy was typically higher for GSH sensitive ADCs;[81, 86] surprisingly however, when these maytansinoids were linked by disulfide or thioether to anti-HER2 antibody trastuzumab they showed similar *in vivo* efficacy.[87]

Non-Stimuli Sensitive Linkers

The discovery that the thioether-linked noncleavable control ADC was highly potent lead to Genentech's development of Ado-trastuzumab emtansine, or *Kadcyla*, one of the currently FDA-approved ADCs. It targets HER2 using anti-HER2-antibody trastuzumab for the treatment of HER2-positive metastatic breast cancer, and licensed ImmunoGen's linker-payload technology with microtubule-disrupting maytansinoid DM1 (EC_{50} of about 10^{-11} to 10^{-12} M).[88] As previously established, the payload was conjugated to the trastuzumab antibody via a lysine residue, with a non-stimulus sensitive SMCC linker such that maytansine is released from proteolytic digestion of the mAb. About 3.5 cytotoxic molecules were conjugated per antibody. In a Phase III clinical trial the safety and efficacy of the ADC for HER2-positive, metatstatic or unresectable locally advanced breast cancer patients were tested. A total of 991 patients were randomly assigned a regime of *Kadcyla* or lapatinib with capecitabine. The safety profile of *Kadcyla* showed favorable results, compared to the lapatinib and capecitabine treatment.[89] The patients treated with lapatinib and capecitabine had a median progression-free survival of 6.4 months and median overall survival of 25.1 months, while patients treated with *Kadcyla* had a median progression-free

survival of 9.6 months and median overall survival of 30.9 months. In 2013 following these results ado-tratuzumab emtansine was approved by the FDA for the treatment of HER2+ positive metastatic breast cancer.[89, 90]

Enzyme-Sensitive Linkers—Specific enzyme labile linkers have also been developed for drug release for ADCs. These are achieved through incorporating a peptide linkage in the ADC, which is then cleaved following cell internalization in the lysosome by proteases such as cathepsin. Enzyme-labile linkers offer an advantage to chemically-labile linkers as the latter is often relatively unstable in serum. Peptide linkers are expectantly more robust as these intracellular proteases are typically inactive outside of cells.[91] The ADC Brentuximab vedotin, or *Adcentris*, developed by Seattle Genetics, and approved by the FDA in 2011, targets CD30 using anti-CD30 mAb cAC10 (chimeric IgG1) for the treatment of Hodgkin lymphoma and systemic anaplastic large cell lymphoma (ALCL) with a microtubule-disrupting agent monomethyl auristatin E (MMAE). The cytotoxic agent, MMAE (EC_{50} of about 10^{-9} to 10^{-11} M) was modified with a cathepsin B sensitive valine-citrulline (Val-Cit) dipeptide separated by self-immolative *p*-amino-benzyoxycarbonyl (PABC) linker.[21, 91] The linker was conjugated to the mAb following interchain cysteine sulfhydryl reduction using a maleimide moiety (Figure 14).[92] The Val-Cit-PABC-MMAE is stable in plasma, however following internalization cleavage of Val-Cit dipeptide leads to 1,6-elimination of *p*-aminobenzyl carbamate to yield MMAE. An average DAR of four MMAE molecules conjugated to the mAb was shown to have an desirable antitumor activity.[23] Brentuximab vedotin showed a half-life of 14 days in mice, while the non-modified mAb had a half-life of 16.7 days. In Phase I clinical trials, about 86% of patients showed tumor regression.[93, 94] In a Phase II single arm study with 102 treated Hodgkin's lymphoma patients, the observed response rates were 75%, with complete response of 34%, leading to FDA's accelerated approval for treatment of HL and ALCL.[94, 95]

Traceless Linkers

An alternative linker strategy that has been reported is "traceless" linkers, in which the drug is conjugated to the antibody directly such that the release regenerates the same products prior to conjugation. These links are called traceless as it regenerates the full antibody and free drug following exposure to appropriate stimuli. One traceless linker strategy involves a derivative of highly potent cemadotin (Cem), that when modified with a thiol ($CemCH_2$-SH) was found similarly potent in the low nanomolar range (IC50 18-83.9 nM for $CemCH_2$-SH vs 0.5-14.8 nM for Cem depending on cell line).[96] The cemadotin derivative was site-specifically conjugated to reduced cysteine sulfhydryls at the C-terminal position of each εCH4 domain of the small immune protein (SIP) format human antibody F8 to give a DAR of 2 (Figure 15). The C-terminal interchain disulfide was mildly reduced using tris(2-carboxyethyl)phosphine hydrochloride, then activated with Ellman's reagent before reaction with $CemCH_2$-SH. Upon exposure to GSH, the disulfide of the linkerless SIP(F8)-SS-CH_2Cem conjugate is reduced to afford free $CemCH_2$-SH and intact antibody. F8 is a vascular–targeting antibody specific to a tumor angiogenesis marker that is the extra-domain A (EDA) of fibronectin. This approach is thus a non-internalizing ADC, but relies on accumulation and release of the drug in the surrounding tumor tissue to cause cell death. As such the linker process is expected to amplify with accumulation, increased tumor cell death

releases higher concentration of reducing agents into the extracellular matrix. *In vivo* analysis of SIP(F8)-SS-CH_2Cem in mice bearing immunocompetent murine F9 teratocarcinoma tumors showed prolonged survival and superior retardation of tumor growth compared to free CemCH2-SH and non-specific ADC SIP(KSF)-SS-CH_2Cem. This suggests the SIP(F8)-SS-CH_2Cem ADC was able to bind to the EDA of fibronectin and release CemCH2-SH into the extracellular milieu to induce cell death of the F9 tumor cells.

From the same group, a similar traceless linker ADC strategy was generated from the site-specific incorporation of either a cysteine (1,2-aminothiol moiety) on the N-terminus or a 1,2-aminothiol on the C-terminus of genetic recombinant antibodies for reaction with an aldehyde-containing cemadotin drug Cem-CHO (Figure 15).[97] The resultant thiazolidine linker is hydrolyzed to release the free drug. The versatility of traceless designs has some limitations including the fact that the drug requires an inherent functionalizable handle or retained potency with chemical modification. The stimuli-sensitive nature of the conjugate is highly dependent on that functional group, such that if the linker shows non-ideal function, other drug derivatives may need testing. While genetic recombinant methods have concerns with scalability, benefits of this method include simplification in other manufacturing steps including drug production and ADC formulation. Further, because the species following release are the original drug and antibody, advantages include potentially lower immunogenicity.

Outlook using Nanoparticle Conjugates

While ADCs have a lot of promise, the field would greatly benefit from a variety of advances in increasing drug load, improving drug conjugation methods, linker sensitivity, and drug release. One concern with mAb based ADCs is that their long circulation half-lives due to FcRn (neonatal Fc receptor) mediated recycling can lead to a greater probability of off-target binding.[98] Additionally, a potential problem with IgGs is that their large size can restrict tumor penetration.[99] Smaller antibody fragments including mAb domains, single-chain variable domain fragments (scFvs, or diabodies), and minibodies can potentially improve tumor diffusion and limit extravasation from vasculature, however have short circulation half-lives and thus tumor exposure is insufficient. Another motivation for employing fragments over full IgGs is that bivalent high affinity binding may contribute to limited penetration of mAbs in tumors, which may be avoided with monovalent binding Fabs.[100] It is also possible that the antibody crosslinks antigens, resulting in signaling pathway activation. However, at present the long circulation half-life of a mAb compared to fragments compensates for the limited tumor penetration in terms of overall success.[12]

The conjugation of antibodies and antibody fragments with nanoparticles[101] may offer a variety of advantages to traditional ADCs. First, the payload could be non-covalently encapsulated in the hydrophobic interior of the particle rather than covalently attached, thereby eliminating a need for sophisticated drug linker and cleavage strategies that can impact the structure of the drug catabolite. The non-covalent encapsulation in a particle can also increase the DAR and provides more facile variation in therapeutic of use. Second, the circulation time and extent of tumor accumulation could be modified according to the size and surface characteristics of the nanoparticle, which may offer advantages in the case of

antibody fragments. Third, due to increased drug load, the required quantity of mAbs may be reduced, drastically affecting the cost of these therapies. The extent of antibody-nanoparticle conjugates currently reported for drug delivery applications are somewhat limited, however some synthetic methods towards generating these species have led to some useful developments.

Due to their high specificity, antibodies are one of the most used biomolecules to provide specificity and bioactivity to various nanoparticles including inorganic nanoparticles, dendrimers, micelles and nanogels. In the following paragraphs, we describe three different concepts for linking Abs to nanoparticles, which are based on: *i*) direct covalent linkage between the surface of the nanoparticles and non-modified antibodies *via* amine or acid side chains, *ii*) chemical modification of the antibodies with a bifunctional linker, which are then utilized to react with functional groups on the surface of the nanoparticles, or *iii*) using adapter molecules (Figure 16).

Antibody immobilization using their reactive amino acids is the most straightforward method. Among them, the lysine amine groups of mAbs are the most used functional groups to covalently immobilize them on the surface of nanoparticles. Their widespread popularity is due to the following factors: *i*) they are residues abundant in most proteins, *ii*) they are usually located on the surface of the mAbs, and *iii*) they are very reactive with a wide variety of functional groups on the surfaces of nanoparticles. For example, immunoglobulin G (IgG) was successfully conjugated to highly fluorescent semiconducting polymer dots (Pdots), which had carboxylate on their surfaces, using the standard carbodiimide coupling chemistry.[102] These Pdots can effectively and specifically label cell surface receptors and subcellular structures in both live and fixed cells, without any detectable nanospecific binding. Similarly, the Anti-HER2 mAb was also conjugated to the 30 nm diameter iron oxide magnetic nanoparticles using a covalent link with the amine groups of the antibody and the carboxyl groups of nanoparticles.[103] These magnetic nanoparticles were successfully used to capture cancer cells with 1:10,000,000 enrichment of cancer cells over normal cells. Recently, antibodies have also been conjugated to polymeric nanoparticles containing aldehyde groups on their surfaces via the formation of Schiff bases with mAb surface amines. After the addition of $NaCNBH_3$, monoclonal antibody (mAb CC49) was stably attached on the surface of nanogels via reductive amination.[104] Surface plasmon resonance (SPR) analysis clearly showed that these CC49-nanogels could effectively bind to its antigen bovine submaxillary mucin (BSM). One more interesting example shows that the reactive epoxide groups on the surface of magnetic polymer microspheres can be also used for direct conjugation by ring opening reactions by the amine groups on the antibody.[105] Flow cytometry results showed that these modified microspheres became specific to CD4 molecules expressed on CD4+ lymphocytes. Thus, these microspheres are promising to be used in various biomedical applications including diagnosis and monitoring of human diseases.

The aspartic and glutamic acid residues of mAbs have also been used for the direct conjugation with nanoparticles. Anti-[human epidermal growth factor receptor 2] monoclonal antibody herceptin was attached to the amine-functionalized silica/titania hollow nanoparticles (HNPs) by conventional *N*-ethyl-*N*′-(3-dimethylaminopropyl)

carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) activation.[106] Compared to unmodified HNP, HER-HNPs could be efficiently internalized by SK-BR-3 human breast cancer cells due to herceptin conjugation. HER-HNPs containing the drug camptothecin showed decreased cell viability than both free CPT and camptothectin-loaded HNP.

However, these afore-described conjugation strategies with direct covalent linkage between the mAb and nanoparticles surface is not necessarily the most appropriate coupling methodology as it results in randomly oriented mAbs on the surface, since the distribution of amine groups within the three-dimensional structure of an antibody is nearly uniform throughout the surface topology, and the stoichiometry of conjugation sites between mAb and nanoparticle is difficult to control. Often the steric hindrance of the nanoparticle can disrupt the mAb-antigen interaction and decrease antigen-binding capacity. In effort to avoid this, modification of antibodies on a limited number of sites is used so that fewer conjugations are made between the particle and mAb.

The reaction between thiols on mAbs and thiol-reactive moieties on the surface of nanoparticles is able to retain high activity of mAbs after conjugation due to the usually low or nonexistent level of free cysteine residues on the mAb surface compared to lysine residues. To achieve this, a reactive thiol must first be introduced in small quantities on the mAb surface. The imidoester group of 2-iminothiolane, Traut's reagent, is commonly reacted with surface lysines to form a stable charge linkage with mAb and free thiol for further coupling.[107] Using this method, TRC105 antibodies were successfully attached to both mesoporous silica nanoparticles and nanographenes whose surfaces were extensively modified with maleimide groups.[108, 109] TRC105-mesoporous silica nanoparticles and TRC-C105-nanographene conjugates showed specific binding with CD105 (endoglin). While this method doesn't control the location of conjugation, it potentially better controls the stoichiometry of conjugation compared to direct amine lysine conjugation methods, and avoids structural instability from interchain sulfhydryl reduction. However, another disadvantage of using Traut's reagent is that the generated free thiol group can be easily oxidized prior to conjugation.

A variety of bifunctional linkers have been used recently to modify antibodies with reactive groups for conjugation to particles. For example, the maleimide-activated antibody was conjugated with thiol groups on oleyl cysteineamide (OCA) pre-functionalized poly(lactic-co-glycolic acid) (PLGA), nanoparticle.[110] Herein, succinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate] (LC-SMCC Figure 17), was first conjugated to amine groups of anti-EGFR mAb centuximab using the NHS ester functionality of LC-SMCC generating an amide linkage and introducing a maleimide group. The maleimide was then reacted with thiol groups on the surface of OCA-modified PLGA nanoparticles. Alternatively, Trastuzumab has been modified with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, Figure 17) and conjugated to polymer micelles containing furan groups on the surfaces, as maleimide can react with furan via Diels-Alder reaction.[111] Another interesting example is that antibiotin IgG was modified with succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic, Figure 17).[112] The HyNic-modified IgG was able to attach covalently to 4-formylbenzoate (4FB) functionalized polymersomes to form a bis-aryl hydrazone bond. More recently, the copper-free reaction

using a strain-promoted alkyne-azide cycloaddition reaction is becoming popular as it overcomes the potential cytotoxicity due to the copper ions.[113] For that, *N*-succinimidyl polyethylene glycol dibenzocyclooctyne (NHS-PEG4-DBCO, Figure 17) was designed, in which the NHS ester end of NHS-PEG4-DBCO is able to react with amine groups from mAbs to form an amide linkage, while the DBCO group at the other end can react with azide groups functionalized on the surface of quantum dot nanoparticles.[114] Similarly, tetrazine-norbornene cycloaddition has been demonstrated using norbornene-modified quantum dots reacted with tetrazine-modified EGF protein, and this reaction could also be for a strategy for antibody conjugation to nanoparticles.[115]

Most chemical mAb conjugation techniques through either direct linkage or modification with bifunctional linker exhibit low efficiencies and random surface orientation. To overcome this, adapter biomolecules can be used for oriented immobilization. The streptavidin-biotin approach has been demonstrated for antibody attachment to nanoparticles, where mAb was modified with streptavidin then conjugated to biotinylated polymeric nanoparticle.[116, 117] While mAb site-specific engineering would be required to for streptavidin conjugation to be oriented, this approach can restrict the number of mAb attachments to the particle. One oriented approach is the use of proteins that specifically bind to the Fc region of antibodies. IgG binding domains, such as Protein A, Protein G and Protein Z, which can be used for site-specific conjugation of antibodies with nanoparticles. Considering that this binding interaction is non-covalent, photoreactive crosslinkers can be introduced into these domains such that site-specific crosslinking can be made between these proteins and Fc region of the IgGs.[118] A third approach uses the specific DNA hybridization for the antibody conjugation.[119] Antibodies were covalently conjugated with a DNA sequence, then hybridized to the complementary antisense sequences attached to the surface of nanoparticles.

CONCLUSIONS

A promising targeted approach to cancer therapy is antibody drug conjugates to increase specific delivery of potent cytotoxins to tumor cells to avoid dose-limiting toxicity of healthy cells that occurs in traditional chemotherapy. Optimization of various parameters including target identification, stable and controlled drug conjugation, payload release, and drug potency has lead to a few clinical successes and even greater interest. The complex combination of these parameters for a specific antibody-drug combination to be delivered to a specific tissue drives the pursuit of an optimal ADC platform. However, a general conjugation method and linker design may not be appropriate for all disease types. Nonetheless, innovation in conjugation methods and development of new linkers will lead to further improvements in ADCs for cancer therapeutics. Of the structural design requirements, the linker design and site-selective conjugation to antibody seem to be the two critical factors. Antibody-based nanoscale delivery systems have the potential to simplify many of these complexities, as these have the potential to circumvent the stringent requirements for the linker chemistry and provide the opportunity to vastly increase the drug-antibody ratio. Overall, the future of antibody-drug combinations, in the form of ADCs or novel nanoparticle-mediated conjugates, seems bright where the capacity of mAbs to act

as targeting missiles is effectively harnessed in diseases where tissue-specific drug delivery is critical.

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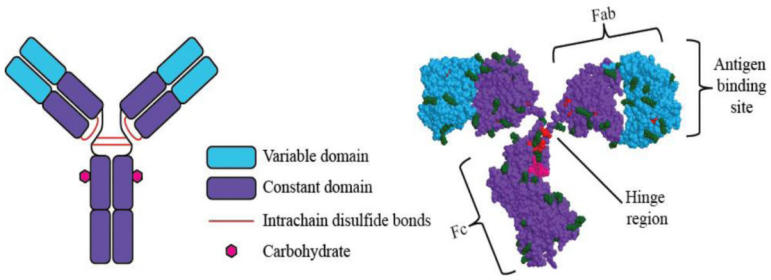


Figure 1.

Schematic representation of antibody (on left) and structuestructure of mouse IgG2 antibody (on right). Green, red and pink color on IgG2 structure represents lysine, cysteine and carbohydrate chains, respectively. PDB ID:1IGT

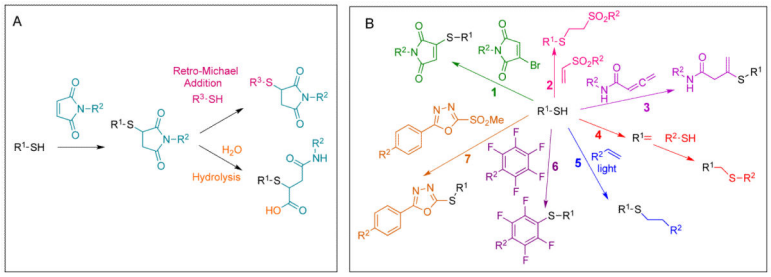


Figure 2.

(A) Retro-Michael addition and hydrolysis degradation of maleimide and (B) cysteine conjugation methods.

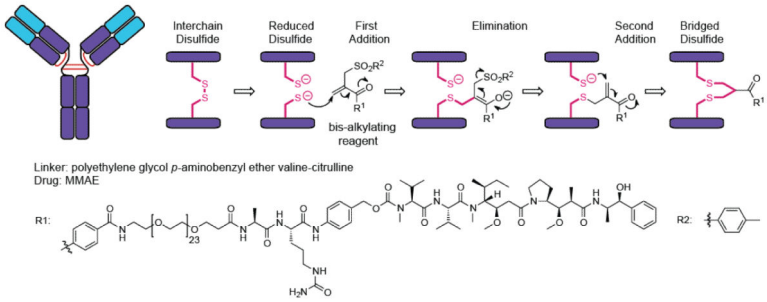


Figure 3.

Bis-alkylating reagent forms bridged conjugation to interchain disulfide to impart structural stability with drug conjugation

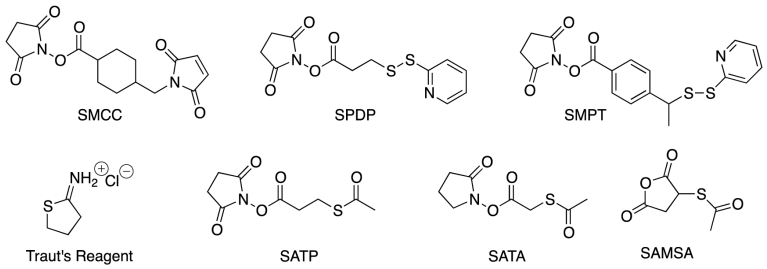


Figure 4.

Common reagents for mAb lysine conjugation and mAb thiolation

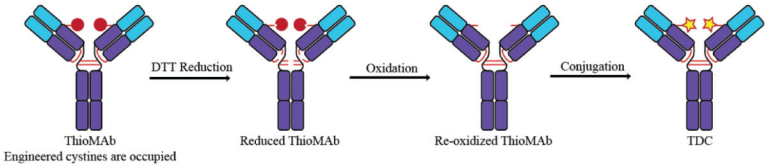


Figure 5.
Drug conjugation steps for ThioMabThioMAb.

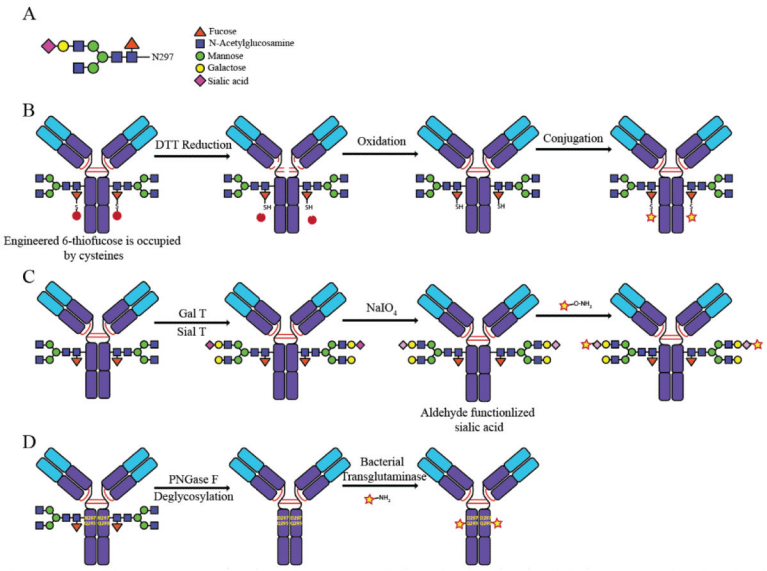


Figure 6.

A) Graphical representaton of N-glycans on IgG, B) metabolic engineering of mAb carbohydrate, C) transglutaminase-based chemo-enzymatic conjugation, and D) sialic acid incorporation and functionalization in glycan.

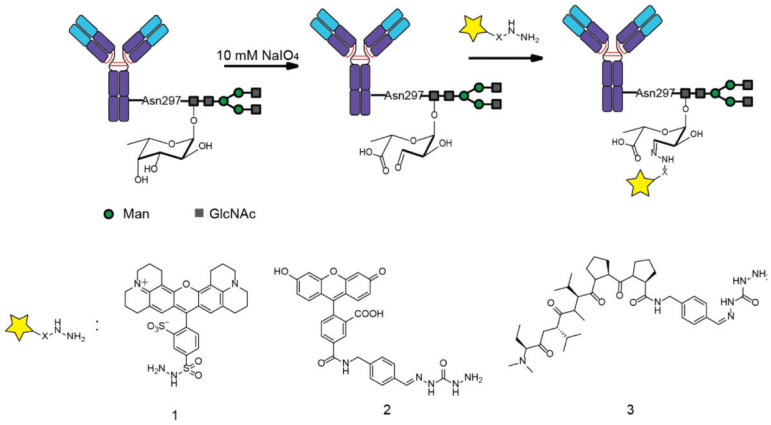


Figure 7.

Fucose-Specific conjugation of hydrazine derivatives to antibody.

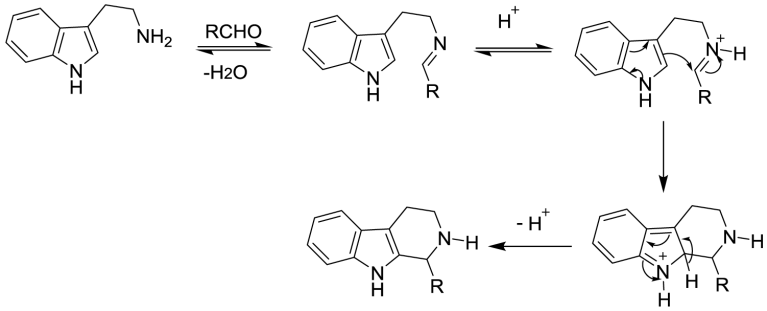
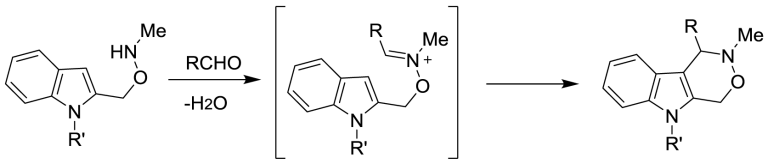


Figure 8.

Mechanism of Pictet-Spengler reaction



R: Protein; R': small molecule

Figure 9.

Oxyamine-based Pictet-Spengler ligation between protein aldehyde and small molecule

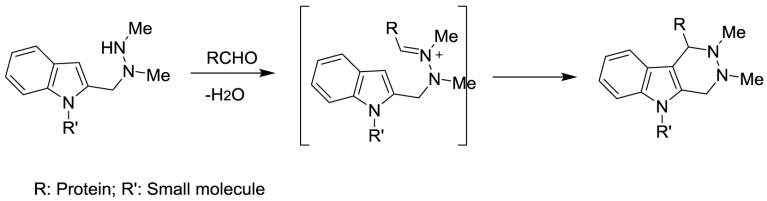


Figure 10.

Hydrazino-Pictet-Spengler ligation between small molecule and protein aldehyde

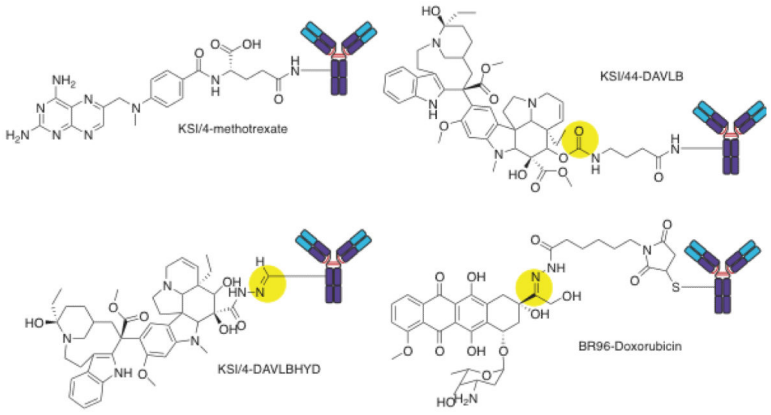


Figure 11.

Early generation ADCs with murine antibody (KS1/4) and chimeric antibody (BR96).

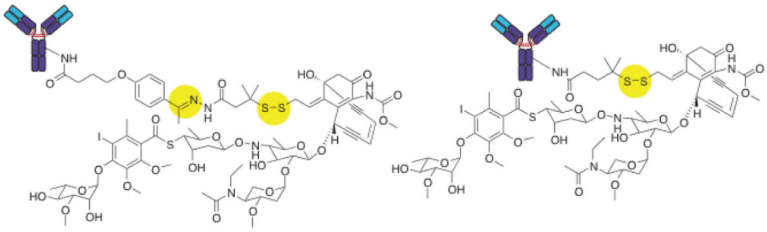


Figure 12.

Structure of anti-CD33 calicheamicin conjugates: hydrazone containing *Mylotarg* (left) and non-hydrazone containing (right).

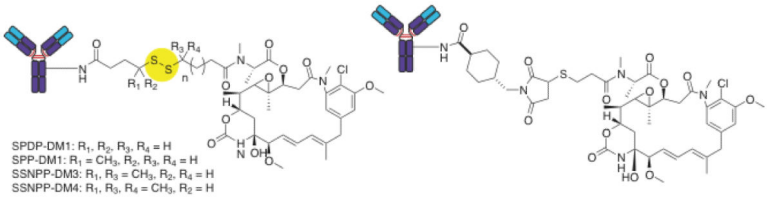


Figure 13.

mAb-maytansinoid conjugates: GSH cleavable (left) and non-cleavable (right).

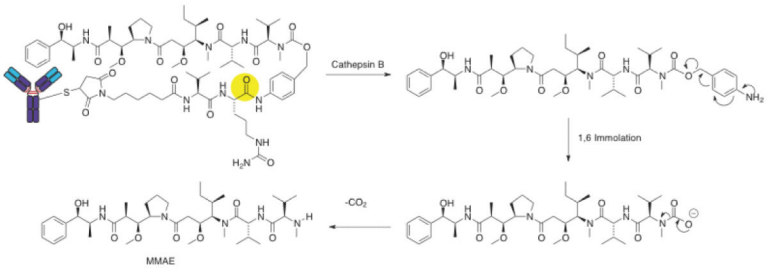


Figure 14.
Structure of *Adcentris* and release of MMAE.

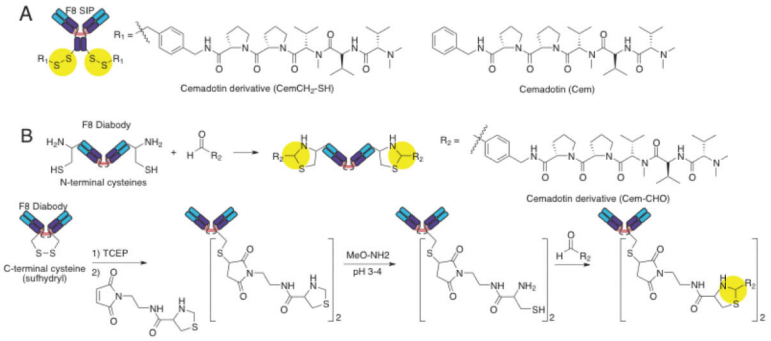


Figure 15.

A) Structure of linkerless SIP(F8)-SS-CH_2Cem and B) Preparation scheme to N-terminal and C-terminal thiazolidine.

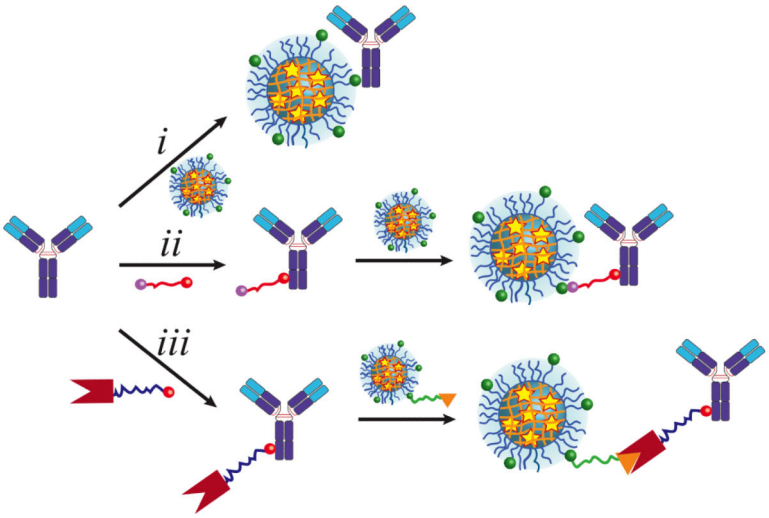


Figure 16.
Scheme presents three different concepts for linking mAbs to nanoparticles

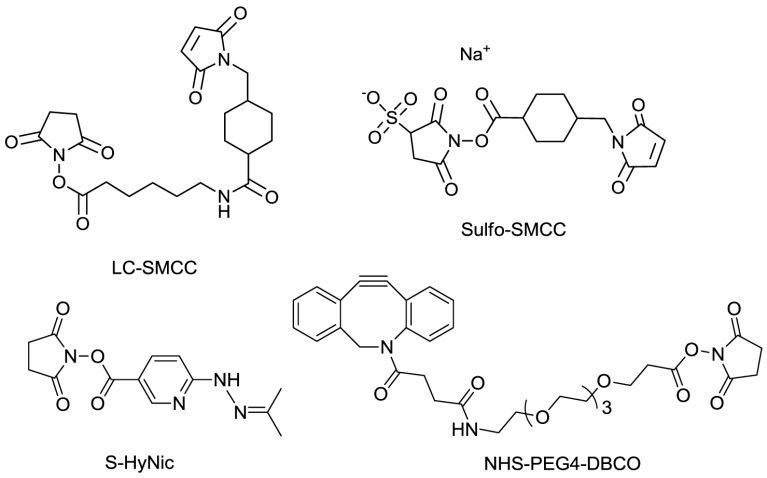
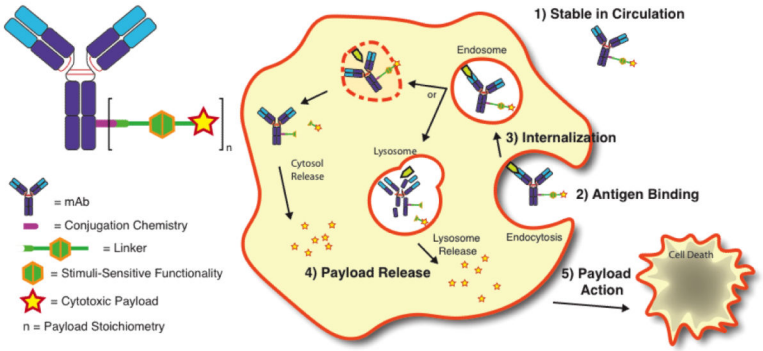


Figure 17.
Bifunctional reagents for introduction of reactive groups onto antibodies.



Scheme 1.

Illustration of the ADC structural components and its delivery pathway requirements

Table 1

FDA approved, withdrawn, and Phase III clinical trial ADCs (www.clinicaltrials.gov).

ADC	Pipeline	Drug	Indication	Antigen	Conjugation method	Linker	Drug release
Ado-Trastuzumab emtansine (*Kadcyla*)	Approved	Maytansinoid DM1	HER2+ MBC	HER2	Lysine	Non-cleavable	Proteolytic Degradation
Brentuximab vedotin (*Adcentris*)	Approved	Auristatin Derivative MMAE	HL, ALCL	CD30	Cysteine	Valine-citulline	Cathepsin B
Gemtuzumab ozogamicin	Withdrawn	Calicheamicin	AML	CD33	Lysine	Hydrazone	Low pH
Inotuzumab ozogamicin	Phase III	Calicheamicin	ALL, NHL	CD22	Lysine	Hydrazone	Low pH
Brentuximab vedotin (*Adcentris*)	Phase III	Auristatin Derivative MMAE	T-cell lymphoma	CD30	Cysteine	Valine-citulline	Cathepsin B
Ado-Trastuzumab emtansine (*Kadcyla*)	Phase III	Maytansinoid DM1	Gastric cancer	HER2	Lysine	Non-cleavable	Proteolytic Degradation

Abbreviations: Metastatic Breast (MBC), Hodgkin's Lymphoma (HL), Anaplastic Large Cell Lymphoma (ALCL), Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin's Lymphoma (NHL), Acute Myelogenous Leukemia (AML).