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Research review paper

Current methods for the synthesis of homogeneous antibody–drug conjugates

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ABSTRACT

Development of efficient and safe cancer therapy is one of the major challenges of the modern medicine. Over the last few years antibody–drug conjugates (ADCs) have become a powerful tool in cancer treatment with two of them, Adcetris® (brentuximab vedotin) and Kadcyla® (ado-trastuzumab emtansine), having recently been approved by the Food and Drug Administration (FDA). Essentially, an ADC is a bioconjugate that comprises a monoclonal antibody that specifically binds tumor surface antigen and a highly potent drug, which is attached to the antibody *via* either cleavable or stable linker. This approach ensures specificity and efficacy in fighting cancer cells, while healthy tissues remain largely unaffected.

Conventional ADCs, that employ cysteine or lysine residues as conjugation sites, are highly heterogeneous. This means that the species contain various populations of the ADCs with different drug-to-antibody ratios (DARs) and different drug load distributions. DAR and drug-load distribution are essential parameters of ADCs as they determine their stability and efficacy. Therefore, various drug-loaded forms of ADCs (usually from zero to eight conjugated molecules per antibody) may have distinct pharmacokinetics (PK) *in vivo* and may differ in clinical performance. Recently, a significant progress has been made in the field of site-specific conjugation which resulted in a number of strategies for synthesis of the homogeneous ADCs. This review describes newly-developed methods that ensure homogeneity of the ADCs including use of engineered reactive cysteine residues (THIOMAB), unnatural amino acids, aldehyde tags, enzymatic transglutaminase- and glycotransferase-based approaches and novel chemical methods. Furthermore, we briefly discuss the limitation of these methods emphasizing the need for further improvement in the ADC design and development.

Contents

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1. Introduction

The idea behind targeted anticancer therapies originates from the 'magic bullet concept' which was introduced at the beginning of the 20th century by Paul Ehrlich, the father of modern immunology and chemotherapy. Ehrlich proposed that in order to reduce adverse effects of toxic molecules on healthy tissues drugs should be selectively delivered to disease-causing cells (Strebhardt and Ullrich, 2008). Realization of Ehrlich's vision became possible when production of monoclonal antibodies, that provide excellent specificity and high affinity of binding to antigens, was developed in the mid-70s (Kohler and Milstein, 1975). Monoclonal antibodies against tumor specific antigens can be labeled either with a particle emitting radioisotope (radioimmunotherapy, RIT) or with a highly potent drug resulting in antibody–drug conjugates (ADCs). Both strategies allow one to specifically destroy cancer cells. Nowadays, two radio-immunoconjugates, ^{131}I-tositumab (Bexxar®, GlaxoSmithKline) and ^{90}Y-ibritumomab tiuxetan (Zevalin®, Bayer Schering Pharma AG/Spectrum Pharmaceuticals) are approved for treatment of non-Hodgkin's lymphoma (Bodet-Milin, 2013; Chamarthy et al., 2011). Currently,^{177}Lu and ^{211}At radio-immunoconjugates targeting colon cancer are intensively investigated (Eriksson et al., 2012, 2014). Conjugation of cytotoxic payloads to monoclonal antibodies, that bind tumor cell surface antigens, enables to target and deliver drugs to cancer cells leaving normal cells largely unaffected. Importantly, this approach takes advantage of highly potent cytotoxic molecules that would be too toxic for use in conventional chemotherapy. Therefore, ADCs constitute a precise and powerful tool in fighting cancer. The research in the ADC field has been extremely intense in the past 10 years. This resulted in the approval of two ADC therapeutics, brentuximab vedotin (Adcetris®, Seattle Genetics) and ado-trastuzumab emtansine (Kadcyla®, Genentech) by the Food and Drug Administration (FDA) in 2011 and 2013, respectively. Furthermore, approximately 40 ADCs are currently undergoing clinical trials. Despite the tremendous progress in ADC technology, further improvement is necessary to ensure safety and efficacy of ADC-based products. One of the main challenges in ADC design is homogeneity of ADC molecules. Currently available ADCs are heterogeneous as they have zero to eight drug molecules per antibody. It has been reported that heterogeneity of ADC species can influence its pharmacokinetics (PK) and *in vivo* performance (Hamblett et al., 2004; Jackson et al., 2014; Junutula et al., 2008a; Strop et al., 2013). Therefore, biotechnology companies and academic units are intensely focused on establishing novel reliable methods for site-specific conjugation of cytotoxic agents to monoclonal antibodies (Table 1). The outcome of their effort has recently been summarized in a few excellent reviews. Agarwal and Bertozzi (2015) and Cal et al. (2014) in their articles discuss details of chemical aspects of site-specific conjugation methods. Behrens and Liu (2014) and Panowksi et al. (2014) give a general overview on well-defined ADC design and production. In our review we describe novel approaches towards homogeneous ADC, including those that are not discussed in above-mentioned reviews.

2. Conventional conjugation methods and their limitations

Essentially, an ADC contains three main components: a monoclonal antibody, a cytotoxic agent and a synthetic linker that is required to attach the drug to the antibody. Conventional conjugation methods employ surface-exposed lysine or interchain cysteine residues as attachment sites for linker-drug molecules. A human IgG comprises about 100 lysine residues. Mass spectrometry analysis of the huN901-DM1 antibody–drug conjugate revealed that potentially 40 of them can be modified with the DM1 cytotoxic drug (Wang et al., 2005). Lysine conjugation results in zero to eight drug molecules per antibody. This implicates that a tremendous number of over one million different ADC species can be generated using this unspecific approach (Wang et al., 2005).

Cysteine conjugation occurs after reduction of four interchain disulfide bonds, which leads to eight thiol groups that are available for linker-drug molecules. In this strategy, drugs are coupled to even number of cysteines (2, 4, 6 or 8) (Hamblett et al., 2004; Sun et al., 2005; Willner et al., 1993).

Table 1
Current site-specific conjugation methods.

Company/institution	Conjugation strategy	Antibody engineering	Chemistry (non-enzymatic reactions)	DAR
Genentech Seattle Genetics	Conventional lysine and cysteine conjugation Lewis Phillips et al. (2008) and Senter and Sievers (2012)	Not required	Thiol–melimide Primary amine-NHS-ester (coupling linker-drug to a native antibody)	3–4
Sutro Biopharma Ambrx	Incorporation of unnatural amino acids into antibodies Axup et al., 2012 and Zimmerman et al., 2014	Required	Click chemistry oxime ligation (coupling linker-drug to an incorporated unnatural amino acid)	2
National Cancer Institute	Incorporation of selenocysteine into antibodies Hofer et al. (2009)	Required	Selenol-maleimide Selenol-iodoacetamide (coupling linker-drug to an incorporated selenocysteine)	2
Rinat-Pfizer	*Streptoverticillium mobaraense* transglutaminase (mTG) Specifically recognizes and modifies genetically introduced glutamine tag (LLQGA) with a primary amine-containing linker-drug module Strop et al. (2013)	Required	–	1.8–2
Sanofi-Genzyme	Glycoengineering Site-specific introduction of sialic acid with the use of galactosyl- and sialytransferases Zhou et al. (2014a)	Not required	Oxime ligation (coupling linker-drug to a modified Fc glycans)	~1.6
Innate Pharma	Microbial transglutaminase (MTGase) Enzymatic conjugation of a primary amine-containing linker/linker-drug module to glutamine specifically recognized by MTGase Dennler et al. (2014)	Required	Thiol–maleimide Click chemistry (coupling drug to linker-antibody)	2
Redwood Bioscience	Formylglycine generating enzyme (FGE) Converts cysteine located in the CXPXR consensus sequence to formylglycine (FGly) Drake et al. (2014)	Required	Hydrazino-*iso*-Pictet-Spengler ligation (coupling linker-drug to FGly)	2
UCL Cancer Institute	Next generation maleimides (NGMs) Rebridge reduced interchain disulfide bonds of a native antibody Schumacher et al. (2014)	Not required	Reaction between thiols and leaving groups of the NGM linker-drug (coupling linker-drug to a native antibody)	1 2 3 4
PolyTherics	Bis-alkylating reagents Rebridge reduced interchain disulfide bonds of a native antibody Badescu et al. (2014)	Not required	Micheal addition and elimination reactions (coupling linker-drug to a native antibody)	2 4

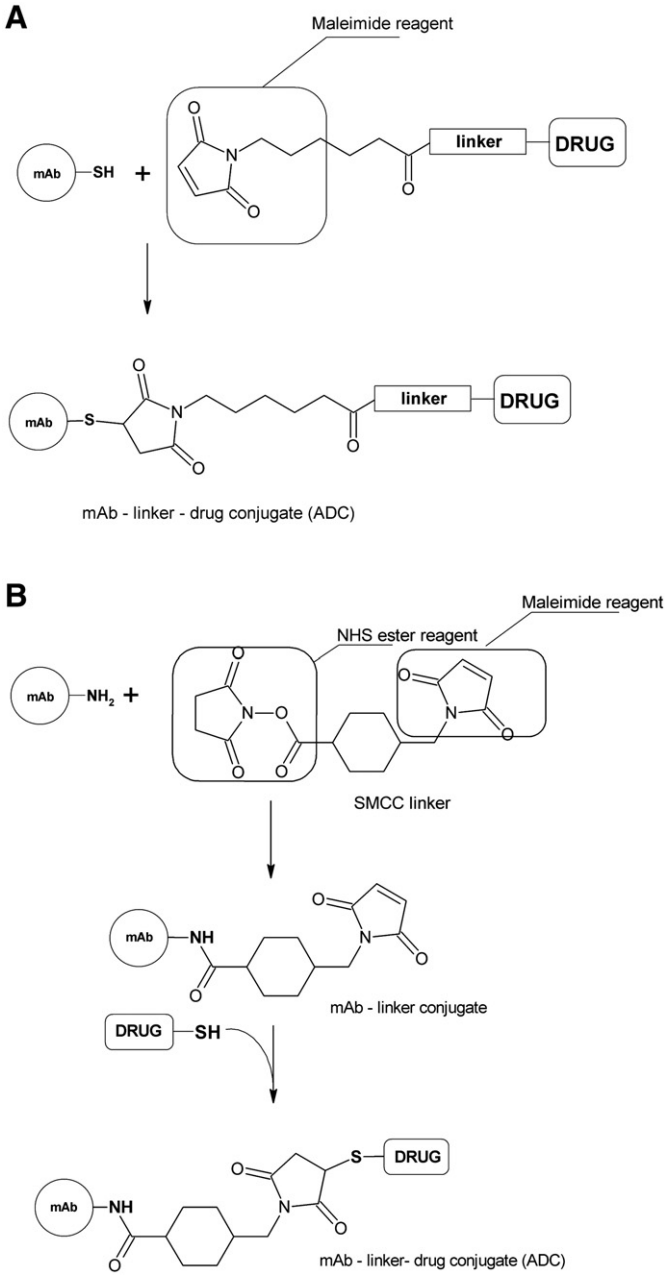

Fig. 1. Conventional conjugation methods. A. Cysteine conjugation relies on a chemical reaction between the cysteine thiol group and the maleimide group present in the linker. B. Lysine conjugation takes advantage of SMCC, which contains an amine-reactive N-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. The NHS-ester groups react with primary amines, including lysines to form stable amide bonds, which results in a linker-modified antibody. In the second reaction, the thiol group present in the drug module forms a nonreducible thioether bond with the maleimide group of the linker.

Since several isomers are observed at each drug substitution level, over a hundred differently drug-loaded species are present in ADC mixtures. Although both approaches generate heterogeneous ADCs, linking drugs through interchain cysteine residues generates significantly fewer ADC species than using lysines. Two FDA-approved ADC therapeutics, brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®), are produced by lysine and cysteine conjugations, respectively.

Brentuximab vedotin (Adcetris®) used for the treatment of Hodgkin lymphoma and systemic anaplastic large cell lymphoma (ALCL) was generated by linking a highly cytotoxic inhibitor of microtubule polymerization, monomethyl auristatin E (MMAE), to the anti-CD30 monoclonal antibody cAC10. These two components are coupled through a cathepsin-cleavable linker [*e.g.* valine–citrulline (vc) dipeptide linker] that undergoes proteolysis in lysosomes releasing MMAE molecules inside target cells (Doronina et al., 2003). The covalent bond between the linker-drug module and the antibody employs modification of disulfide bonds that link the antibody's heavy and light chains together (Senter and Sievers, 2012; Sun et al., 2005; van de Donk and Dhimolea, 2012) (Fig. 1A). Importantly, removing disulfide bonds from an antibody does not affect its functions (Andersen and Reilly, 2004). In addition, interchain disulfide bridges are more prone to reduction than intrachain disulfide bridges (Schroeder et al., 1981; Willner et al., 1993). This allows one to generate free thiol groups under mild reducing conditions leaving antibody intact. The thiol groups can be then used as conjugation sites for cytotoxic drug molecules. Adcetris® contains mainly 2, 4 and 6 molecules of vcMMAE per antibody and less than 10% (for each) of unconjugated antibodies and the ADCs with eight drugs (Senter and Sievers, 2012; Sun et al., 2005). Despite heterogeneity, brentuximab vedotin successfully passed pivotal phase 2 clinical trials with the objective response rate (ORR) of 75% (including 34% of complete responses) for Hodgkin lymphoma patients (Younes et al., 2012) and ORR of 86% (including 57% of complete responses) for patients with relapsed or refractory systemic ALCL (Pro et al., 2012).

Trastuzumab, better known under its trade name Herceptin® (Genentech), is an anti-HER2 monoclonal antibody that is used in HER2-positive metastatic breast cancer. It was approved by the FDA in 1998 having become the first monoclonal antibody used in the targeted cancer therapy. Because the combination of trastuzumab with chemotherapy regiment [*e.g.* with microtubule-targeting drugs (Lewis Phillips et al., 2008)] enhances its antitumor effects, a trastuzumab-based ADC was developed by Genentech. Ado-trastuzumab emtansine (Kadcyla®) is made up of trastuzumab and a highly potent derivative of maytansine DM1 linked together by the use of SMCC [Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate] (Barginear et al., 2012; Lewis Phillips et al., 2008; LoRusso et al., 2011; Peddi and Hurvitz, 2013) (Fig. 1B). In this case, the DAR ranges from 3.2 to 3.8 DM1 molecules per antibody with a smooth, uniform distribution of the cytotoxic payload (Kim et al., 2014; Lewis Phillips et al., 2008).

Gemtuzumab ozogamicin (Mylotarg®, Wyeth/Pfizer) was approved for the treatment of acute myeloid leukemia (AML) by the FDA in 2000 having become the first ADC in clinical use. However, due to the lack of clinical benefits and possible toxicity observed in the post-approval clinical trials, Mylotarg® was withdrawn from the US and European markets in 2010 (Ricart, 2011). Similarly to Kadcyla, Mylotarg® was generated through lysine conjugation. However, unlike Kadcyla®, Mylotarg® contained 50% of the antibody conjugated to four to six drug molecules with the remaining antibody having been unconjugated (Bross et al., 2001). This characteristic of Mylotarg®, along with insufficient stability of a hydrazone linker, might have contributed to its failure in the therapy of AML.

Apart from cysteines and lysines, other residues, including N-terminal serine and threonine, can be used for site-specific protein modification. These hydroxyl-containing amino acids can be converted to a highly reactive carbonyl group through periodate oxidation and then reacted with an aminooxy- or hydrazide-functionalized compounds resulting in oxime or hydrazone linkage, respectively (Gaertner and Offord, 1996; Geoghegan and Stroh, 1992; Zhou et al., 2014b). So far, this strategy was successfully applied for site-specific PEGylation of interleukin-8, G-CSF and interferon β-1b (Gaertner and Offord, 1996; Zhou et al., 2014b).

3. Why homogeneity of ADCs is important?

Conventional methods that are used for ADC synthesis result in a heterogeneous mixture of ADC species that differ in the drug-to-antibody ratio (DAR) and drug load distribution/location (Hamblett

et al., 2004; Wang et al., 2005). Typically, zero to eight drug molecules can be attached to the antibody. Consequently, heterogeneous ADCs may contain both unconjugated and overloaded antibodies. Unconjugated antibodies compete with drug-loaded species for antigen binding that can diminish the activity of ADC therapeutics. On the other hand, a high degree of the antibody modification may result in antibody aggregation, increased toxicity, decreased stability and shorter half-life of ADCs in the circulation. Experimental work has revealed that the optimal DAR for most ADCs is four drug molecules per antibody as this ratio represents a compromise between cytotoxicity and pharmacokinetic stability of ADCs (Hamblett et al., 2004; Senter and Sievers, 2012). The characterization of an ADC composed of a highly cytotoxic drug monomethyl auristatin E (MMAE) and the anti-CD30 monoclonal antibody cAC10 demonstrated that although *in vitro* tumor cell killing activity of this ADC increased with increasing drug load (IC_{50} values drug load 8 < drug load 4 < drug load 2), the *in vivo* antitumor activity of a species containing four MMAE molecules was comparable with a species containing eight MMAE molecules at equal antibody doses. In addition, the higher drug-loaded species exhibited faster renal clearance (Hamblett et al., 2004).

Recently, a significant role of a drug-conjugation site has been reported (Shen et al., 2012; Strop et al., 2013). A study carried out by

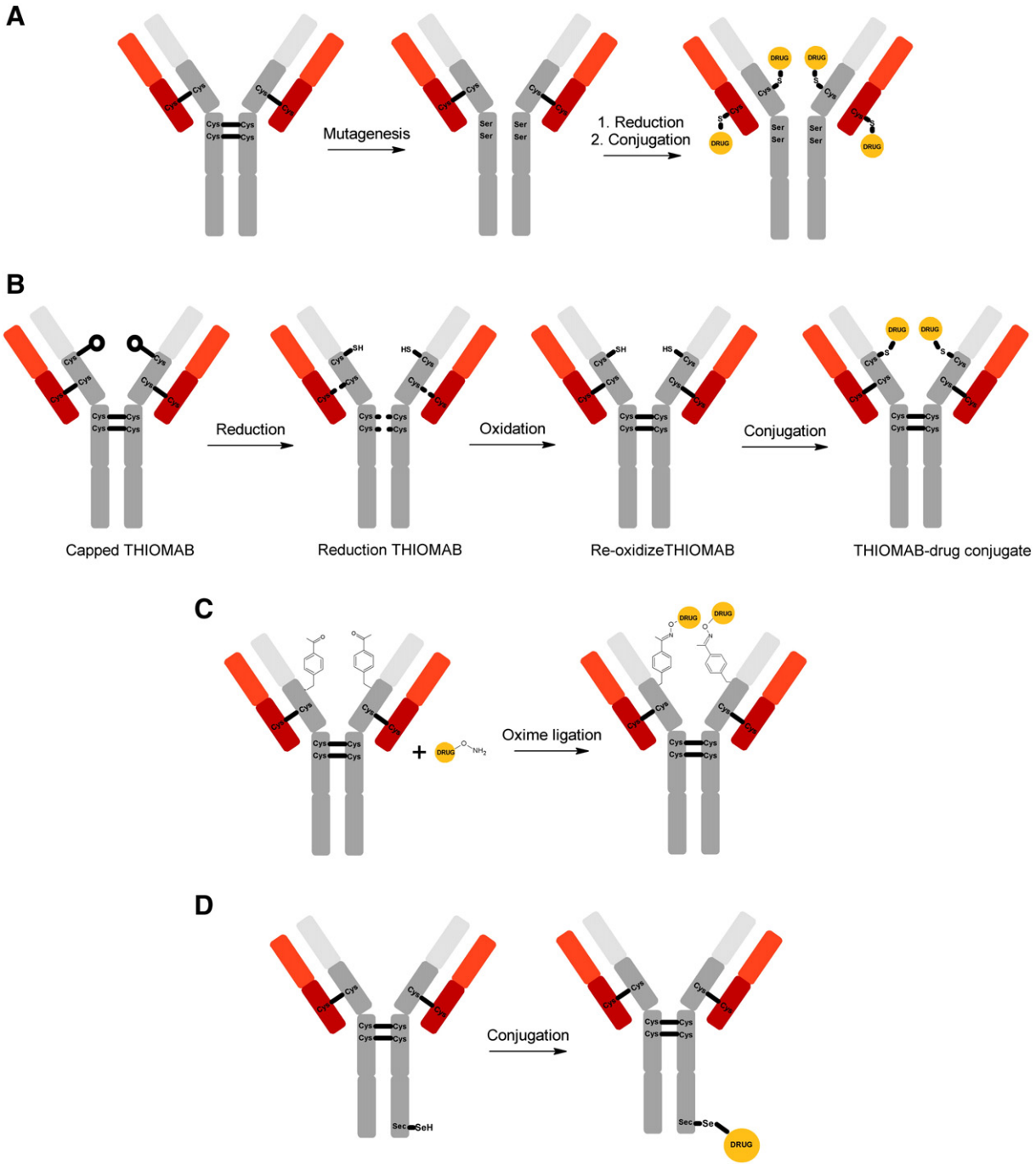

Fig. 2. Antibody engineering-based methods. A. Interchain cysteine to serine substitutions enable to conjugate cytotoxin to the remaining cysteines, which significantly reduces the heterogeneity of ADCs B. Conjugation of cytotoxic drugs to THIOMABs. Capped THIOMABs (glutathione or cysteine attached to the engineered cysteine) undergo reduction and partial oxidation prior to the conjugation reaction C and D. Incorporation of unnatural amino acid (*e.g.* p-acetylphenylalanine, pAcPhe) or selenocysteine (Sec) allows for site-specific conjugation of cytotoxic agent.

Shen et al. (2012), demonstrated that the physicochemical properties of the conjugation site, including solvent-accessibility and a net electric charge of the local environment, can have a functional impact on ADC stability and biological activity *in vivo*. A thiol-reactive maleimide linker was used to couple MMAE to the cysteines that were engineered at three different sites into the therapeutic anti-HER2 antibody (the THIOMAB technology described in Section 4.1.2). Plasma stability, pharmacokinetics and efficacy of the obtained conjugates were analyzed, revealing significant differences between the variants. The highest rate of drug release was observed for the variant having MMAE conjugated to a highly solvent accessible cysteine, whereas conjugation to a partially buried cysteine located in a positively charged region resulted in the most stable ADC variant (Shen et al., 2012).

These findings allowed one to propose the model assuming, that conjugation sites with high solvent accessibility promote a rapid loss of conjugated thiol-reactive linkers in plasma due to maleimide exchange with reactive thiols in albumin, free cysteine or glutathione (Alley et al., 2008). On the contrary, partially solvent accessible site with a positively charged environment supports linker stability by preventing this exchange reaction (Shen et al., 2012). Importantly, this model applies to the ADCs that contain melamine linkers, and other linkers may be differently sensitive to the physicochemical properties of conjugation site. The importance of conjugation site was also described by Strop et al. (2013) who compared two distinct conjugation sites on the anti-M1S1 antibody C16. The sites were located on the heavy and light chains of the antibody. The analysis of pharmacokinetic properties of these two ADC species showed that in the ADC species utilizing conjugation site on the light chain was more stable in rat serum than the ADC species utilizing conjugation site on the heavy chain (Strop et al., 2013). In this case, chemical stability of a transglutaminase linkage was preserved in rat and mouse sera, suggesting another conjugation site-dependent mechanism that contributes to drug loss.

Optimization of a drug-to-antibody ratio and drug load distribution/location emerges as an important consideration for ADC design. Ideally, the final ADC-based product should exclusively contain an optimally drug-loaded form of the conjugate. Overall, only homogeneous and reproducible ADCs can provide a therapeutic tool that has predictable properties and batch-to-batch consistency.

4. Site-specific conjugation methods

4.1. Antibody engineering-based methods

4.1.1. Reducing the number of interchain disulfide bonds

Cysteine conjugation can result in homogeneous ADCs when all interchain cysteines are coupled to drug molecules. A good example of such ADC is a conjugate of the anti-CD30 monoclonal antibody cAC10 and monomethyl auristatin E (MMAE) developed by Doronina et al. (2003) and Francisco et al. (2003). This cAC10-vcMMAE conjugate contained eight drug molecules per antibody, which represents the highest drug load that can be obtained by the use of interchain cysteines as conjugation sites (Doronina et al., 2003; Francisco et al., 2003). However, the optimal *in vivo* performance was observed with the four drug-loaded form (Hamblett et al., 2004). The first attempt to generate homogeneous ADCs with a fixed stoichiometry of two and four drugs per antibody at defined sites was described by McDonagh et al. (2006). Selected interchain cysteines derived from three or two bisulfide bonds were mutated to serines which resulted in the variants of the antibody cAC10 with either two or four remaining accessible cysteines (Fig. 2A). Following reduction, the variants with the reduced number of interchain cysteines were conjugated to vcMMAE. Received highly homogeneous species were compared to the heterogeneous ADCs, which were based on the native cAC10 antibody (McDonagh et al., 2006).

The study demonstrated that the engineered ADCs with defined sites and stoichiometries of drug attachment had similar antitumor activity, tolerability and pharmacokinetics as the ADCs with the same (average) DAR but heterogeneous drug attachment sites. Nevertheless, decreasing the number of drug molecules coupled to the antibody from four to two led to drop in efficacy and increase in tolerability, which was consistent with previous reports (Hamblett et al., 2004). Overall, these observations suggested that the stoichiometry of drug attachment is a more critical determinant of ADC properties than is the site of drug attachment and conjugate homogeneity (McDonagh et al., 2006). Noticeably, this hypothesis may be limited to conjugates generated with the use of interchain disulfide bonds of IgG_1 which are all located in the highly solvent accessible hinge region (Liu and May, 2012).

4.1.2. Engineered cysteine mutants

The THIOMAB strategy is based on reactive cysteine substitutions at carefully selected positions in the constant domains of the antibody Fab region which is not involved in antigen binding. This allows one to obtain conjugates with defined site and stoichiometry and preserves interchain disulfide bridges intact. The key success factor of this strategy is the identification of proper substitution sites in which introducing reactive cysteine residue does not interfere with antibody function and structure. For this purpose, the Phage Elisa for Selection of Reactive Thiols (PHESELECTOR) was developed (Junutula et al., 2008b). Reactive cysteine residues were introduced at various positions into a trastuzumab-Fab (hu4D5Fab) used as a model system. The variants were displayed on phage and screened for reactive cysteines that do not interfere with antigen binding. This screening tool allowed one to identify 10 residues that should be suitable for cysteine substitution and site-specific conjugation (Junutula et al., 2008a, 2008b).

The antibodies containing engineered reactive cysteine residues at identified positions were named THIOMABs. The THIOMAB variant of anti-MUC16 antibody, with heavy chain alanine 114 (HC-A114) substituted with cysteine, was used to generate the anti-MUC16 THIOMAB-drug conjugate (TDC) which was then compared with the conventional anti-MUC16 antibody–drug conjugate (ADC). Importantly, the anti-MUC16 THIOMAB exhibited equivalent antigen binding to the original anti-MUC16 antibody which further confirmed the results of the PHESELECTOR screen. The site-specific conjugation of vcMMEA to the anti-MUC16 THIOMAB was achieved by the reduction and partial re-oxidation of this THIOMAB which gave two free thiol groups at position 114 in the heavy chains followed by the reaction of these thiols with the maleimide group present in the linker-drug module (Fig. 2B). The conventional cysteine conjugation method was utilized to couple MMAE to the original anti-MUC16 antibody. *In vitro* and *in vivo* cytotoxicity assays showed that both conjugates, the anti-MUC16 TDC and ADC, had comparable antitumor activity, although the ADC had almost twofold higher drug load than the TCD (~3.5 drug molecules per anti-MUC16 antibody, ~2 drug molecules per anti-MUC16 THIOMAB). Safety studies of the anti-MUC16 TDC and ADC carried out in rats and cynomolgus monkeys showed that the adverse effects, including impaired functions of the liver and low white blood cell count, were far more pronounced in the case of anti-MUC16 ADC treatment (Junutula et al., 2008a). Moreover, renal clearance of anti-MUC16 TDC is slower than that of the anti-MUC16 ADC in a rat model. Similar results demonstrating that the TDC version of trastuzumab-DM1 conjugate was equally efficient at the same dose as its ADC counterpart and yet less toxic to animals suggest that the THIOMAB strategy provides homogeneous conjugates with improved therapeutic index in comparison to the conventional ADCs (Junutula et al., 2010).

4.1.3. Unnatural amino acids incorporation

The genetic code encodes 20 common amino acids, but it can be expanded to accommodate additional amino acids. This can be accomplished by the generation of an orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pair, that site-specifically incorporates a desired unnatural amino acid (*e.g.* p-acetylphenylalanine, pAcPhe) into nascent polypeptides in the response to an amber stop codon (UAG) placed in a gene of interest (Lemke, 2014; Wang, 2003; Wang et al., 2001).

Recently, the genetic incorporation of unnatural amino acids has become a promising tool in ADC design (Kim et al., 2013). This approach was successfully employed by Axup et al. (2012), who generated site-specific auristatin conjugates of trastuzumab (a monoclonal anti-HER2 antibody). The amber stop codon was introduced in the heavy chain of full-length IgG of trastuzumab at position 121 replacing alanine (HC-A121X). The antibody was co-expressed with an orthogonal *Escherichia coli* tyrosyl tRNA/aaRS pair in Chinese hamster ovary (CHO) cell culture. In these settings, pAcPhe was loaded onto the amber tRNA by the aaRS and then specifically incorporated in the amber site in the heavy chain of trastuzumab. Following purification, the antibody was coupled to the auristatin F derivative containing a terminal alkoxy-amine group by an oxime ligation through the engineered pAcPhe residues (Fig. 2C). The analysis of antitumor potency and pharmacokinetics of this site-specific ADC confirmed its efficacy, specificity and stability in blood serum.

Very recently, the use of unnatural amino acid in ADC production was further developed by Zimmerman et al. (2014) (Sutro Biopharma), who discovered a novel variant of the *Methanococcus jannaschii* tyrosyl tRNA synthetase with a high activity and specificity towards the unnatural amino acid para-azidomethyl-L-phenylalanine (pAMF) and established a cell-free protein expression platform for production of high yields of antibodies containing a site-specific incorporation of pAMF.

4.1.4. Selenocysteine incorporation

Incorporation of selenocysteine can be an alternative to the unnatural amino acids platform. Selenocysteine (Sec) is called the 21st amino acid and exists in all kingdoms of life as a component of selenoproteins (Johansson et al., 2005). Currently, there are 25 known selenoproteins in mammals and selenocysteine that have been found in the active site of those that have been attributed a catalytic function (Kryukov et al., 2003). Selenocysteine contains selenium in the place of sulfur (pKa 5.2) which makes it more reactive towards electrophiles (*e.g.* maleimide or iodoacetamide) in acidic conditions (pH 5.2) than its classical counterpart, cysteine (pKa 8.3) (Kim et al., 2013). This chemical property of selenocysteine was used to selectively couple maleimide and idoacetamide containing agents to the antibodies with genetically engineered selenocysteine (Hofer et al., 2009; Li et al., 2014) (Fig. 2D). Selenocysteine is incorporated into nascent polypeptides in the response to the opal stop codon (UGA) when a stem–loop structure, known as the Sec insertion sequence (SECIS), is present in the 3′ untranslated regions (UTRs) in eukaryotes and in archaea, or immediately downstream of UGA in bacteria (Kryukov et al., 2003). Furthermore, selenocysteine can be engineered into a classical protein by insertion of the UGA codon and the SECIS at the 3′ of the gene encoding this protein. The proof of concept experiments involving conjugation of fluorescent probes, biotin and biotin-polyethylene glycol (biotin-PEG) to antibodies resulted in the fully functional conjugates having defined sites and stoichiometries of agent attachment, which demonstrates that incorporation of selenocysteine provides a novel technology for generating homogeneous ADCs (Hofer et al., 2009; Li et al., 2014).

4.2. Enzymatic methods

4.2.1. Glycotransferases

IgGs are N-glycosylated at the conserved asparagine 297 (N297) within the CH2 domain of the Fc fragment. The human Fc N-linked glycans consist of a variety of glycoforms, which are referred as G0, G1 and G2 and contain 0, 1 and 2 terminal galactoses, respectively. Qasba and coworkers developed a mutant of β1,4-galactosyltransferase, β1,4Gal-T1-Y289L, which facilitates transfer of modified galactose having a chemical handle at the C2 position (GalNAz or 2-keto-Gal) onto the G0 glycoform to enable site-specific IgG modification (Boeggeman et al., 2009; Ramakrishnan and Qasba, 2002). Recently, an additional mutation was introduced to the mutant resulting in a double mutant that exhibits higher catalytic activity in the presence of Mg^{2+} than in the presence of Mn^{2+} that can be toxic to cells. This allows labeling of surface glycans on living cells, which can be further used in glycome studies (Mercer et al., 2013). Importantly, enzymatic modification of the Fc glycans followed by a chemically-driven attachment of a cytotoxic molecule provides a feasible way to generate site-specific ADCs (Boeggeman et al., 2009; Zhu et al., 2014). Initially, this strategy was used to link biotinylated or a fluorescent dye carrying derivatives to therapeutic monoclonal antibodies. The first enzymatic step involved a release of all terminal galactoses using β1,4-galactosidase from *Streptococcus pneumonia*, which resulted in a homogeneous population of G0 glycoform. Then, degalactosylated glycans were modified by the attachment of galactose containing chemically reactive functional group, *e.g.* C2-keto-Gal or GalNAz catalyzed by β1,4Gal-T1-Y289L. Functionalized biotin and fluorescent dyes were subsequently linked to the modified glycans using an appropriate chemistry, *e.g.* oxime ligation (Boeggeman et al., 2009). Most notably, in a recent study this approach has been evaluated for generation of an ADC comprising a newly identified monoclonal antibody against HER2 receptor, m860, and auristatin F (Zhu et al., 2014). The resulting highly homogeneous bioconjugate exhibited cell-killing activity specifically against HER2-positive tumor cells demonstrating that glycoengineering technology can be potentially applied in ADC design and production.

Glycoengineering has also been employed in a recently described method for the synthesis of site-specific ADCs, in which sialic acid was used as a chemical handle for a selective conjugation (Zhou et al., 2014a). This was achieved by the incorporation of sialic acid units onto the native glycans of trastuzumab using a mixture of β1,4-galactosyltransferase (Gal T) and α2,6-sialyltransferase (Sial T). Prior to reaction with aminooxy-functionalized linker-drug, sialic acid residues were oxidized under mild conditions, which led to the conversion of the acidic groups to aldehyde groups. The resulting modified antibody was reacted with the cytotoxic module *via* the oxime ligation (Fig. 3A). The described procedure was evaluated by conjugating trastuzumab to two different cytotoxins, monomethylauristatin E (MMAE) and dolastatin 10 (Zhou et al., 2014a). The obtained conjugates were significantly more homogeneous when compared to their counterparts that were generated using the conventional conjugation through interchain cysteines (described in Section 2). Moreover, the glycoengineered ADCs exhibited a comparable antitumor activity to that of the conventional ADCs despite lower drug load (Zhou et al., 2014a).

4.2.2. Transglutaminases

Microbial transglutaminase (MTGase) catalyzes the formation of an isopeptide bond between the γ-carbonyl amine group of glutamines and the primary amine of lysine that is accompanied by the release of ammonia (Griffin et al., 2002). The coupling activity of microbial (bacterial) transglutaminase has been applied to modify antibodies, including synthesis of homogeneous ADCs (Dennler et al., 2014; Jeger et al., 2010; Strop, 2014; Strop et al., 2013). Dennler et al. (2014) has recently proposed a chemo-enzymatic conjugation strategy, which yielded highly homogeneous trastuzumab-MMAE conjugate with a DAR of 2. This strategy involved two steps: enzymatic, employing peptide-N-glycosidase F (PGNase F) and MTGase followed by chemical reaction, with the use of strain-promoted azide-alkyne cycloaddition (SPAAC) chemistry. PGNase was used to remove a glycan attached to asparagine residue (N297) adjacent to the conjugation site, glutamine 295 (Q295). Glutamine 295 within the heavy chain (HC) of IgGs was previously shown to be specifically recognized by MTGase, which enabled a site-specific conjugation with an exact DAR of 2. A small azide linker, containing a primary amine group, was coupled to Q295 of the deglycosylated antibody by MTGase. This enzymatic reaction was followed by a Cu(I)-free cycloaddition of the alkyne unit-containing auristatin (MMAE) module (Fig. 3B). The SPAAC reaction was relatively fast (a few hours) with a 2.5-fold excess of the cytotoxic module and resulted in uniform and functional ADCs (Dennler et al., 2014).

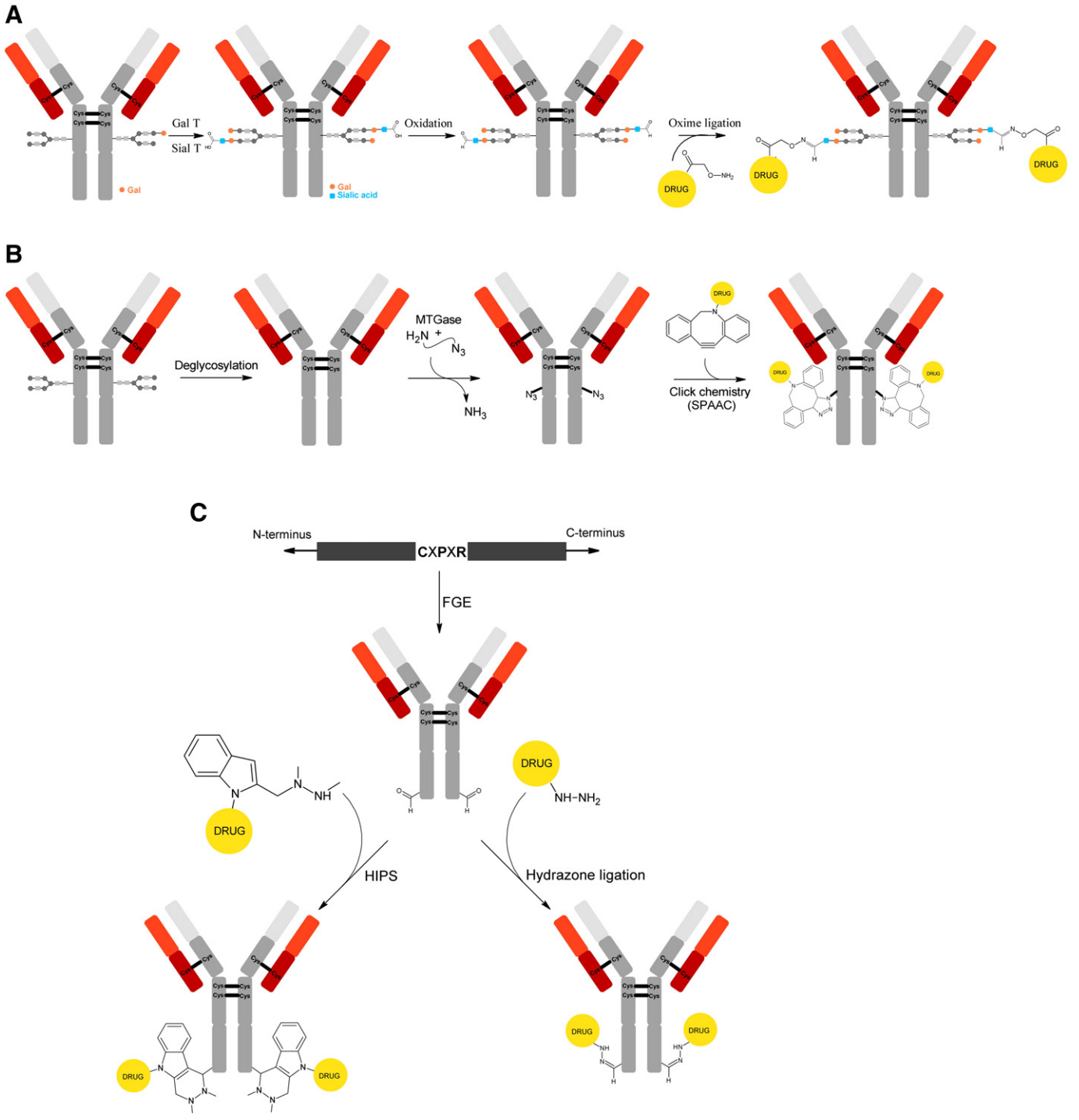

Fig. 3. Enzymatic methods A. Modification of that native glycans on asparagine 297 in the Fc region using galactosyl- (Gal T) and sialyltransferases (Sial T) results in the incorporation of sialic acid units. Following oxidation of sialic acid, drug molecule is coupled to the aldehyde group through oxime ligation. B. Microbial transglutaminase (MTGase) incorporates a small azide linker specifically onto glutamine 295. Cytotoxic molecule is conjugated to the linker using click chemistry (SPAAC). C. Genetically engineered aldehyde tag (CXPXR) is recognized and modified by formylglycine-generating enzyme (FGE). Introduced formylglycine (FGly) can be then subjected to click chemistry or hydrazone ligation with a cytotoxic module.

4.2.3. Formylglycine-generating enzyme (FGE)

Type I sulfatases, the enzymes that hydrolyze sulfate esters, are activated by the oxidation of their active site cysteine to the aldehyde-containing C_α-formylglycine residue. This unusual co-translational modification is conferred by the formylglycine (FGly)-generating enzymes (FGEs). FGEs recognize and modify a short consensus sequence, CXPXR (where X is any amino acid), in the context of heterologous proteins. This observation was applied to generate a novel platform for site-specific ADCs based on the incorporation of FGly into monoclonal antibodies (Drake et al., 2014; Rabuka et al., 2012). Introducing the aldehyde tag sequence (*e.g.* LCTPSR) into a protein of interest along with co-expression of FGE allows one to produce the aldehyde-tagged protein in mammalian or bacterial expression system (Rabuka et al., 2012). Following the production of the modified protein, a chemical approach must be employed to couple a cytotoxic agent to the aldehyde moiety of FGly. In the proof of concept experiments that demonstrated the feasibility of this approach, aminooxy- or hydrazide-functionalized molecules were successfully attached to the model proteins (Rabuka et al.,

2012). Recently, the aldehyde tag strategy was further developed by Drake et al. (2014), who used hydrazino-*iso*-Pictet-Spengler (HIPS) chemistry to couple cytotoxic maytansine to the aldehyde-tag containing trastuzumab (Fig. 3C). The HIPS ligation results in the formation of a stable, covalent C–C bond, which is more stable in physiological condition than hydrazone or oxime ligation products (Agarwal et al., 2013). Furthermore, the study showed that the aldehyde tag can be introduced in the various locations within the antibody without affecting the stability and antitumor activity of the obtained ADCs (Drake et al., 2014).

4.3. Chemical approaches

The majority of methods for site-specific conjugation of cytotoxic drugs to antibodies described above involved a modification of these antibodies prior to conjugation reaction. Recently, a universal chemically-driven strategy for the synthesis of homogeneous ADCs has been proposed. This novel approach takes advantage of bis-sulfone reagents that undergo bis-alkylation to conjugate both thiols of the two cysteine residues that were obtained through the reduction of native disulfide bonds (Badescu et al., 2014; Del Rosario et al., 1990) (Fig. 4A). Previously, this method has been successfully applied for the site-specific conjugation of PEG to the therapeutic proteins, including human interferon α-2b and a human CD receptor-blocking antibody fragment (Fab) (Shaunak et al., 2006). To validate the use of bis-sulfone reagents in ADC production, a cytotoxic payload (MMAE) was loaded onto bis-sulfone module containing a small PEG spacer of 24 repeat units, which was used in order to increase solubility and improve pharmacokinetics of the conjugates (Badescu et al., 2014). The resulting cytotoxic modules were coupled to the reduced trastuzumab (the therapeutic anti-HER2 antibody). Chemical conjugation yielded 78% of ADCs with a drug-to-antibody ratio (DAR) of 4 and less than 1% of unconjugated antibody. Notably, by adjusting conditions of the conjugation reaction, the desired average DAR can be obtained. The MMAE-trastuzumab conjugates retained antigen binding and stability, and exhibited antitumor activity both *in vitro* and *in vivo*. A similar method, relying on the next generation maleimides (NGMs) for site-specific conjugation in a controlled manner, was described in a recent study by Schumacher et al. (2014). The NGMs are maleimides, which are substituted in the 3- and 4-positions with leaving groups, including bromide anion (Br^-) and thiophenol anion (PhS^-). This chemical modification of maleimides facilitates a reaction with two nucleophilic thiol groups derived from a reduced disulfide bridge (Schumacher et al., 2014). Recently, the strategy based on the reaction between leaving groups and interchain cysteines has been further developed. Maruani et al. (2015) used a dibromopyridazinedione construct with two orthogonal clickable handles to enable herceptin modification with both a cytotoxic drug and a fluorophore. This approach resulted in a highly stable and homogeneous (DAR 4:1) ADC. Therefore, a chemoselective dual click strategy might be successfully applied in ADC production (Maruani et al., 2015).

4.4. Photoactive protein Z

Conjugation of a photoactive protein Z to antibodies has recently emerged as a new, unconventional approach towards the synthesis of uniform ADCs. Protein Z is a small (58 amino acids), helical protein derived from the IgG-binding B domain of protein A. Notably, protein Z, called also Z domain, binds most of IgG isotypes specifically within the CH2–CH3 region of the Fc fragment with high affinity (Nilsson et al., 1987). Recently, 13 variants of protein Z with a UV active unnatural amino acid, benzoylphenylalanine (BPA), engineered into different locations have been constructed (Hui and Tsourkas, 2014). BPA enables to covalently couple protein Z to the antibody of interest upon exposure to long wavelength UV light (365 nm) (Fig. 4B). The variants were evaluated in terms of their efficiencies of photo-conjugation to various native IgGs. Two of them, L17BPA and K35BPA, underwent coupling reaction with the highest efficiency, ranging from 65% to 95% within 1 h of UV exposure, and therefore are good candidates for ADC design (Hui and Tsourkas, 2014). The technology, which allows to introduce a click-chemistry compatible azide group onto C-terminus of photoactive

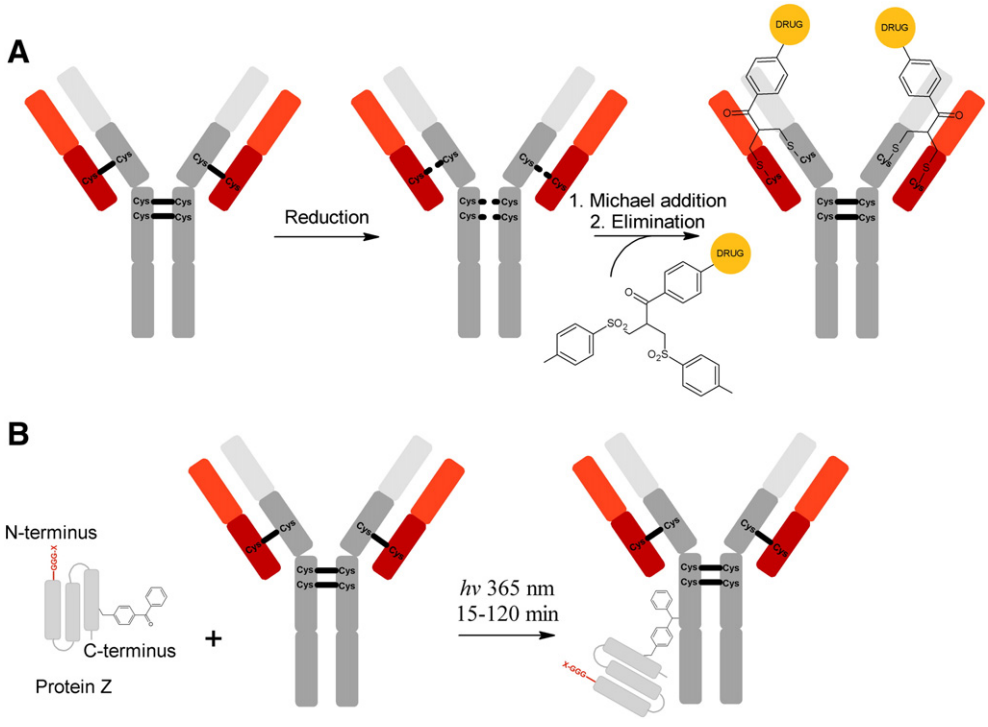

Fig. 4. Chemical approach (A) and photoactivation of protein Z (B). A. A bis-sulfone reagent containing a cytotoxic moiety conjugates both thiol groups derived from a reduced native disulfide bond. B. Photoactive protein Z is conjugated specifically to the Fc region of the antibody upon exposure to long wavelength UV. Coupling a cytotoxic drug to the photoactive protein Z would enable to generate site-specific ADC by the use of UV light.

protein Z by expressed protein ligation (EPL) has recently been described (Hui et al., 2014). Therefore, development of ADCs based on the optimized photoactive protein Z seems to be a matter of time.

5. Conclusions and perspectives

According to a number of studies, homogeneity of ADCs is a crucial determinant of their potency and safety. Synthesis of uniform ADCs limits batch-to-batch variability, which is important for manufacturing process as well as for clinical applications. Commercially available ADCs, Kadcyla® and Adcetris® are a breakthrough in the treatment of metastatic breast cancer and CD-30 positive lymphomas, respectively. Nevertheless, their therapeutic potential may be not fully exploited due to heterogeneity. Since publication of the first studies demonstrating that site-specific conjugation improves the therapeutic index of ADCs (Junutula et al., 2008a; McDonagh et al., 2006), a number of approaches towards generation of homogeneous ADCs have been developed. Initially, these strategies involved engineering of native antibodies either by removing or introducing cysteines (described in Section 4.1). Antibody engineering was further investigated, which resulted in the methods that were based on incorporation of genetically encoded unnatural amino acids or selenocysteine (Axup et al., 2012; Hofer et al., 2009). In the recent years, several enzymatic methods have also been proposed (described in Section 4.2). However, the majority of these methods still requires antibodies to be modified either by single-site mutation or introduction of genetically engineered tags (*e.g.* aldehyde tag and glutamine tag), which makes them relatively complex (Dennler et al., 2014; Drake et al., 2014; Rabuka et al., 2012; Strop et al., 2013). Additionally, the position of introduced amino acid or amino acid sequence needs to be carefully optimized to ensure stability of conjugates and avoid aggregation of antibodies (Drake et al., 2014; Shen et al., 2012; Strop et al., 2013). Novel chemical methods provide a rapid synthesis of homogeneous ADCs that are based on native, non-engineered antibodies (described in Section 4.3). Importantly, they are universal as, similarly to the conventional cysteine conjugation, they employ interchain cysteines as conjugation sites (Badescu et al., 2014; Schumacher et al., 2014). Noticeably, in contrast to the removal of disulfide bridges that takes place in the conventional cysteine conjugation, the use of bis-sulfone reagents and the next generation maleimides (NGMs) causes re-bridging of disulfide bonds, which leaves the antibody structurally intact and may help to preserve its effector functions (Romans et al., 1977; Seegan et al., 1979).

A variety of conjugation approaches that have been developed in a relatively short period of time show that ADC design is a very challenging field (Perez et al., 2014). Apart from the drug–antibody coupling strategies, new targeting molecules, including antibody-derived fragments and single-domain antibodies, are evaluated for clinical use. Thanks to advanced antibody engineering techniques, alternative formats of antibodies can be conveniently produced. The most popular antibody configurations include Fabs, scFvs, diabodies, triabodies and minibodies (Alvarenga et al., 2014; Nelson, 2010). A distinct type of antibody fragment, called nanobody or VHH, can be derived from a single-chain camelid antibody comprising a single variable domain and two constant domains. Importantly, it has been demonstrated that diabodies, minibodies as well as nanobodies can be successfully used as cytotoxic drug carriers (Cortez-Retamozo et al., 2004; De Meyer et al., 2014; Kim et al., 2008; Perrino et al., 2014). Currently, Ablynx Inc. and Spirogen are collaborating on novel pyrrolobenzodiazepines (PBDs)-nanobody conjugates for cancer treatment. Moreover, bispecific antibodies and bispecific antibody fragments, which bind two distinct antigens or epitopes on the same antigen, have recently emerged as very promising targeting molecules (Spiess et al., 2015). Biotechnology and pharmaceutical companies worldwide are developing a new class of ADC therapeutics based on bispecific antibodies (Garber, 2014; Lameris et al., 2014). Intensive research work on different aspects of ADCs leads to a further improvement of cytotoxic conjugates, which is necessary to accelerate their passage from a proof of concept stage to clinical application.

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References

Agarwal, P., Bertozzi, C.R., 2015. Site-specific antibody–drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. Bioconjug. Chem. 26, 176–192.
Agarwal, P., Kudirka, R., Albers, A.E., Barfield, R.M., de Hart, G.W., Drake, P.M., Jones, L.C., Rabuka, D., 2013. Hydrazino-Pictet-Spengler ligation as a biocompatible method for the generation of stable protein conjugates. Bioconjug. Chem. 24, 846–851.
Alley, S.C., Benjamin, D.R., Jeffrey, S.C., Okeley, N.M., Meyer, D.L., Sanderson, R.J., Senter, P.D., 2008. Contribution of linker stability to the activities of anticancer immunoconjugates. Bioconjug. Chem. 19, 759–765.
Alvarenga, L., Zahid, M., Tommaso, A., Juste, M., Aubrey, N., Billiald, P., Muzard, J., 2014. Engineering venom's toxin-neutralizing antibody fragments and its therapeutic potential. Toxins (Basel) 6, 2541–2567.
Andersen, D.C., Reilly, D.E., 2004. Production technologies for monoclonal antibodies and their fragments. Curr. Opin. Biotechnol. 15, 456–462.
Axup, J.Y., Bajjuri, K.M., Ritland, M., Hutchins, B.M., Kim, C.H., Kazane, S.A., Halder, R., Forsyth, J.S., Santidrian, A.F., Stafin, K., et al., 2012. Synthesis of site-specific antibody–drug conjugates using unnatural amino acids. Proc. Natl. Acad. Sci. 109, 16101–16106.
Badescu, G., Bryant, P., Bird, M., Henseleit, K., Swierkosz, J., Parekh, V., Tommasi, R., Pawlisz, E., Jurlewicz, K., Farys, M., et al., 2014. Bridging disulfides for stable and defined antibody drug conjugates. Bioconjug. Chem. 25, 1124–1136.
Barginear, M.F., John, V., Budman, D.R., 2012. Trastuzumab-DM1: a clinical update of the novel antibody–drug conjugate for HER2-overexpressing breast cancer. Mol. Med. 18, 1473–1479.
Behrens, C.R., Liu, B., 2014. Methods for site-specific drug conjugation to antibodies. MAbs 6, 46–53.
Bodet-Milin, C., 2013. Radioimmunotherapy of B-cell non-Hodgkin's lymphoma. Front. Oncol. 3, 1–13.
Boeggeman, E., Ramakrishnan, B., Pasek, M., Manzoni, M., Puri, A., Loomis, K.H., Waybright, T.J., Qasba, P.K., 2009. Site specific conjugation of fluoroprobes to the remodeled Fc N-Glycans of monoclonal antibodies using mutant glycosyltransferases: application for cell surface antigen detection. Bioconjug. Chem. 20, 1228–1236.
Bross, P.F., Beitz, J., Chen, G., Chen, X.H., Duffy, E., Kieffer, L., Roy, S., Sridhara, R., Rahman, A., Williams, G., Pazdur, R., 2001. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin. Cancer Res. 7, 1490–1496.
Cal, P.M.S.D., Bernardes, G.J.L., Gois, P.M.P., 2014. Cysteine-selective reactions for antibody conjugation. Angew. Chem. Int. Ed. Engl. 53, 10585–10587.
Chamarthy, M.R., Williams, S.C., Moadel, R.M., 2011. Radioimmunotherapy of non-Hodgkin's lymphoma: from the "magic bullets" to "radioactive magic bullets". Yale J. Biol. Med. 84, 391–407.
Cortez-Retamozo, V., Backmann, N., Senter, P.D., Wernery, U., De Baetselier, P., Muyldermans, S., Revets, H., 2004. Efficient cancer therapy with a nanobody-based conjugate. Cancer Res. 64, 2853–2857.
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol. 32, 263–270.
Del Rosario, R.B., Wahl, R.L., Brocchini, S.J., Lawton, R.G., Smith, R.H., 1990. Sulfhydryl site-specific crosslinking and labeling of monoclonal antibodies by a fluorescent equilibrium transfer alkylation crosslink reagent. Bioconjug. Chem. 1, 51–59.
Dennler, P., Chiotellis, A., Fischer, E., Brégeon, D., Belmant, C., Gauthier, L., Lhospice, F., Romagne, F., Schibli, R., 2014. Transglutaminase-based chemo-enzymatic conjugation approach yields homogeneous antibody–drug conjugates. Bioconjug. Chem. 25, 569–578.
Doronina, S.O., Toki, B.E., Torgov, M.Y., Mendelsohn, B.A., Cerveny, C.G., Chace, D.F., DeBlanc, R.L., Gearing, R.P., Bovee, T.D., Siegall, C.B., et al., 2003. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat. Biotechnol. 21, 778–784.
Drake, P.M., Albers, A.E., Baker, J., Banas, S., Barfield, R.M., Bhat, A.S., de Hart, G.W., Garofalo, A.W., Holder, P., Jones, L.C., et al., 2014. Aldehyde tag coupled with HIPS chemistry enables the production of ADCs conjugated site-specifically to different antibody regions with distinct *in vivo* efficacy and PK outcomes. Bioconjug. Chem. 25, 1331–1341.
Eriksson, S.E., Ohlsson, T., Nilsson, R., Tennvall, J., 2012. Treatment with unlabeled mAb BR96 after radioimmunotherapy with 177Lu-DOTA-BR96 in a syngeneic rat colon carcinoma model. Cancer Biother. Radiopharm. 27, 175–182.
Eriksson, S.E., Elgström, E., Bäck, T., Ohlsson, T., Jensen, H., Nilsson, R., Lindegren, S., Tennvall, J., 2014. Sequential radioimmunotherapy with 177Lu- and 211At-labeled monoclonal antibody BR96 in a syngeneic rat colon carcinoma model. Cancer Biother. Radiopharm. 29, 238–246.
Francisco, J.A., Cerveny, C.G., Meyer, D.L., Mixan, B.J., Klussman, K., Chace, D.F., Rejniak, S.X., Gordon, K.A., DeBlanc, R., Toki, B.E., et al., 2003. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood 102, 1458–1465.
Gaertner, H.F., Offord, R.E., 1996. Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. Bioconjug. Chem. 7, 38–44.
Garber, K., 2014. Bispecific antibodies rise again. Nat. Rev. Drug Discov. 13, 799–801.

Geoghegan, K.F., Stroh, J.G., 1992. Site-directed conjugation of nonpeptide groups to peptides and proteins *via* periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. Bioconjug. Chem. 3, 138–146.
Griffin, M., Casadio, R., Bergamini, C.M., 2002. Transglutaminases: nature's biological glues. Biochem. J. 396, 377–396.
Hamblett, K.J., Senter, P.D., Chace, D.F., Sun, M.M.C., Lenox, J., Cerveny, C.G., Kissler, K.M., Bernhardt, S.X., Kopcha, A.K., Zabinski, R.F., et al., 2004. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clin. Cancer Res. 10, 7063–7070.
Hofer, T., Skeffington, L.R., Chapman, C.M., Rader, C., 2009. Molecularly defined antibody conjugation through a selenocysteine interface. Biochemistry 48, 12047–12057.
Hui, J.Z., Tsourkas, A., 2014. Optimization of photoactive protein z for fast and efficient site-specific conjugation of native IgG. Bioconjug. Chem. 25, 1709–1719.
Hui, J.Z., Al Zaki, A., Cheng, Z., Popik, V., Zhang, H., Luning Prak, E.T., Tsourkas, A., 2014. Facile method for the site-specific, covalent attachment of full-length IgG onto nanoparticles. Small 10, 3354–3363.
Jackson, D., Atkinson, J., Guevara, C.I., Zhang, C., Kery, V., Moon, S.J., Virata, C., Yang, P., Lowe, C., Pinkstaff, J., et al., 2014. *In vitro* and *in vivo* evaluation of cysteine and site specific conjugated herceptin antibody–drug conjugates. PLoS One 9, e83865.
Jeger, S., Zimmermann, K., Blanc, A., Grünberg, J., Honer, M., Hunziker, P., Struthers, H., Schibli, R., 2010. Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase. Angew. Chem. Int. Ed. 49, 9995–9997.
Johansson, L., Gafvelin, G., Arnér, E.S.J., 2005. Selenocysteine in proteins — properties and biotechnological use. Biochim. Biophys. Acta 1726, 1–13.
Junutula, J.R., Raab, H., Clark, S., Bhakta, S., Leipold, D.D., Weir, S., Chen, Y., Simpson, M., Tsai, S.P., Dennis, M.S., et al., 2008a. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat. Biotechnol. 26, 925–932.
Junutula, J.R., Bhakta, S., Raab, H., Ervin, K.E., Eigenbrot, C., Vandlen, R., Scheller, R.H., Lowman, H.B., 2008b. Rapid identification of reactive cysteine residues for site-specific labeling of antibody-Fabs. J. Immunol. Methods 332, 41–52.
Junutula, J.R., Flagella, K.M., Graham, R.A., Parsons, K.L., Ha, E., Raab, H., Bhakta, S., Nguyen, T., Dugger, D.L., Li, G., et al., 2010. Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. Clin. Cancer Res. 16, 4769–4778.
Kim, K.M., McDonagh, C.F., Westendorf, L., Brown, L.L., Sussman, D., Feist, T., Lyon, R., Alley, S.C., Okeley, N.M., Zhang, X., et al., 2008. Anti-CD30 diabody-drug conjugates with potent antitumor activity. Mol. Cancer Ther. 7, 2486–2497.
Kim, C.H., Axup, J.Y., Schultz, P.G., 2013. Protein conjugation with genetically encoded unnatural amino acids. Curr. Opin. Chem. Biol. 17, 412–419.
Kim, M.T., Chen, Y., Marhoul, J., Jacobson, F., 2014. Statistical modeling of the drug load distribution on trastuzumab emtansine (kadcyla), a lysine-linked antibody drug conjugate. Bioconjug. Chem. 25, 1223–1232.
Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497.
Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigó, R., Gladyshev, V.N., 2003. Characterization of mammalian selenoproteomes. Science 300, 1439–1443.
Lameris, R., de Bruin, R.C.G., Schneiders, F.L., van Bergen en Henegouwen, P.M.P., Verheul, H.M.W., de Gruijl, T.D., van der Vliet, H.J., 2014. Bispecific antibody platforms for cancer immunotherapy. Crit. Rev. Oncol. Hematol. 92, 153–165.
Lemke, E.A., 2014. The exploding genetic code. ChemBioChem 15, 1691–1694.
Lewis Phillips, G.D., Li, G., Dugger, D.L., Crocker, L.M., Parsons, K.L., Mai, E., Blättler, W.A., Lambert, J.M., Chari, R.V.J., Lutz, R.J., et al., 2008. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res. 68, 9280–9290.
Li, X., Yang, J., Rader, C., 2014. Antibody conjugation *via* one and two C-terminal selenocysteines. Methods 65, 133–138.
Liu, H., May, K., 2012. Structural variations, chemical modifications and possible impacts to stability and biological function disulfide bond structures of IgG molecules. MAbs 4, 17–23.
LoRusso, P.M., Weiss, D., Guardino, E., Girish, S., Sliwkowski, M.X., 2011. Trastuzumab emtansine: a unique antibody–drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. Clin. Cancer Res. 17, 6437–6447.
Maruani, A., Smith, M.E.B., Miranda, E., Chester, K.A., Chudasama, V., Caddick, S., 2015. A plug-and-play approach to antibody-based therapeutics *via* a chemoselective dual click strategy. Nat. Commun. 6, 6645.
McDonagh, C.F., Turcott, E., Westendorf, L., Webster, J.B., Alley, S.C., Kim, K., Andreyka, J., Stone, I., Hamblett, K.J., Francisco, J.A., et al., 2006. Engineered antibody–drug conjugates with defined sites and stoichiometries of drug attachment. Protein Eng. Des. Sel. 19, 299–307.
Mercer, N., Ramakrishnan, B., Boeggeman, E., Verdi, L., Qasba, P.K., 2013. Use of novel mutant galactosyltransferase for the bioconjugation of terminal N-Acetylglucosamine (GlcNAc) residues on live cell surface. Bioconjug. Chem. 24, 144–152.
Nelson, A.L., 2010. Antibody fragments: Hope and hype. MAbs 2, 77–83.
Nilsson, B., Moks, T., Jansson, B., Abrahmsén, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T.A., Uhlén, M., 1987. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng. 1, 107–113.
Panowksi, S., Bhakta, S., Raab, H., Polakis, P., Junutula, J.R., 2014. Site-specific antibody drug conjugates for cancer therapy. MAbs 6, 34–45.
Peddi, P.F., Hurvitz, S.A., 2013. Trastuzumab emtansine: the first targeted chemotherapy for treatment of breast cancer. Future Oncol. 9, 319–326.
Perez, H.L., Cardarelli, P.M., Deshpande, S., Gangwar, S., Schroeder, G.M., Vite, G.D., Borzilleri, R.M., 2014. Antibody–drug conjugates: Current status and future directions. Drug Discov. Today 19, 869–881.
Perrino, E., Steiner, M., Krall, N., Bernardes, G.J.L., Pretto, F., Casi, G., Neri, D., 2014. Curative properties of noninternalizing antibody–drug conjugates based on maytansinoids. Cancer Res. 74, 2569–2578.
Pro, B., Advani, R., Brice, P., Bartlett, N.L., Rosenblatt, J.D., Illidge, T., Matous, J., Ramchandren, R., Fanale, M., Connors, J.M., et al., 2012. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study. J. Clin. Oncol. 30, 2190–2196.
Rabuka, D., Rush, J.S., deHart, G.W., Wu, P., Bertozzi, C.R., 2012. Site-specific chemical protein conjugation using genetically encoded aldehyde tags. Nat. Protoc. 7, 1052–1067.
Ramakrishnan, B., Qasba, P.K., 2002. Structure-based design of β1,4-Galactosyltransferase I (β4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens β4Gal-T1 donor specificity. J. Biol. Chem. 277, 20833–20839.
Ricart, A.D., 2011. Antibody–drug conjugates of calicheamicin derivative: gemtuzumab ozogamicin and inotuzumab ozogamicin. Clin. Cancer Res. 17, 6417–6427.
Romans, D.G., Tilley, C.A., Crookston, M.C., Falk, R.E., Dorrington, K.J., 1977. Conversion of incomplete antibodies to direct agglutinins by mild reduction: evidence for segmental flexibility within the Fc fragment of immunoglobulin G. Proc. Natl. Acad. Sci. 74, 2531–2535.
Schroeder, D.D., Tankersly, D.L., Lundblad, J.L., 1981. A new preparation of modified immune serum globulin (human) suitable for intravenous administration. I. Standardization of the reduction and alkylation reaction. Vox Sang. 40, 373–382.
Schumacher, F.F., Nunes, J.P.M., Maruani, A., Chudasama, V., Smith, M.E.B., Chester, K.A., Baker, J.R., Caddick, S., 2014. Next generation maleimides enable the controlled assembly of antibody–drug conjugates *via* native disulfide bond bridging. Org. Biomol. Chem. 12, 7261–7269.
Seegan, G.W., Smith, C.A., Schumaker, V.N., 1979. Changes in quaternary structure of IgG upon reduction of the interheavy-chain disulfide bond. Proc. Natl. Acad. Sci. 76, 907–911.
Senter, P.D., Sievers, E.L., 2012. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. Nat. Biotechnol. 30, 631–637.
Shaunak, S., Godwin, A., Choi, J.W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M., Brocchini, S., 2006. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nat. Chem. Biol. 2, 312–313.
Shen, B.Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K.L., Tien, J., Yu, S.F., Mai, E., et al., 2012. Conjugation site modulates the *in vivo* stability and therapeutic activity of antibody–drug conjugates. Nat. Biotechnol. 30, 184–189.
Spiess, C., Zhai, Q., Carter, P.J., 2015. Alternative molecular formats and therapeutic applications for bispecific antibodies. Mol. Immunol. (in press (available at http://www.sciencedirect.com/science/article/pii/S016158901500005X).
Strebhardt, K., Ullrich, A., 2008. Paul Ehrlich's magic bullet concept: 100 years of progress. Nat. Rev. Cancer 8, 473–480.
Strop, P., 2014. Versatility of Microbial Transglutaminase. Bioconjug. Chem. 25, 855–862.
Strop, P., Liu, S.H., Dorywalska, M., Delaria, K., Dushin, R.G., Tran, T.T., Ho, W.H., Farias, S., Casas, M.G., Abdiche, Y., et al., 2013. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chem. Biol. 20, 161–167.
Sun, M.M.C., Beam, K.S., Cerveny, C.G., Hamblett, K.J., Blackmore, R.S., Torgov, M.Y., Handley, F.G.M., Ihle, N.C., Senter, P.D., Alley, S.C., 2005. Reduction–alkylation strategies for the modification of specific monoclonal antibody disulfides. Bioconjug. Chem. 16, 1282–1290.
Van de Donk, N.W.C.J., Dhimolea, E., 2012. Brentuximab vedotin. MAbs 4, 458–465.
Wang, L., 2003. Amersham Prize winner. Expanding the genetic code. Science 302, 584–585.
Wang, L., Brock, A., Herberich, B., Schultz, P.G., 2001. Expanding the genetic code of *Escherichia coli*. Science 292, 498–500.
Wang, L., Amphlett, G., Blättler, W.A., Lambert, J.M., Zhang, W., 2005. Structural characterization of the maytansinoid–monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. Protein Sci. 14, 2436–2446.
Willner, D., Trail, P.A., Hofstead, S.J., King, H.D., Lasch, S.J., Braslawsky, G.R., Greenfield, R.S., Kaneko, T., Firestone, R.A., 1993. (6-Maleimidocaproyl)hydrazone of doxorubicin-a new derivative for the preparation of immunoconjugates of doxorubicin. Bioconjug. Chem. 4, 521–527.
Younes, A., Gopal, A.K., Smith, S.E., Ansell, S.M., Rosenblatt, J.D., Savage, K.J., Ramchandren, R., Bartlett, N.L., Cheson, B.D., de Vos, S., et al., 2012. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. J. Clin. Oncol. 30, 2183–2189.
Zhou, Q., Stefano, J.E., Manning, C., Kyazike, J., Chen, B., Gianolio, D.A., Park, A., Busch, M., Bird, J., Zheng, X., et al., 2014a. Site-specific antibody-drug conjugation through glycoengineering. Bioconjug. Chem. 25, 510–520.
Zhou, Z., Zhang, J., Sun, L., Ma, G., Su, Z., 2014b. Comparison of site-specific PEGylations of the N-terminus of interferon beta-1b: Selectivity, efficiency, and *in vivo*/vitro activity. Bioconjug. Chem. 25, 138–146.
Zhu, Z., Ramakrishnan, B., Li, J., Wang, Y., Feng, Y., Prabakaran, P., Colantonio, S., Dyba, M.A., Qasba, P.K., Dimitrov, D.S., 2014. Site-specific antibody–drug conjugation through an engineered glycotransferase and a chemically reactive sugar. MAbs 6, 1190–1200.
Zimmerman, E.S., Heibeck, T.H., Gill, A., Li, X., Murray, C.J., Madlansacay, M.R., Tran, C., Uter, N.T., Yin, G., Rivers, P.J., et al., 2014. Production of site-specific antibody–drug conjugates using optimized non-natural amino acids in a cell-free expression system. Bioconjug. Chem. 25, 351–361.