Published in final edited form as:

Annu Rev Biochem. 2019 June 20; 88: 433–459. doi:10.1146/annurev-biochem-062917-012911.

Glycoengineering of Antibodies for Modulating Functions

Lai-Xi Wang, Xin Tong, Chao Li, John P. Giddens, Tiezheng Li

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA;

Abstract

Antibodies are immunoglobulins that play essential roles in immune systems. All antibodies are glycoproteins that carry at least one or more conserved *N*-linked oligosaccharides (*N*-glycans) at the Fc domain. Many studies have demonstrated that both the presence and fine structures of the attached glycans can exert a profound impact on the biological functions and therapeutic efficacy of antibodies. However, antibodies usually exist as mixtures of heterogeneous glycoforms that are difficult to separate in pure glycoforms. Recent progress in glycoengineering has provided useful methods that enable production of glycan-defined and site-selectively modified antibodies for functional studies and for improved therapeutic efficacy. This review highlights major approaches in glycoengineering of antibodies with a focus on recent advances in three areas: glycoengineering through glycan biosynthetic pathway manipulation, glycoengineering through in vitro chemoenzymatic glycan remodeling, and glycoengineering of antibodies for site-specific antibody–drug conjugation.

Keywords

antibody; glycosylation; glycoengineering; chemoenzymatic; antibody–drug conjugate

INTRODUCTION

Antibodies, also known as immunoglobulins, play essential roles in the immune system. They are used by the immune system to fight against invasive pathogens through direct neutralization, by activation of the complement system for cell lysis, and/or by facilitating phagocytosis of pathogens. There are five distinct classes of antibodies in humans: IgG, IgM, IgA, IgE, and IgD. They share similar domain structures but differ in the flexibility of the hinge region and their oligomeric states. Antibodies, as represented by IgG-type monoclonal antibodies (mAbs), are an important class of therapeutic proteins widely used for the treatment of cancer and autoimmune diseases (1–3). IgG is also the most abundant isotype of antibodies in blood circulation, accounting for approximately 75% of human immunoglobulins. A typical IgG antibody is composed of two light and two heavy chains that are associated to form three protein domains: two identical Fab regions specific for antigen binding and an Fc domain (constant or crystallizable IgG fragment) responsible for engaging various Fc receptors in antibody effector functions. The Fab domain and the Fc

domain are connected by a flexible hinge region (Figure 1a). The IgG–Fc domain is a homodimer, in which the two third constant domains (C_H3 domains) are paired through noncovalent interactions, while each of the two second constant domains (C_H2 domains) carries an *N*-linked oligosaccharide (*N*-glycan) at the conserved *N*-glycosylation site (Asn-297) (4–6).

Structural analysis of *N*-glycans released from the Fc domain of human polyclonal IgG and recombinant mAbs has indicated that the Fc glycans are of the typical biantennary complex type with a considerable level of structure heterogeneity (7, 8). More than 30 different Fc oligosaccharides in which the core heptasaccharide bears 0, 1, or 2 terminal galactose moieties (named G0, G1, and G2 glycoforms, respectively) have been characterized, and most are fucosylated (defined as G0F, G1F, and G2F, respectively) (Figure 1b). The bisecting *N*-acetylglucosamine (GlcNAc) and sialylated glycoforms are minor species in normal human IgGs. The present review uses the recommended standard symbols for monosaccharides in all graphical presentations of glycans (9).

Ample experimental data have shown that glycosylation can profoundly affect the biological functions and therapeutic efficacy of antibodies (10–17). Moreover, recent studies have further demonstrated that the fine structures of glycans, particularly those attached to the Fc domain, can differentially tune the immunological properties of a given antibody. For example, Fc core fucosylation significantly reduces antibody-dependent cellular cytotoxicity (ADCC) owing to low affinity to the FcγIIIa receptor (18), a finding that led to the development of low-fucose-content antibody variants with improved anticancer activity (19, 20). By contrast, the terminal α2,6-sialylated Fc glycoform, a minor component in intravenous immunoglobulin (IVIG), is responsible for the anti-inflammatory activity of IVIG in animal models (21–24). In the case of IgE, an exciting recent discovery reported by Anthony and coworkers (25) indicates that a single oligomannose-type *N*-glycan at the IgE Fc domain is indispensable for initiation of anaphylaxis. These studies showcase the significance of antibody glycosylation for biological function.

Natural and recombinant antibodies are usually produced as heterogeneous mixtures of glycoforms that are extremely difficult to separate or enrich to isolate pure forms (Figure 1b). Thus, methods that can lead to the production of structurally well-defined homogeneous glycoforms of antibodies are needed for both functional studies and the development of more efficient antibody-based therapeutics (1). Several aspects of antibody glycosylation have been explored to control and modulate the glycosylation pattern of antibodies. One is the genetic approach that focuses on controlling protein glycosylation by manipulating the *N*-glycan biosynthetic pathways in different host expression systems. This approach has been applied to produce optimal glycoforms of some therapeutic antibodies with improved therapeutic efficacy (26–32). Another promising strategy is in vitro glycan remodeling via chemoenzymatic synthesis that could lead to highly homogeneous antibody glycoforms (33–36). In addition, glycoengineering of Fc *N*-glycans is emerging as an attractive method for site-specific antibody–drug conjugation (37). The present review highlights recent advances in these three major areas of glycoengineering of antibodies.

GLYCOENGINEERING OF ANTIBODIES THROUGH BIOSYNTHETIC PATHWAY MANIPULATION

Expression of recombinant antibodies in conventional mammalian host expression systems such as the Chinese hamster ovary (CHO) cell line usually leads to the production of mixtures of heterogeneous glycoforms (38–40), which are usually not optimal for their therapeutic efficacy. Engineering of the glycan biosynthetic pathway represents a major approach to controlling and optimizing antibody glycosylation to improve their therapeutic outcomes (1, 11, 12). In particular, this approach has been extensively used to produce antibody glyco-variants with enhanced therapeutic efficacy in different cell- or disease-related model systems.

Biosynthetic Pathway Glycoengineering to Produce Low-Fucose or Nonfucosylated Antibodies

Because Fc core fucosylation of antibodies adversely affects the ADCC functions of IgG antibodies, biosynthetic pathway glycoengineering has been used to produce antibodies with low-fucose content or with complete knockout of the core fucose. Biosynthesis of *N*-glycoproteins, including antibodies, occurs in the endoplasmic reticulum and Golgi apparatus. The general biosynthetic pathway is illustrated in Figure 2a. Briefly, a large dolichol-linked oligosaccharide precursor, Glc₃Man₉GlcNAc₂, is assembled in multiple steps on the cytoplasmic face and later on the lumen of the endoplasmic reticulum. The precursor oligosaccharide is then transferred to a consensus (Asn-X-Ser/Thr) *N*-glycosylation site in a nascent protein under the catalysis of an oligosaccharyltransferase. After initial trimming of the precursor oligosaccharide to monoglucosylated and nonglucosylated forms, which are key intermediates involved in lectin-mediated protein folding processes, the correctly folded glycoprotein is translocated from the endoplasmic reticulum to the Golgi apparatus for further glycosylation processing. Various glycosidases and glycosyltransferases are involved, leading to the formation of different glycoforms (Figure 2a). Core fucosylation is fulfilled through catalysis of the unique α 1,6-fucosyltransferase (FUT8), which recognizes the GlcNAcMan₃GlcNAc₂ glycoform as the acceptor substrate and uses sugar nucleotide GDP-L-fucose as the donor substrate (see Figure 2a). The de novo biosynthesis of GDP-L-fucose involves several steps starting with mannose-1-phosphate (Figure 2b).

Cell lines capable of producing modified glycosylation patterns of glycoproteins were first generated by random mutagenesis, followed by selection with various plant lectins (41). This approach generated Lec mutant cell lines that produced glycoproteins with more defined glycosylation patterns compared with those produced by wild-type CHO cells (42). The mutant Lec13, which carries a mutation in the GDP-mannose 4,6-dehydratase (GMD) gene, produces glycoproteins with significantly less fucosylation than those of wild-type CHO cells (43, 44). Another mammalian cell line, YB2/O derived from rat myeloma cells, produces antibodies with low content of core fucose, owing to its naturally lower expression of FUT8 (45). The YB2/O cell line was used to produce two mAbs, roledumab and ublituximab, which are currently in phase II clinical trials (46, 47).

In another classic example of producing low-fucose-content antibodies, Bailey and coworkers (26) demonstrated that CHO cells overexpressing GnTIII, a glycosyltransferase that attaches the bisecting GlcNAc moiety to *N*-glycans, produced glycosylated antibodies with bisecting GlcNAc and low-fucose content. These antibodies showed significantly increased ADCC activity compared with the prototype antibody. Further studies demonstrated that altering the localization pattern of GnTIII using the localization motif of Golgi α -mannosidase II further decreased the core-fucosylation level to yield antibodies containing ~70% of nonfucosylated glycoforms (48). This GnTIII overexpression technology, acquired by Roche in 2005, was used to produce the glycoengineered anti-CD20 antibody obinutuzumab, which was approved by the US Food and Drug Administration in 2013 for treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia (49).

While the expression of GnTIII did reduce the fucose level of IgG, it did not abolish core fucosylation. Attempting to completely block core fucosylation, Satoh and coworkers (27, 50) produced an FUT8 knockout cell line using sequential homologous recombination. The engineered cell line was capable of producing a nonfucosylated anti-CD20 antibody that showed a 100-fold increase in ADCC activity compared with the nonglycoengineered antibody. This stable cell line was used to produce several candidate therapeutic antibodies, including mogamulizumab that was approved for the treatment of adult T cell leukemia/lymphoma in Japan (51).

As an alternative approach to producing nonfucosylated antibodies, Satoh and coworkers (52) also attempted to engineer the biosynthetic pathway of GDP-L-fucose, the essential donor substrate for FUT8 (Figure 2b). They demonstrated that core fucosylation was inhibited in a GMD knockout cell line. Because GMD is essential for *de novo* biosynthesis of GDP, its knockout eliminates all sources for GDP-fucose (52).

As another approach to depleting GDP-fucose levels, Misaghi and coworkers (53) generated a GDP-4-keto-6-D-deoxymannose epimerase/GDP-4-keto-6-L-galactose reductase (FX) knockout cell line using CRISPR/Cas9 technology. This knockout cell line was able to produce completely nonfucosylated antibodies. Interestingly, the fully fucosylated antibodies could be produced by adding L-fucose to the culture, thus enabling comparison of antibodies with or without fucose.

Instead of directly inhibiting GDP-fucose production by knocking out the genes involved in fucose biosynthesis, Sandig and coworkers (54) produced nonfucosylated antibodies by heterologous expression of the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase, which converts GDP-4-keto-6-deoxy-D-mannose to GDP-D-rhamnose. This GDP-rhamnose provides feedback inhibition to GMD activity, leading to low levels of GDP-fucose. Of the antibodies produced by this method, 98% lacked fucose and demonstrated increased ADCC activity (54).

In another study, Song and coworkers (55) produced a GDP-fucose transporter knockout cell line using techniques including CRISPR-Cas9, TALEN, and ZFN, and the resultant cell line could produce antibodies lacking core fucose. By knocking out the transporter gene, the cells were unable to transport GDP-fucose into the Golgi, which thereby inhibited

fucosylation. This knockout cell line was used to produce nonfucosylated anti-HER2 antibodies with enhanced ADCC (55).

In contrast to genetic knockout approaches, monosaccharide analogs have been used as inhibitors to interrupt GDP-fucose biosynthesis and thus block antibody fucosylation. Senter and coworkers (56) produced nonfucosylated antibodies using fucose analogs 2-fluorofucose and 5-alkynylfucose that inhibited the biosynthesis pathways of GDP-fucose. Nonfucosylated antibodies produced in CHO cells fed with these inhibitors showed enhanced ADCC. This method was used to produce an anti-CD40 antibody, SEA-CD40, which is currently undergoing phase I clinical trials (56). Allen and coworkers (57) later showed that the 6,6,6-trifluorofucose (fucostatin I) and a fucose-1-phosphonate analog of 6,6,6-trifluorofucose (fucostatin II) were more potent GMD inhibitors, and they were able to produce nonfucosylated antibodies with enhanced ADCC activity. More recently, Goddard-Borger and coworkers (58) found that 6,6,6-trifluorofucose inhibitor significantly decreased the core-fucosylation level of secreted IgG1 isotype mAb in hybridoma cell lines to produce low-fucose-content mAb.

Hossler and coworkers (59) reported a novel glycoengineering study that explored competitive core arabinosylation to reduce the core-fucosylation level during antibody production. They found that inclusion of a high concentration (10 mM) of D-arabinose in the cell culture resulted in almost complete arabinosylation (>98%) at the core of the produced antibody. This study suggests that D-arabinose, which is different from L-fucose in lacking the 5-methyl group in the structure, can be efficiently incorporated into the biosynthetic pathway to compete with core fucosylation during antibody expression. Interestingly, these fully arabinosylated antibodies showed a similar increase in ADCC activity as that of nonfucosylated antibodies (59). Results suggest that the methyl group present in L-fucose may play an essential structural role for the adverse effects of core fucosylation on Fc γ IIIa receptor binding and ADCC activity. Removal of a methyl group from L-fucose, i.e., its conversion to D-arabinose, may be sufficient to increase the binding affinity of antibodies to Fc γ IIIa receptor and thus to enhance ADCC activity (59).

In addition to mammalian cell line glycoengineering, nonmammalian cell lines, including yeast (31), insect (60, 61), fungi (62) and plant (29, 63, 64), that lack endogenous FUT8 activity were also explored for the production of nonfucosylated antibodies. While these nonmammalian cells lack the biosynthetic pathway to core fucosylation, they produce many nonmammalian glycoforms that could be immunogenic, such as the hypermannosylated glycoforms found in yeast and the xylose and α -1,3-fucosylated glycoforms found in plants. These limitations have been addressed by many studies that knock out the genes responsible for nonhuman glycoforms and overexpress genes that lead to more mature humanized *N*-glycans (31, 65–70). The major genetic approaches for glycoengineering to generate specific and enriched antibody glycoforms are summarized in Figure 3.

Biosynthetic Pathway Glycoengineering to Produce Antibodies with Increased Galactose or Sialic Acid Content

Probably owing to steric hindrance, the Fc *N*-glycans of a typical recombinant IgG antibody usually carry a low content of terminal galactose (approximately 80% are G0F and G1F

glycoforms) and an even lower content (less than 10%) of terminal sialylation. Because terminal galactosylation appears to be important for complement-dependent cytotoxicity (CDC) (71) and Fc sialylation is responsible for the anti-inflammatory activity of IVIG (72), there have been efforts to enhance the production of these specific glycoforms for various functional studies and to develop more efficient therapeutics.

Jefferis and coworkers (73) first showed that amino acid mutations in the Fc region of the antibody, including F241A, F243A, V264A, D265A, and R301A mutations, increased the accessibility of the glycosylation site to both galactosyltransferase and sialyltransferase, leading to more processed glycoforms. Betenbaugh and coworkers (74) produced the hypergalactosylated IgG glycoforms using a combined approach of ST3GAL4 and ST3GAL6 knockout and protein engineering of the Fc region. Using this approach with a single-point F241A mutation on IgG-Fc, they produced IgGs with 80% of G2F and, when further combined with a FUT8 knockout system, IgGs with 65% of the G2 glycoform. Further mutation of IgG-Fc at three other sites (F243A, V262E, and V264E) led to the production of 80% G2 glycoforms (74).

Raymond and coworkers (75) showed that, by overexpression of α -2,6-sialyltransferase and β -1,4-galactosyltransferase in combination with an F243A mutation at the Fc domain, antibody could be produced with more than 80% Fc sialylation in which the majority was in the α -2,6-sialylated form. Further work by Betenbaugh and coworkers (76) showed that antibodies with 77% α -2,6-sialylation could be achieved by overexpressing α -2,6-sialyltransferase and knocking out ST3GAL4 and ST3GAL6 (both genes responsible for α -2,3 sialyltransferase activity) with CRISPR/Cas9 in combination with four point mutations (F241A, F243A, V262E, V264E). The functions of these Fc sialylation-enriched antibody glycoforms have not yet been investigated.

GLYCOENGINEERING OF ANTIBODIES THROUGH IN VITRO CHEMOENZYMATIC GLYCAN REMODELING

Glycoengineering through biosynthetic pathway manipulations have enabled production of certain pure or enriched antibody glycoforms for functional studies and for further therapeutic development. Nevertheless, the quality and diversity of glycoforms that can be accessed through genetic manipulations are quite limited, owing to the complexity and restriction of the glycan biosynthetic pathways. Parallel to in vivo genetic approaches, in vitro chemoenzymatic glycan remodeling of antibodies using glycosidases and glycosyltransferases has been employed to trim or extend the sugar chains in an intact antibody. Application of this approach generated a panel of enriched Fc glycoforms, including G0F, G2F, and bisecting GlcNAc G2F of an intact antibody (77). These glycosylation-defined glycoforms were used to characterize the effects of Fc glycosylation on antibody Fc receptor binding (77). More recently, Bosques and coworkers (24) performed global enzymatic galactosylation and sialylation of IVIG to produce fully sialylated IVIG that showed enhanced anti-inflammatory activity in animal models. Despite these advances, the success of this glycosyltransferase-based strategy is heavily dependent on the existing *N*-

glycans in the antibody and the efficiency of the respective glycosidases and glycosyltransferases for sugar chain trimming and extension, respectively.

In contrast to the glycosyltransferase-based strategy that enables sugar chain elongation by adding monosaccharides one at a time, another chemoenzymatic synthesis method is based on using endoglycosidases for oligosaccharide block remodeling. This *ab initio* chemoenzymatic glycan remodeling approach has attracted tremendous interest in recent years for glycoprotein synthesis and antibody glycan remodeling (33–36, 78). It consists of two key steps: deglycosylation using endoglycosidase to remove heterogeneous *N*-glycans and subsequent enzymatic transfer *en bloc* of a preassembled *N*-glycan by a mutant endoglycosidase. This approach is characterized by its high convergence and efficiency for glycan remodeling. In this section, we highlight its development for antibody glycan remodeling with examples from recent publications.

Early Work Toward Chemoenzymatic Synthesis of Homogeneous IgG-Fc Glycoforms

In 2008, Wang and coworkers (79) performed the first endoglycosidase-catalyzed glycan remodeling on a recombinant human IgG1 Fc domain. Yeast-expressed IgG-Fc was first deglycosylated with Endo-H, and then various enzymatic transglycosylation reactions with synthetic glycan oxazolines were examined (Figure 4). Endo-A, a bacterial endoglycosidase, was able to transfer a synthetic glycan from the oxazoline derivative to the GlcNAc-Fc under mild conditions without needing to denature the protein. A subsequent study indicated that Endo-A was remarkably efficient in using various modified *N*-glycan core oxazolines for Fc glycosylation remodeling, leading to the formation of a series of structurally defined Fc glycoforms that were used to probe binding with Fc γ receptors (80). SPR binding studies indicate that sugar residues on the core could differentially affect the affinity of Fc for the Fc γ IIIa receptor. One important finding was that bisecting GlcNAc did enhance the affinity of Fc for Fc γ IIIa receptor (80). Later, Wang and coworkers (81) generated glycosynthase mutants from Endo-D, an Endo- β -*N*-acetylglucosaminidase from *Streptococcus pneumoniae*, which could use both GlcNAc-Fc and fucosylated GlcNAc-Fc as substrate for transglycosylation without product hydrolysis. Despite these advances, however, Endo-A, Endo-D, and Endo-M mutants could not transfer full-length complex-type *N*-glycan to the Fc domain. Thus, for full-scale application, new enzymes/glycosynthase mutants capable of transferring mature *N*-glycans were needed to transform full-length glycans and intact antibodies.

Glycoengineering of Intact Monoclonal Antibodies by Endo-S and Endo-S Glycosynthase Mutants

Major progress was made in 2012 when Wang and coworkers (82) reported the discovery of the novel glycosynthase mutants Endo-S-D233A and Endo-S-D233Q from Endo-S, a GH18 endoglycosidase from *Streptococcus pyogenes*. Asp-233 was identified as an essential residue that promotes formation of the sugar oxazolinium ion intermediate during endoglycosidase-catalyzed hydrolysis. As depicted in Figure 5, site-specific mutation at the Asp-233 residue of Endo-S rendered the enzyme incapable of catalyzing hydrolysis. However, the mutant can still use the synthetic glycan oxazoline (which was viewed as a transition state mimic) for glycosylation of an acceptor. Two glycosynthase mutants, Endo-

S-D233A and Endo-S-D233Q, were generated, and both glycosylated the GlcNAc- or core-fucosylated GlcNAc moiety of a deglycosylated antibody. These findings paved the way for glycan remodeling of intact therapeutic IgG antibodies to obtain new glycoforms with natural or selectively modified Fc glycans (Figure 6) (82). For example, glycan remodeling of rituximab, an anticancer monoclonal antibody, led to efficient generation of a fully sialylated glycoform and a nonfucosylated glycoform of rituximab in high yield. Compared with commercial antibodies, nonfucosylated G2 glycoforms showed more than 20-fold enhanced affinity for the Fc γ IIIa receptor. In addition, azido tag could be selectively introduced at the Fc glycan, providing an opportunity for further chemoselective modification by click chemistry (82).

The discovery of the glycosynthase mutants Endo-S-D233A and Endo-S-D233Q for enzymatic glycan remodeling of intact antibodies opens a way to generate specific antibody glycoforms for functional studies (71, 83–88). For example, Wong and coworkers (83) applied these Endo-S mutants for Fc glycan remodeling of an intact antibody to yield a series of antibody glycoforms to probe their biological activities, which led to the identification of a sialylated biantennary *N*-glycan as an optimized glycoform for the enhancement of ADCC and CDC. Shirai and coworkers (84) assembled a relatively large glycoform library of anti-HER2 antibodies and used it to study the effects of Fc glycosylation specifically on Fc γ receptor binding, ADCC activity, and CDC activity of the antibody. Davis and coworkers (85) applied the enzymatic glycan remodeling approach for transforming an anti-HER2 antibody with various tags as chemical reporters, which would be useful for fluorescent labeling of antibodies or for antibody–drug conjugation.

Expanding the Antibody Glycoengineering Toolbox with Endo-S2, Endo-F3, and Their Mutants

The pair of wild-type Endo-S and its glycosynthase mutant (Endo-S-D233A or Endo-S-D233Q) is particularly useful for Fc glycan remodeling of intact antibodies. However, Endo-S is limited by its strict substrate specificity. Endo-S can act only on typical biantennary complex-type Fc *N*-glycans and possesses only marginal activity on high-mannose or hybrid-type *N*-glycans (89, 90). As a continuous effort to expand the scope of glycan remodeling, Wang and coworkers (91) generated novel glycosynthase mutants from Endo-S2, an endoglycosidase from *S. pyogenes* of serotype M49 (90, 92). Endo-S2 shows approximately 37% sequence identity to Endo-S but demonstrates much broader substrate specificity for glycans than does Endo-S. Studies have shown that Endo-S2 is capable of deglycosylating all the major types of Fc *N*-glycans, including high-mannose type, complex type, and hybrid type (90). Sequence alignment identified amino acid D184 homologous to the previously identified D233 of Endo-S. Interestingly, mutation at D184 provided an array of mutants showing transglycosylation activity with diminished hydrolytic activity. Among them, the D184M mutant demonstrated the highest catalytic efficiency and was able to transform an intact antibody into not only complex-type, but also high-mannose- and hybrid-type glycoforms (Figure 7) (91). As a related study, Wong and coworkers (93) also generated several new mutants of Endo-S2, including T138Q, which also behaved as glycosynthase. In addition, Wang and coworkers (94) performed site-specific covalent immobilization of Endo-S2 and its glycosynthase mutant D184M and found that the immobilized enzymes

could also efficiently function for glycan remodeling. This technology enabled streamlined chemoenzymatic glycan remodeling of antibodies without the need to separate the intermediate and enzymes.

As an example of application, Wang and coworkers (95) constructed a focused library of homogeneous glycoforms of antibody rituximab and used them to investigate the effects of Fc glycosylation on effector function. The side-by-side comparative studies of the different glycoforms included in vitro Fc γ receptor binding analysis, cell-based ADCC assays, and in vivo cellular depletion experiments. Experimental data clearly indicated that core fucosylation of the antibodies adversely affected Fc γ IIIa receptor binding, in vitro ADCC, and in vivo IgG-mediated cellular depletion, regardless of their sialylation status. However, the effect of sialylation on ADCC was dependent on core fucosylation. In the presence of core fucosylation, sialylation significantly decreased ADCC in a cell-based assay and suppressed antibody-mediated cell killing in vivo. By contrast, sialylation did not significantly impact ADCC activity in the absence of core fucosylation (95).

Wang and coworkers (96) also reported the generation of novel glycosynthase mutants from Endo-F3, another bacterial GH18 family endoglycosidase. Interestingly, the resulting D165A and D165Q mutants were highly selective for core-fucosylated *N*-glycan and transferred both bi- and triantennary *N*-glycans to intact antibodies (Figure 7). The D165A and D165Q mutants of Endo-F3 were the first glycosynthases capable of transferring highly branched *N*-glycans. Previously reported glycosynthase mutants from Endo-S and Endo-S2 enzymes could not act on triantennary Fc *N*-glycans.

Wong and coworkers (97) recently demonstrated that wild-type Endo-S2 transferred complex-type *N*-glycan to an antibody from a ground-state sialoglycopeptide in a kinetic controlled manner, when a large excess (more than 1,000 molar equivalents) of the sialoglycopeptide was used. Manabe et al. (98) obtained similar results via an Endo-CC mutant from a bacterial endoglycosidase. To explore the potential of enzymes capable of using ground-state substrate for glycosylation, Iwamoto and coworkers (99) generated new double mutants, including D233Q/Q303L and D233Q/E350Q from Endo-S. These mutants showed unusual transglycosylation activity and used sialoglycopeptide as substrate for glycosylation of deglycosylated antibodies with a relatively small excess of the donor substrate (99).

However, in addition to Fc glycosylation, some mAbs are also glycosylated at the Fab domains. Moreover, approximately 20% of circulating IVIG carries *N*-glycans at the Fab domains. Studies have shown that Fab glycosylation can play important roles in immunity, antigen recognition, and serum half-life of the antibodies (16, 100). Hitherto, it had been a difficult task to selectively modify Fc or Fab glycans of an intact antibody or a multiply glycosylated protein. Recently, Wang and coworkers (101) reported efficient chemoenzymatic synthesis that allows discriminative glycoengineering of both Fc and Fab glycans of cetuximab, a therapeutic antibody. Cetuximab is a chimeric mouse-human anti-EGFR monoclonal antibody that is widely used for the treatment of colorectal, head, and neck cancers (102). It is glycosylated in both Fab and Fc domains at the N88 and N297 sites, respectively, of the heavy chain with tremendous heterogeneity in the *N*-glycan structures

(103, 104). The authors showed that Fc and Fab glycans could be independently and discriminatively remodeled to create distinct Fc and Fab glycoforms by taking advantage of the substrate specificity of three endoglycosidases (Endo-S, Endo-S2, and Endo-F3) and their glycosynthase mutants as well as the unique substrate selectivity of *Lactobacillus casei* α 1,6-fucosidase. They generated an optimal homogeneous glycoform of cetuximab in which the heterogeneous and immunogenic Fab *N*-glycans were replaced with a sialylated *N*-glycan and the core-fucosylated Fc *N*-glycans were remodeled with a nonfucosylated and fully galactosylated *N*-glycan. Glycoengineered cetuximab demonstrated increased affinity for the Fc γ IIIa receptor and showed significantly enhanced ADCC activity (101). Taken together, these endoglycosidase-catalyzed glycan remodeling methods provide a general platform for glycoengineering of antibodies to afford diverse homogeneous antibody glycoforms, which are valuable tools for various biological studies and for development of efficient antibody-based therapeutics.

GLYCOENGINEERING FOR SITE-SPECIFIC ANTIBODY–DRUG CONJUGATION

Antibody–drug conjugation is emerging as a very promising strategy for selective delivery of highly toxic drugs to target cells. Antibody–drug conjugates combine the specificity of antibodies and the high potency of drugs to achieve targeted cell killing (37, 105–115). Three antibody–drug conjugates were recently approved by the US Food and Drug Administration, including Seattle Genetics’s Adcetris® (CD30-specific brentuximab–drug conjugate) used for treatment of relapsed Hodgkin lymphoma (116), Genentech/Roche’s Kadcyla® (HER2-specific trastuzumab–drug conjugate) used for the treatment of metastatic breast cancer (117), and Pfizer/Wyeth’s Besponsa™ (CD22-specific inotuzumab–drug conjugate) for relapsed or refractory B cell precursor acute lymphoblastic leukemia (118). Currently, many antibody–drug conjugates are in clinical trial and/or preclinical development.

Three key components constitute an antibody–drug conjugate construct: the antibody, the drug (payload), and the linker that conjugates the two. Multiple factors can affect the outcome and efficacy of an antibody–drug conjugate: (a) An antibody of choice must be highly specific for a unique and/or highly expressed antigen and should be efficiently internalized upon antigen binding. (b) The payload (drug) must be highly potent and can be readily modified for antibody conjugation. (c) The linker should be flexible and, hopefully, can enhance the water solubility of most hydrophobic drugs for conjugation. (d) Conjugation chemistry should be efficient and consistent. In addition, antibody–drug conjugates must be stable in circulation without prematurely releasing the drug, but upon internalization into cells, the drug should be rapidly released via an appropriate mechanism to reach its intracellular target.

Two fundamental methods were used to prepare the first generation of antibody–drug conjugates, including the three mentioned above that were recently approved: nonselective acylation of lysine residues with an activated ester of a modified drug and thiol-alkylation of in situ-generated cysteine residues from the hinge region with a maleimide-modified drug.

However, these methods lack site selectivity and typically end up with heterogeneous mixtures of products with differing drug/antibody ratios as well as conjugation sites (regioisomers) (119, 120). These parameters significantly impact the properties of antibody–drug conjugates such as their pharmacokinetics, antigen binding, stability, toxicity, and immunogenicity. Therefore, there is an urgent need to develop a site-specific conjugation methodology to produce structurally homogeneous antibody–drug conjugates that demonstrate well-defined properties, can be manufactured more consistently, and can be developed with fewer regulatory hurdles.

In one of the several approaches to obtain structurally defined homogeneous antibody–drug conjugates (121, 122), the protein part is engineered to introduce additional cysteines, unnatural amino acids, or peptide tags at predetermined site(s), such that the tagged antibody can then react with a modified drug molecule by chemoselective or enzymatic ligation (122–126). This approach usually requires redesign and expression of the target antibody with the engineered tags. Another approach is to engineer the Fc glycans of the antibody for site-specific ligation (85, 127–135). All IgG antibodies carry a highly conserved *N*-glycan at the Asn-297 of the IgG-Fc domain. The advantage of this approach is that site-selective modification at this conserved *N*-glycan does not change the protein structure and thus usually will not affect the antibody's inherent affinity for antigens. In addition, it can be readily applied to both existing commercial antibodies and newly developed recombinant antibodies. Below, we highlight recent advances in glycoengineering of antibodies for site-specific antibody–drug conjugation.

Glyco-Conjugation Through Mild Oxidation of Fc Glycans Coupled with Chemoselective Drug Conjugation

Oligosaccharides containing vicinal *cis* diol structures can undergo a mild oxidative ring-opening reaction by periodate treatment to generate aldehyde groups, which can be further functionalized with other groups, including hydrazides and aminooxy groups for bioconjugation. Several terminal residues, including fucose, galactose, and sialic acids that contain vicinal *cis* diols, can be oxidized selectively with mild periodate oxidation. This concept was first applied to antibody modification in the 1980s, demonstrating that biotinylated and radiolabeled antibodies could be produced by periodate oxidation of antibody oligosaccharides followed by chemoselective conjugation. However, antibody glycosylation is tremendously heterogeneous. For example, a majority of therapeutic mAbs produced in CHO or HEK293 cell lines exist as a mixture of G0F, G1F, and G2F Fc glycoforms, where G and F represent galactose and core fucose, respectively, and the numbers (0–2) indicate the terminal galactose moieties of the Fc glycans. As a result, direct oxidation of natural or recombinant antibodies usually led to heterogeneous mixtures of the conjugates. To have better control of the homogeneity of antibody–drug conjugates, Neri and coworkers (127) developed a CHO cell line that could control Fc *N*-glycosylation at the G0F glycoform, where fucose could be selectively oxidized. Thus, treatment of the G0F antibody with 10-mM NaIO₄ selectively oxidized the fucose moiety to provide an aldehyde derivative, which was used to append a fluorophore or a drug moiety through hydrazone formation (Figure 8a). The prepared antibody–drug conjugate, which contains an analog of dolastatin, demonstrated potent cell cytotoxicity against cancer cells in *in vitro* assays (127).

In contrast to core fucose, oxidation of sialic acid can take place under relatively mild conditions because their vicinal diols are present as a glycerol moiety that is less hindered and more susceptible to periodate oxidation. However, sialic acid content in most recombinant mAbs is relatively low. For example, mAbs produced in CHO cell lines usually contain <5% sialic acids in their Fc *N*-glycans. To address this problem, Zhou and coworkers (128) applied a chemoenzymatic glycan remodeling method to introduce sialic acid in the Fc *N*-glycans in trastuzumab. First, Fc *N*-glycans in trastuzumab were extended by glycosylation with β 1,4-galactosyltransferase and α 2,6-sialyltransferase using UDP-Gal and CMP-Sia, respectively, as donor substrates, yielding mainly monosialylated Fc glycoforms of trastuzumab owing to α 2,6-sialyltransferase site selectivity. Second, the sialylated Fc *N*-glycans were oxidized with a low concentration (1 mM) of NaIO₄ to generate the aldehyde moiety. Finally, coupling of the aldehyde group with an aminoxy-functionalized cytotoxic agent through oxime formation yielded the corresponding antibody–drug conjugate with a drug/antibody ratio of ~1.6 (Figure 8b). This study examined three different antibodies and two cytotoxic agents. The resulting glyco-conjugated antibody–drug conjugates showed selective cytotoxicity against antigen-positive tumor cells in cell-based analysis and also demonstrated significantly enhanced antitumor efficacy over the unmodified antibody in a HER2-positive tumor animal model (128). The results suggest that enzymatic remodeling of Fc glycans to incorporate sialic acid moiety, combined with oxidation and oxime ligation, provides a promising method to generate antibody–drug conjugates with well-defined product profiles. Notably, the use of a low concentration (1 mM) of periodate for efficient oxidation of the sugar residues was critical, as the use of a relatively high periodate concentration (more than 5 mM) also resulted in oxidation of the methionine residues at the Fc domain, which adversely affected the binding of the antibody with the neonatal Fc receptor. Reduced affinity of antibody–drug conjugates with the neonatal Fc receptor might result in their much shorter half-life in vivo. Thus, careful control of the oxidant's concentration and reaction time should be exercised to avoid oxidation of the methionine residues in the protein backbone when this method is used to make antibody–drug conjugates.

Glycoengineering to Incorporate Tags for Bioorthogonal Conjugation

In addition to the functionalization of antibody glycans via periodate oxidation that might cause side reactions on methionine and other residues, alternative chemoenzymatic glycoengineering methods to incorporate a handle in the antibody glycans that could be readily used for bioorthogonal reactions with modified cytotoxic agents have also been explored to produce antibody–drug conjugates. As an example, Boons and coworkers (129) performed enzymatic remodeling on the Fc *N*-glycans of an anti-CD22 monoclonal antibody to introduce an azide-tagged sialic acid, which was then reacted with a modified cytotoxic agent via a strain-promoted azide-alkyne cycloaddition to afford the antibody–drug conjugate. To maximize incorporation of sialic acid residues, truncated Fc *N*-glycans containing mainly G0F and G1F forms were first extended to the G2F glycoform by adding galactose moieties to G0F and G1F via β 1,4GalT-catalyzed galactosylation. Then, mammalian α 2,6-sialyltransferase was used to introduce a modified sialic acid carrying a 9-azide group to the Fc *N*-glycans of the antibody. A copper-free strain-promoted alkyne-azide cycloaddition reaction between the azide-tagged antibody and doxorubicin (a cytotoxic

agent) containing an activated cyclooctyne moiety was used to yield the antibody–drug conjugate (Figure 9a). Mammalian α 2,6-sialyltransferase was efficient in transforming galactose-terminated Fc *N*-glycans to introduce four azide-modified sialic acid moieties per antibody. The synthesized antibody–drug conjugate showed dose-dependent and targeted cell killing, demonstrating how this method provides an attractive approach for constructing structurally well-defined antibody–drug conjugates.

As an alternative approach for introducing a clickable tag into antibodies via enzymatic glycoengineering, Qasba and coworkers (136–142) generated novel β 1,4-galactosyltransferase mutants that transferred tag-modified GalNAc moieties to terminal GlcNAc moieties in the glycans of glycoproteins and antibodies. Early work by Ramakrishnan & Qasba (130) demonstrated that a novel mutant of β 1,4-galactosyltransferase I, namely Y289L, efficiently transferred GalNAc to an acceptor from UDP-GalNAc. Hsieh-Wilson and coworkers (131) found that the Y289L mutant could accommodate C2-modified UDP-Gal and was able to transfer a C2-keto-Gal from UDP-C2-keto-Gal to *O*-GlcNAc-glycosylated proteins. The resulting keto-tagged proteins could be detected by reaction with an aminooxy-modified biotin for chemiluminescence. Hsieh-Wilson and coworkers (143) further reported that an azide-modified UDP-GalNAc, UDP-GalNAz, could also be recognized by this mutant enzyme as a substrate for tagging *O*-GlcNAc moiety. This finding provided a method for direct fluorescence detection and cellular imaging of *O*-GlcNAc-glycosylated proteins, as the azide-labeled proteins could be readily probed through bioorthogonal reactions (143).

Qasba and coworkers (133, 136, 137, 140, 141) first applied this chemoenzymatic strategy for glycoengineering of antibodies. To introduce the modified sugar, C2-keto-Gal, the IgG1 antibody was treated with sialidase and galactosidase to remove the sialic acid and galactose residues, respectively, then the C2-keto-Gal moiety was attached to the exposed GlcNAc residues in the Fc *N*-glycans by the mutant enzyme. The introduced keto functional group allowed site-specific labeling of the antibody with various probes, e.g., fluorescent tags, via chemoselective oxime formation reactions (136, 137, 141).

Dimitrov and coworkers (132) applied this chemoenzymatic synthesis approach for generating antibody–drug conjugates through site-selective conjugation of a drug at the Fc glycans. Thus, m860, a newly discovered anti-HER2 antibody, was modified with a reactive keto-Gal moiety using Y289L. Then, the functionalized antibody was reacted with aminooxy auristatin F to conjugate the drug selectively to Fc *N*-glycans via oxime formation (Figure 9b). The antibody–drug conjugate thus prepared exhibited potent cell-killing activity against HER2-positive cancer cells, including trastuzumab-resistant breast cancer cells (132). This chemoenzymatic method could be generally applicable for preparing antibody–drug conjugates with different antibodies and payloads. As a related application, Lewis and coworkers (144) used it for selective radiolabeling of antibodies at Fc *N*-glycans. The prostate-specific membrane antigen-targeting antibody J591 was selected, and after degalactosylation, Y289L was used to incorporate an azide-modified GalNAc (GalNAz) at the Fc *N*-glycans. Subsequent strain-promoted click conjugation of desferrioxamine (the chelator-modified dibenzocyclooctyne) and the azide-tagged antibody, followed by radiolabeling of the chelator-modified antibody with ^{89}Zr , afforded the ^{89}Zr -labeled

antibody conjugate. The radiolabeled antibody conjugate demonstrated high stability and immune reactivity in vitro and showed highly selective tumor cell uptake in an athymic nude mouse model (144). The antibody–drug conjugation using click reactions such as the azide–alkyne cycloaddition create unnatural linkages, which may be immunogenic and result in undesired immune response. Yet, little information is available on this aspect of antibody–drug conjugates. Future studies should be directed to careful evaluation of the immunogenicity of such antibody–drug conjugates.

Glyco-Conjugation by Chemoenzymatic Glycan Remodeling

Recently, Huang and coworkers (134, 135) applied Endo-S-catalyzed Fc glycan remodeling technology for antibody–drug conjugation. In this approach, the antibody trastuzumab was deglycosylated using wild-type Endo-S, then an azide-tagged glycan oxazoline generated in situ was used as a substrate for glycosynthase Endo-S-D233Q and was transferred to the deglycosylated trastuzumab to yield an azide-tagged antibody that was finally conjugated with cytotoxic agents including MMAE by click chemistry to afford glycosite-specific antibody–drug conjugates (Figure 10a). A dual-drug antibody–drug conjugate was also prepared by lysine conjugation on top of the chemoenzymatic transformation. Cell-based assays indicated that release of small-molecule drugs from antibody–drug conjugates relied on the cleavable Val-Cit linker fragment embedded in the structure (134). Independently, Davis and coworkers also applied Endo-S-catalyzed glycan remodeling technology for Fc glycan site-specific introduction of chemical reporters on sialic acid moieties, which could be further functionalized to carry various cargo compounds, including fluorescent probes and cytotoxic agents (85). In another related study, van Delft and coworkers (145) reported an alternative chemoenzymatic method that combines endoglycosidase-catalyzed deglycosylation and galactosyltransferase-catalyzed attachment of a keto- or azide-labeled monosaccharide to tag the antibody. After these enzymatic modifications, strain-promoted click chemistry was used to link the cytotoxic agents to the antibody to afford the antibody–drug conjugate (Figure 10b).

In addition to in vitro chemoenzymatic transformations, clickable tags can be introduced to Fc *N*-glycans through metabolic glycoengineering for making antibody–drug conjugates. During their study of screening for various monosaccharide derivatives as potential inhibitors for core fucosylation, Senter and coworkers (146) found that a 6-thiofucose peracetate could be incorporated efficiently into Fc *N*-glycans to produce 5-thiofucose-tagged antibodies in CHO cells. The cysteine disulfide–masked 5-thiofucose derivative in the product could be selectively reduced to free thiols, which could be conjugated with maleimide-linked MMAE to form site-specific antibody–drug conjugates.

Overall, conjugation of drugs specifically at the Fc *N*-glycans offers several advantages. First, because the site of conjugation is far from the Fab domains, it would not usually interfere with their antigen binding. Second, the glycan is relatively hydrophilic, so it could well balance the hydrophobic nature of most small-molecule cytotoxic agents, thus reducing the possibility of antibody aggregation. Third, site-specific conjugation would also facilitate optimization, characterization, and reproducibility of the final products, which can significantly reduce the hurdles toward regulatory approval.

CONCLUSIONS

Recent development of various glycoengineering methods, including in vivo genetic manipulation of *N*-glycan biosynthetic pathways, in vitro chemoenzymatic glycan remodeling, and Fc glycan site-specific antibody–drug conjugation, has made it possible to produce a range of homogeneous antibody glycoforms and site-specifically modified antibodies for functional studies. Some glycoengineered antibodies have been approved as new therapeutic agents for treatment of human diseases. Further studies along these lines are expected to expand the toolbox of accessible glycoforms, which will be highly valuable for detailed study of the structure–activity relationship of antibody functions and for the development of better antibody-based therapeutics.

ACKNOWLEDGMENTS

We thank other members of the Wang laboratory for stimulating discussions during the writing of this review. The National Institutes of Health is acknowledged for financial support of this work (grant numbers R01 GM096973 and R01 GM080374).

DISCLOSURE STATEMENT

L.-X. Wang is the founder and a member of GlycoT Therapeutics, LLC. Other coauthors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

Glossary

Antibody

class of secretory proteins, also called immunoglobulins, that are used in the adaptive immune system to neutralize pathogens

Fab

antigen-binding fragment

Antigen

any foreign substance specifically recognized by antibodies

Fc

crystallizable fragment

***N*-glycan**

sugar moiety covalently attached by an *N*-glycosidic bond to a side chain carboxamide of asparagine residue on a protein

Glycoform

glycoprotein variants that possess the same polypeptide backbone but differ in the nature and site of glycosylation

Glycosylation

attachment of a carbohydrate moiety to another molecule through formation of a glycosidic bond

Chemoenzymatic synthesis

combined chemical and enzymatic approach to the synthesis of natural and unnatural compounds

Glycoengineering

specific chemical or biological alteration of glycan structures in a glycoconjugate

Glycoprotein

covalent conjugate of a protein and a mono- or oligosaccharide

Nonfucosylated antibody

antibody carrying no core fucose moiety on its Fc domain

Transglycosylation

glycoside hydrolase-catalyzed reaction in which the released sugar is transferred to an acceptor other than water to form a new glycoside

Glycosynthase

glycosidase mutant that lacks intrinsic hydrolytic activity but can use an activated species as a donor substrate for glycosylation

LITERATURE CITED

1. Yu X, Marshall MJE, Cragg MS, Crispin M. 2017 Improving antibody-based cancer therapeutics through glycan engineering. *BioDrugs* 31:151–66 [PubMed: 28466278]
2. Ecker DM, Jones SD, Levine HL. 2015 The therapeutic monoclonal antibody market. *mAbs* 7:9–14 [PubMed: 25529996]
3. Aggarwal RS. 2014 What's fueling the biotech engine—2012 to 2013. *Nat. Biotechnol* 32:32–39 [PubMed: 24406926]
4. Mimura Y, Church S, Ghirlando R, Ashton PR, Dong S, et al. 2000 The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol. Immunol* 37:697–706 [PubMed: 11275255]
5. Mimura Y, Sondermann P, Ghirlando R, Lund J, Young SP, et al. 2001 Role of oligosaccharide residues of IgG1-Fc in FcγRIIb binding. *J. Biol. Chem* 276:45539–47 [PubMed: 11567028]
6. Sondermann P, Huber R, Oosthuizen V, Jacob U. 2000 The 3.2-Å crystal structure of the human IgG1 Fc fragment-FcγRIII complex. *Nature* 406:267–73 [PubMed: 10917521]
7. Wormald MR, Rudd PM, Harvey DJ, Chang SC, Scragg IG, Dwek RA. 1997 Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* 36:1370–80 [PubMed: 9063885]
8. Takahashi N, Nakagawa H, Fujikawa K, Kawamura Y, Tomiya N. 1995 Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. *Anal. Biochem* 226:139–46 [PubMed: 7540366]
9. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, et al. 2015 Symbol nomenclature for graphical representations of glycans. *Glycobiology* 25:1323–24 [PubMed: 26543186]
10. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. 2007 The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu. Rev. Immunol* 25:21–50 [PubMed: 17029568]
11. Jefferis R. 2005 Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog* 21:11–16 [PubMed: 15903235]

12. Jefferis R 2009 Glycosylation as a strategy to improve antibody-based therapeutics. *Nat. Rev. Drug Discov* 8:226–34 [PubMed: 19247305]
13. Nimmerjahn F, Ravetch JV. 2008 Anti-inflammatory actions of intravenous immunoglobulin. *Annu. Rev. Immunol* 26:513–33 [PubMed: 18370923]
14. Anthony RM, Wermeling F, Ravetch JV. 2012 Novel roles for the IgG Fc glycan. *Ann. N.Y. Acad. Sci* 1253:170–80 [PubMed: 22288459]
15. Reusch D, Tejada ML. 2015 Fc glycans of therapeutic antibodies as critical quality attributes. *Glycobiology* 25:1325–34 [PubMed: 26263923]
16. van de Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y. 2016 The emerging importance of IgG Fab glycosylation in immunity. *J. Immunol* 196:1435–41 [PubMed: 26851295]
17. Alter G, Ottenhoff THM, Joosten SA. 2018 Antibody glycosylation in inflammation, disease and vaccination. *Semin. Immunol* 39:102–10 [PubMed: 29903548]
18. Ferrara C, Grau S, Jäger C, Sondermann P, Brünker P, et al. 2011 Unique carbohydrate–carbohydrate interactions are required for high affinity binding between FcγRIII and antibodies lacking core fucose. *PNAS* 108:12669–74 [PubMed: 21768335]
19. Niwa R, Shoji-Hosaka E, Sakurada M, Shinkawa T, Uchida K, et al. 2004 Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. *Cancer Res* 64:2127–33 [PubMed: 15026353]
20. Illidge T, Cheadle EJ, Donaghy C, Honeychurch J. 2014 Update on obinutuzumab in the treatment of B-cell malignancies. *Expert Opin. Biol. Ther* 14:1507–17 [PubMed: 25190612]
21. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006 Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313:670–73 [PubMed: 16888140]
22. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. 2008 Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320:373–76 [PubMed: 18420934]
23. Schwab I, Mihai S, Seeling M, Kasperkiewicz M, Ludwig RJ, Nimmerjahn F. 2014 Broad requirement for terminal sialic acid residues and FcγRIIB for the preventive and therapeutic activity of intravenous immunoglobulins in vivo. *Eur. J. Immunol* 44:1444–53 [PubMed: 24505033]
24. Washburn N, Schwab I, Ortiz D, Bhatnagar N, Lansing JC, et al. 2015 Controlled tetra-Fc sialylation of IVIG results in a drug candidate with consistent enhanced anti-inflammatory activity. *PNAS* 112:E1297–306 [PubMed: 25733881]
25. Shade KT, Platzer B, Washburn N, Mani V, Bartsch YC, et al. 2015 A single glycan on IgE is indispensable for initiation of anaphylaxis. *J. Exp. Med* 212:457–67 [PubMed: 25824821]
26. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. 1999 Engineered glycoforms of an anti-neuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol* 17:176–80 [PubMed: 10052355]
27. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, Kusunoki M, Iida S, et al. 2004 Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol. Bioeng* 87:614–22 [PubMed: 15352059]
28. Stanley P, Sundaram S, Tang J, Shi S. 2005 Molecular analysis of three gain-of-function CHO mutants that add the bisecting GlcNAc to N-glycans. *Glycobiology* 15:43–53 [PubMed: 15329358]
29. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, et al. 2006 Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat. Biotechnol* 24:1591–97 [PubMed: 17128273]
30. Strasser R, Castilho A, Stadlmann J, Kunert R, Quendler H, et al. 2009 Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous β1,4-galactosylated N-glycan profile. *J. Biol. Chem* 284:20479–85 [PubMed: 19478090]
31. Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, et al. 2006 Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nat. Biotechnol* 24:210–15 [PubMed: 16429149]

32. Zhou Q, Shankara S, Roy A, Qiu H, Estes S, et al. 2008 Development of a simple and rapid method for producing non-fucosylated oligomannose containing antibodies with increased effector function. *Biotechnol. Bioeng* 99:652–65 [PubMed: 17680659]
33. Li C, Wang LX. 2018 Chemoenzymatic methods for the synthesis of glycoproteins. *Chem. Rev* 118:8359–413 [PubMed: 30141327]
34. Fairbanks AJ. 2017 The ENGases: versatile biocatalysts for the production of homogeneous *N*-linked glycopeptides and glycoproteins. *Chem. Soc. Rev* 46:5128–46 [PubMed: 28681051]
35. Danby PM, Withers SG. 2016 Advances in enzymatic glycoside synthesis. *ACS Chem. Biol* 11:1784–94 [PubMed: 27176929]
36. Wang LX, Lomino JV. 2012 Emerging technologies for making glycan-defined glycoproteins. *ACS Chem. Biol* 7:110–22 [PubMed: 22141574]
37. Agarwal P, Bertozzi CR. 2015 Site-specific antibody–drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjug. Chem* 26:176–92 [PubMed: 25494884]
38. Zauner G, Selman MH, Bondt A, Rombouts Y, Blank D, et al. 2013 Glycoproteomic analysis of antibodies. *Mol. Cell. Proteom* 12:856–65
39. Stadlmann J, Weber A, Pabst M, Anderle H, Kunert R, et al. 2009 A close look at human IgG sialylation and subclass distribution after lectin fractionation. *Proteomics* 9:4143–53 [PubMed: 19688751]
40. Wuhler M, Stam JC, van de Geijn FE, Koeleman CA, Verrips CT, et al. 2007 Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. *Proteomics* 7:4070–81 [PubMed: 17994628]
41. Stanley P, Siminovitch L. 1977 Complementation between mutants of CHO cells resistant to a variety of plant lectins. *Somat. Cell Genet* 3:391–405 [PubMed: 601679]
42. Stanley P. 1992 Glycosylation engineering. *Glycobiology* 2:99–107 [PubMed: 1606361]
43. Ripka J, Adamany A, Stanley P. 1986 Two Chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose. *Arch. Biochem. Biophys* 249:533–45 [PubMed: 2428310]
44. Ohyama C, Smith PL, Angata K, Fukuda MN, Lowe JB, Fukuda M. 1998 Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. *J. Biol. Chem* 273:14582–87 [PubMed: 9603974]
45. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, et al. 2003 The absence of fucose but not the presence of galactose or bisecting *N*-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem* 278:3466–73 [PubMed: 12427744]
46. Yver A, Homery MC, Fuseau E, Guemas E, Dhainaut F, et al. 2012 Pharmacokinetics and safety of role-dumab, a novel human recombinant anti-RhD antibody with an optimized Fc for improved engagement of FCγRIII, in healthy volunteers. *Vox Sang* 103:213–22 [PubMed: 22568808]
47. Sharman JP, Farber CM, Mahadevan D, Schreeder MT, Brooks HD, et al. 2017 Ublituximab (TG-1101), a novel glycoengineered anti-CD20 antibody, in combination with ibrutinib is safe and highly active in patients with relapsed and/or refractory chronic lymphocytic leukaemia: results of a phase 2 trial. *Br. J. Haematol* 176:412–20 [PubMed: 27982425]
48. Ferrara C, Brunker P, Suter T, Moser S, Puntener U, Umana P. 2006 Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous β1, 4-*N*-acetylglucosaminyltransferase III and Golgi α-mannosidase II. *Biotechnol. Bioeng* 93:851–61 [PubMed: 16435400]
49. Ratner M. 2014 Genentech's glyco-engineered antibody to succeed Rituxan. *Nat. Biotechnol* 32:6–7 [PubMed: 24406911]
50. Yamane-Ohnuki N, Satoh M. 2009 Production of therapeutic antibodies with controlled fucosylation. *mAbs* 1:230–36 [PubMed: 20065644]
51. de Lartigue J. 2012 Mogamulizumab for the treatment of adult T-cell leukemia/lymphoma. *Drugs Today* 48:655–60 [PubMed: 23110261]

52. Kanda Y, Imai-Nishiya H, Kuni-Kamochi R, Mori K, Inoue M, et al. 2007 Establishment of a GDP-mannose 4,6-dehydratase (GMD) knockout host cell line: a new strategy for generating completely non-fucosylated recombinant therapeutics. *J. Biotechnol* 130:300–10 [PubMed: 17559959]
53. Louie S, Haley B, Marshall B, Heidersbach A, Yim M, et al. 2017 FX knockout CHO hosts can express desired ratios of fucosylated or afucosylated antibodies with high titers and comparable product quality. *Biotechnol. Bioeng* 114:632–44 [PubMed: 27666939]
54. von Horsten HH, Ogorek C, Blanchard V, Demmler C, Giese C, et al. 2010 Production of non-fucosylated antibodies by co-expression of heterologous GDP-6-deoxy-d-lyxo-4-hexulose reductase. *Glycobiology* 20:1607–18 [PubMed: 20639190]
55. Chan KF, Shahreel W, Wan C, Teo G, Hayati N, et al. 2016 Inactivation of GDP-fucose transporter gene (Slc35c1) in CHO cells by ZFNs, TALENs and CRISPR-Cas9 for production of fucose-free antibodies. *Biotechnol. J* 11:399–414 [PubMed: 26471004]
56. Okeley NM, Alley SC, Anderson ME, Boursalian TE, Burke PJ, et al. 2013 Development of orally active inhibitors of protein and cellular fucosylation. *PNAS* 110:5404–9 [PubMed: 23493549]
57. Allen JG, Mujacic M, Frohn MJ, Pickrell AJ, Kodama P, et al. 2016 Facile modulation of antibody fucosylation with small molecule fucostatin inhibitors and cocrystal structure with GDP-mannose 4,6-dehydratase. *ACS Chem. Biol* 11:2734–43 [PubMed: 27434622]
58. McKenzie NC, Scott NE, John A, White JM, Goddard-Borger ED. 2018 Synthesis and use of 6,6,6-trifluoro-l-fucose to block core-fucosylation in hybridoma cell lines. *Carbohydr. Res* 465:4–9 [PubMed: 29874559]
59. Hossler P, Chumsae C, Racicot C, Ouellette D, Ibraghimov A, et al. 2017 Arabinosylation of recombinant human immunoglobulin-based protein therapeutics. *mAbs* 9:715–34 [PubMed: 28375048]
60. Iizuka M, Ogawa S, Takeuchi A, Nakakita S, Kubo Y, et al. 2009 Production of a recombinant mouse monoclonal antibody in transgenic silkworm cocoons. *FEBS J* 276:5806–20 [PubMed: 19740109]
61. Tada M, Tatematsu K-I, Ishii-Watabe A, Harazono A, Takakura D, et al. 2015 Characterization of anti-CD20 monoclonal antibody produced by transgenic silkworms (*Bombyx mori*). *mAbs* 7:1138–50 [PubMed: 26261057]
62. Ward M, Lin C, Victoria DC, Fox BP, Fox JA, et al. 2004 Characterization of humanized antibodies secreted by *Aspergillus niger*. *Appl. Env. Microbiol* 70:2567–76 [PubMed: 15128505]
63. Schähs M, Strasser R, Stadlmann J, Kunert R, Rademacher T, Steinkellner H. 2007 Production of a monoclonal antibody in plants with a humanized *N*-glycosylation pattern. *Plant Biotechnol. J* 5:657–63 [PubMed: 17678502]
64. Strasser R, Stadlmann J, Schähs M, Stiegler G, Quendler H, et al. 2008 Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like *N*-glycan structure. *Plant Biotechnol. J* 6:392–402 [PubMed: 18346095]
65. Wildt S, Gerngross TU. 2005 The humanization of *N*-glycosylation pathways in yeast. *Nat. Rev. Microbiol* 3:119–28 [PubMed: 15685223]
66. Bosch D, Castilho A, Loos A, Schots A, Steinkellner H. 2013 *N*-glycosylation of plant-produced recombinant proteins. *Curr. Pharm. Des* 19:5503–12 [PubMed: 23394562]
67. Castilho A, Gattinger P, Grass J, Jez J, Pabst M, et al. 2011 *N*-glycosylation engineering of plants for the biosynthesis of glycoproteins with bisected and branched complex *N*-glycans. *Glycobiology* 21:813–23 [PubMed: 21317243]
68. Castilho A, Bohorova N, Grass J, Bohorov O, Zeitlin L, et al. 2011 Rapid high yield production of different glycoforms of Ebola virus monoclonal antibody. *PLOS ONE* 6:e26040 [PubMed: 22039433]
69. Zeitlin L, Pettitt J, Scully C, Bohorova N, Kim D, et al. 2011 Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. *PNAS* 108:20690–94 [PubMed: 22143789]
70. Mabashi-Asazuma H, Kuo C-W, Khoo K-H, Jarvis DL. 2013 A novel baculovirus vector for the production of nonfucosylated recombinant glycoproteins in insect cells. *Glycobiology* 24:325–40 [PubMed: 24362443]

71. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, et al. 2015 Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J. Clin. Invest* 125:4160–70 [PubMed: 26436649]
72. Anthony RM, Ravetch JV. 2010 A novel role for the IgG Fc glycan: the anti-inflammatory activity of sialylated IgG Fcs. *J. Clin. Immunol* 30(Suppl. 1):S9–14 [PubMed: 20480216]
73. Lund J, Takahashi N, Pound JD, Goodall M, Jefferis R. 1996 Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fc γ receptor I and influence the synthesis of its oligosaccharide chains. *J. Immunol* 157:4963–69 [PubMed: 8943402]
74. Chung CY, Wang Q, Yang S, Ponce SA, Kirsch BJ, et al. 2017 Combinatorial genome and protein engineering yields monoclonal antibodies with hypergalactosylation from CHO cells. *Biotechnol. Bioeng* 114:2848–56 [PubMed: 28926673]
75. Raymond C, Robotham A, Spearman M, Butler M, Kelly J, Durocher Y. 2015 Production of α 2,6-sialylated IgG1 in CHO cells. *mAbs* 7:571–83 [PubMed: 25875452]
76. Chung CY, Wang Q, Yang S, Yin B, Zhang H, Betenbaugh M. 2017 Integrated genome and protein editing swaps α -2,6 sialylation for α -2,3 sialic acid on recombinant antibodies from CHO. *Biotechnol. J* 12:1600502
77. Hodoniczky J, Zheng YZ, James DC. 2005 Control of recombinant monoclonal antibody effector functions by Fc *N*-glycan remodeling in vitro. *Biotechnol. Prog* 21:1644–52 [PubMed: 16321047]
78. Wang LX, Amin MN. 2014 Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem. Biol* 21:51–66 [PubMed: 24439206]
79. Wei Y, Li C, Huang W, Li B, Strome S, Wang LX. 2008 Glycoengineering of human IgG1-Fc through combined yeast expression and in vitro chemoenzymatic glycosylation. *Biochemistry* 47:10294–304 [PubMed: 18771295]
80. Zou G, Ochiai H, Huang W, Yang Q, Li C, Wang LX. 2011 Chemoenzymatic synthesis and Fc γ receptor binding of homogeneous glycoforms of antibody Fc domain. Presence of a bisecting sugar moiety enhances the affinity of Fc to Fc γ IIIa receptor. *J. Am. Chem. Soc* 133:18975–91 [PubMed: 22004528]
81. Fan SQ, Huang W, Wang LX. 2012 Remarkable transglycosylation activity of glycosynthase mutants of Endo-D, an Endo- β -*N*-acetylglucosaminidase from *Streptococcus pneumoniae*. *J. Biol. Chem* 287:11272–81 [PubMed: 22318728]
82. Huang W, Giddens J, Fan SQ, Toonstra C, Wang LX. 2012 Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. *J. Am. Chem. Soc* 134:12308–18 [PubMed: 22747414]
83. Lin CW, Tsai MH, Li ST, Tsai TI, Chu KC, et al. 2015 A common glycan structure on immunoglobulin G for enhancement of effector functions. *PNAS* 112:10611–16 [PubMed: 26253764]
84. Kuroguchi M, Mori M, Osumi K, Tojino M, Sugawara S, et al. 2015 Glycoengineered monoclonal antibodies with homogeneous glycan (M3, G0, G2, and A2) using a chemoenzymatic approach have different affinities for Fc γ RIIIa and variable antibody-dependent cellular cytotoxicity activities. *PLOS ONE* 10:e0132848 [PubMed: 26200113]
85. Parsons TB, Struwe WB, Gault J, Yamamoto K, Taylor TA, et al. 2016 Optimal synthetic glycosylation of a therapeutic antibody. *Angew. Chem. Int. Ed* 55:2361–67
86. Ahmed AA, Giddens J, Pincetic A, Lomino JV, Ravetch JV, et al. 2014 Structural characterization of anti-inflammatory immunoglobulin G Fc proteins. *J. Mol. Biol* 426:3166–79 [PubMed: 25036289]
87. Smith EL, Giddens JP, Iavarone AT, Godula K, Wang LX, Bertozzi CR. 2014 Chemoenzymatic Fc glycosylation via engineered aldehyde tags. *Bioconjug. Chem* 25:788–95 [PubMed: 24702330]
88. Liu R, Giddens J, McClung CM, Magnelli PE, Wang LX, Guthrie EP. 2016 Evaluation of a glycoengineered monoclonal antibody via LC-MS analysis in combination with multiple enzymatic digestion. *mAbs* 8:340–46 [PubMed: 26514686]
89. Collin M, Olsen A. 2001 EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *EMBO J* 20:3046–55 [PubMed: 11406581]
90. Sjogren J, Cosgrave EF, Allhorn M, Nordgren M, Bjork S, et al. 2015 EndoS and EndoS2 hydrolyze Fc-glycans on therapeutic antibodies with different glycoform selectivity and can be

used for rapid quantification of high-mannose glycans. *Glycobiology* 25:1053–63 [PubMed: 26156869]

91. Li T, Tong X, Yang Q, Giddens JP, Wang LX. 2016 Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. *J. Biol. Chem* 291:16508–18 [PubMed: 27288408]
92. Sjogren J, Struwe WB, Cosgrave EF, Rudd PM, Stervander M, et al. 2013 EndoS2 is a unique and conserved enzyme of serotype M49 group A *Streptococcus* that hydrolyses N-linked glycans on IgG and α_1 -acid glycoprotein. *Biochem. J* 455:107–18 [PubMed: 23865566]
93. Shivatare SS, Huang LY, Zeng YF, Liao JY, You TH, et al. 2018 Development of glycosynthases with broad glycan specificity for the efficient glyco-remodeling of antibodies. *Chem. Commun* 54:6161–64
94. Li T, Li C, Quan DN, Bentley WE, Wang LX. 2018 Site-specific immobilization of endoglycosidases for streamlined chemoenzymatic glycan remodeling of antibodies. *Carbohydr. Res* 458:77–84 [PubMed: 29475193]
95. Li T, DiLillo DJ, Bournazos S, Giddens JP, Ravetch JV, Wang LX. 2017 Modulating IgG effector function by Fc glycan engineering. *PNAS* 114:3485–90 [PubMed: 28289219]
96. Giddens JP, Lomino JV, Amin MN, Wang LX. 2016 Endo-F3 glycosynthase mutants enable chemoenzymatic synthesis of core-fucosylated triantennary complex type glycopeptides and glycoproteins. *J. Biol. Chem* 291:9356–70 [PubMed: 26966183]
97. Liu CP, Tsai TI, Cheng T, Shivatare VS, Wu CY, et al. 2018 Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation. *PNAS* 115:720–25 [PubMed: 29311294]
98. Manabe S, Yamaguchi Y, Abe J, Matsumoto K, Ito Y. 2018 Acceptor range of Endo- β -*N*-acetylglucosaminidase mutant Endo-CC N180H: from monosaccharide to antibody. *R. Soc. Open Sci* 5:171521 [PubMed: 29892355]
99. Iwamoto M, Sekiguchi Y, Nakamura K, Kawaguchi Y, Honda T, Hasegawa J. 2018 Generation of efficient mutants of endoglycosidase from *Streptococcus pyogenes* and their application in a novel one-pot transglycosylation reaction for antibody modification. *PLOS ONE* 13:e0193534 [PubMed: 29474426]
100. Song R, Oren DA, Franco D, Seaman MS, Ho DD. 2013 Strategic addition of an N-linked glycan to a monoclonal antibody improves its HIV-1-neutralizing activity. *Nat. Biotechnol* 31:1047–52 [PubMed: 24097413]
101. Giddens JP, Lomino JV, DiLillo DJ, Ravetch JV, Wang LX. 2018 Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. *PNAS* 115:12023–27 [PubMed: 30397147]
102. Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, et al. 2008 Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose. *N. Engl. J. Med* 358:1109–17 [PubMed: 18337601]
103. Qian J, Liu T, Yang L, Daus A, Crowley R, Zhou Q. 2007 Structural characterization of N-linked oligosaccharides on monoclonal antibody cetuximab by the combination of orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole–quadrupole time-of-flight tandem mass spectrometry and sequential enzymatic digestion. *Anal. Biochem* 364:8–18 [PubMed: 17362871]
104. Janin-Bussat MC, Tonini L, Huillet C, Colas O, Klinguer-Hamour C, et al. 2013 Cetuximab Fab and Fc *N*-glycan fast characterization using IdeS digestion and liquid chromatography coupled to electrospray ionization mass spectrometry. *Methods Mol. Biol* 988:93–113 [PubMed: 23475716]
105. Tsuchikama K, An Z. 2018 Antibody–drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell* 9:33–46 [PubMed: 27743348]
106. Lambert JM, Berkenblit A. 2018 Antibody–drug conjugates for cancer treatment. *Annu. Rev. Med* 69:191–207 [PubMed: 29414262]
107. Sau S, Alsaab HO, Kashaw SK, Tatiparti K, Iyer AK. 2017 Advances in antibody–drug conjugates: a new era of targeted cancer therapy. *Drug Discov. Today* 22:1547–56 [PubMed: 28627385]
108. Deng S, Lin Z, Li W. 2017 Recent advances in antibody–drug conjugates for breast cancer treatment. *Curr. Med. Chem* 24:2505–27 [PubMed: 28554322]

109. Beck A, Goetsch L, Dumontet C, Corvaia N. 2017 Strategies and challenges for the next generation of antibody–drug conjugates. *Nat. Rev. Drug Discov* 16:315–37 [PubMed: 28303026]
110. Kubizek F, Eggenreich B, Spadiut O. 2017 Status quo in antibody–drug conjugates—can glyco-enzymes solve the current challenges? *Protein Pept. Lett* 24:686–95 [PubMed: 28741467]
111. Donaghy H. 2016 Effects of antibody, drug and linker on the preclinical and clinical toxicities of antibody–drug conjugates. *mAbs* 8:659–71 [PubMed: 27045800]
112. Casi G, Neri D. 2015 Antibody–drug conjugates and small molecule–drug conjugates: opportunities and challenges for the development of selective anticancer cytotoxic agents. *J. Med. Chem* 58:8751–61 [PubMed: 26079148]
113. Perez HL, Cardarelli PM, Deshpande S, Gangwar S, Schroeder GM, et al. 2014 Antibody–drug conjugates: current status and future directions. *Drug Discov. Today* 19:869–81 [PubMed: 24239727]
114. Chari RV, Miller ML, Widdison WC. 2014 Antibody–drug conjugates: an emerging concept in cancer therapy. *Angew. Chem. Int. Ed* 53:3796–827
115. Sievers EL, Senter PD. 2013 Antibody–drug conjugates in cancer therapy. *Annu. Rev. Med* 64:15–29 [PubMed: 23043493]
116. Senter PD, Sievers EL. 2012 The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat. Biotechnol* 30:631–37 [PubMed: 22781692]
117. Lambert JM, Chari RV. 2014 Ado-trastuzumab emtansine (T-DM1): an antibody–drug conjugate (ADC) for HER2-positive breast cancer. *J. Med. Chem* 57:6949–64 [PubMed: 24967516]
118. Herrera AF, Molina A. 2018 Investigational antibody–drug conjugates for treatment of B-lineage malignancies. *Clin. Lymphoma Myeloma Leuk* 18:452–68.e4 [PubMed: 29804872]
119. Huang RY, Chen G. 2016 Characterization of antibody–drug conjugates by mass spectrometry: advances and future trends. *Drug Discov. Today* 21:850–55 [PubMed: 27080148]
120. Beck A, Terral G, Debaene F, Wagner-Rousset E, Marcoux J, et al. 2016 Cutting-edge mass spectrometry methods for the multi-level structural characterization of antibody–drug conjugates. *Expert Rev. Proteom* 13:157–83
121. Behrens CR, Liu B. 2014 Methods for site-specific drug conjugation to antibodies. *mAbs* 6:46–53 [PubMed: 24135651]
122. Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. 2014 Site-specific antibody drug conjugates for cancer therapy. *mAbs* 6:34–45 [PubMed: 24423619]
123. Junutula JR, Gerber HP. 2016 Next-generation antibody–drug conjugates (ADCs) for cancer therapy. *ACS Med. Chem. Lett* 7:972–73 [PubMed: 27882192]
124. Strop P, Delaria K, Foletti D, Witt JM, Hasa-Moreno A, et al. 2015 Site-specific conjugation improves therapeutic index of antibody drug conjugates with high drug loading. *Nat. Biotechnol* 33:694–96 [PubMed: 26154005]
125. Dennler P, Chiotellis A, Fischer E, Bregeon D, Belmant C, et al. 2014 Transglutaminase-based chemo-enzymatic conjugation approach yields homogeneous antibody–drug conjugates. *Bioconjug. Chem* 25:569–78 [PubMed: 24483299]
126. Axup JY, Bajjuri KM, Ritland M, Hutchins BM, Kim CH, et al. 2012 Synthesis of site-specific antibody–drug conjugates using unnatural amino acids. *PNAS* 109:16101–6 [PubMed: 22988081]
127. Zuberbuhler K, Casi G, Bernardes GJ, Neri D. 2012 Fucose-specific conjugation of hydrazide derivatives to a vascular-targeting monoclonal antibody in IgG format. *Chem. Commun* 48:7100–2
128. Zhou Q, Stefano JE, Manning C, Kyazike J, Chen B, et al. 2014 Site-specific antibody–drug conjugation through glycoengineering. *Bioconjug. Chem* 25:510–20 [PubMed: 24533768]
129. Li X, Fang T, Boons GJ. 2014 Preparation of well-defined antibody–drug conjugates through glycan remodeling and strain-promoted azide-alkyne cycloadditions. *Angew. Chem. Int. Ed* 53:7179–82
130. Ramakrishnan B, Qasba PK. 2002 Structure-based design of β 1,4-galactosyltransferase I (β 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens β 4Gal-T1 donor specificity. *J. Biol. Chem* 277:20833–39 [PubMed: 11916963]

131. Khidekel N, Arndt S, Lamarre-Vincent N, Lippert A, Poulin-Kerstien KG, et al. 2003 A chemoenzymatic approach toward the rapid and sensitive detection of *O*-GlcNAc posttranslational modifications. *J. Am. Chem. Soc* 125:16162–63 [PubMed: 14692737]
132. Zhu Z, Ramakrishnan B, Li J, Wang Y, Feng Y, et al. 2014 Site-specific antibody–drug conjugation through an engineered glycotransferase and a chemically reactive sugar. *mAbs* 6:1190–200 [PubMed: 25517304]
133. Ramakrishnan B, Boeggeman E, Qasba PK. 2008 Applications of glycosyltransferases in the site-specific conjugation of biomolecules and the development of a targeted drug delivery system and contrast agents for MRI. *Expert Opin. Drug Deliv* 5:149–53 [PubMed: 18248315]
134. Tang F, Yang Y, Tang Y, Tang S, Yang L, et al. 2016 One-pot *N*-glycosylation remodeling of IgG with non-natural sialylglycopeptides enables glycosite-specific and dual-payload antibody–drug conjugates. *Org. Biomol. Chem* 14:9501–18 [PubMed: 27714198]
135. Tang F, Wang LX, Huang W. 2017 Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody–drug conjugates. *Nat. Protoc* 12:1702–21 [PubMed: 28749929]
136. Ramakrishnan B, Boeggeman E, Manzoni M, Zhu Z, Loomis K, et al. 2009 Multiple site-specific in vitro labeling of single-chain antibody. *Bioconjug. Chem* 20:1383–89 [PubMed: 19507852]
137. Boeggeman E, Ramakrishnan B, Pasek M, Manzoni M, Puri A, et al. 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* 20:1228–36 [PubMed: 19425533]
138. Qasba PK, Ramakrishnan B, Boeggeman E. 2008 Structure and function of β -1,4-galactosyltransferase. *Curr. Drug Targets* 9:292–309 [PubMed: 18393823]
139. Qasba PK, Boeggeman E, Ramakrishnan B. 2008 Site-specific linking of biomolecules via glycan residues using glycosyltransferases. *Biotechnol. Prog* 24:520–26 [PubMed: 18426242]
140. Ramakrishnan B, Boeggeman E, Qasba PK. 2007 Novel method for in vitro *O*-glycosylation of proteins: application for bioconjugation. *Bioconjug. Chem* 18:1912–18 [PubMed: 17953440]
141. Boeggeman E, Ramakrishnan B, Kilgore C, Khidekel N, Hsieh-Wilson LC, et al. 2007 Direct identification of nonreducing GlcNAc residues on N-glycans of glycoproteins using a novel chemoenzymatic method. *Bioconjug. Chem* 18:806–14 [PubMed: 17370997]
142. Qasba PK, Ramakrishnan B, Boeggeman E. 2006 Mutant glycosyltransferases assist in the development of a targeted drug delivery system and contrast agents for MRI. *AAPS J.* 8:E190–95 [PubMed: 16584127]
143. Clark PM, Dweck JF, Mason DE, Hart CR, Buck SB, et al. 2008 Direct in-gel fluorescence detection and cellular imaging of *O*-GlcNAc-modified proteins. *J. Am. Chem. Soc* 130:11576–77 [PubMed: 18683930]
144. Zeglis BM, Davis CB, Aggeler R, Kang HC, Chen A, et al. 2013 Enzyme-mediated methodology for the site-specific radiolabeling of antibodies based on catalyst-free click chemistry. *Bioconjug. Chem* 24:1057–67 [PubMed: 23688208]
145. van Geel R, Wijdeven MA, Heesbeen R, Verkade JM, Wasiel AA, et al. 2015 Chemoenzymatic conjugation of toxic payloads to the globally conserved N-glycan of native mAbs provides homogeneous and highly efficacious antibody–drug conjugates. *Bioconjug. Chem* 26:2233–42 [PubMed: 26061183]
146. Okeley NM, Toki BE, Zhang X, Jeffrey SC, Burke PJ, et al. 2013 Metabolic engineering of monoclonal antibody carbohydrates for antibody–drug conjugation. *Bioconjug. Chem* 24:1650–55 [PubMed: 24050213]

Figure 1.

(a) Schematic structure of common IgG antibodies. (b) Typical *N*-glycoforms found at the Asn297 site of the IgG–Fc domain. Monosaccharide symbols comply with the standard symbol nomenclature for representing glycans: galactose (Gal) (*yellow circle*); mannose (Man) (*green circle*); *N*-acetylglucosamine (GlcNAc) (*blue square*); *N*-acetylneuraminic acid (Neu5Ac) (*purple diamond*); fucose (Fuc) (*red triangle*). Other abbreviations: Fab, antigen-binding fragment; Fc, crystallizable fragment.

Figure 2.

(a) Biosynthesis of *N*-glycoproteins in eukaryotic cells. (b) De novo biosynthetic pathway of GDP-L-fucose in mammalian cells. Abbreviations: Alg, asparagine-linked glycosylation enzyme; CNX/CRT, calnexin/calreticulin; Dol, dolichol; Fuc, fucose; FUT8, α 1,6-fucosyltransferase; FX, GDP-4-keto-6-D-deoxymannose epimerase/GDP-4-keto-6-liter-galactose reductase; G-I, α -glucosidase I; G-II, α -glucosidase II; Gal, galactose; GalT, galactosyltransferase; GDP, guanosine diphosphate; GlcNAc, *N*-acetylglucosamine; GMD, GDP-mannose 4,6-dehydratase; GMP, guanosine monophosphate; GnT, *N*-acetylglucosaminyltransferase; Man, mannose; Mns, α -mannosidase; Neu5Ac, *N*-acetylneuraminic acid; NXS/T, the consensus three amino acid sequence for *N*-glycosylation, where X is any amino acid except proline; OST, oligosaccharyltransferase; SiaT, sialyltransferase.

Figure 3.

Glycoengineering methods to produce low-fucose or nonfucosylated mAbs in vivo. (a) Overexpression of GnTIII in CHO cells. (b) Knockout of FUT8 in CHO cells. (c) Knockout of GMD in CHO cells. (d) Knockout of FX in CHO cells. (e) Overexpression of RMD in CHO cells. (f) Knockout of GDP-fucose transporter in CHO cells. (g) Use of fucose analogs that block GDP-fucose production (inhibition of GMD). (h) Expression in cell lines that have low or no endogenous FUT8 expression. Abbreviations: CHO, Chinese hamster ovary; Fuc, fucose; FUT8, α 1,6-fucosyltransferase; GMD, GDP-mannose 4,6-dehydratase; GnT, *N*-acetylglucosaminyltransferase; mAbs, monoclonal antibodies; RMD, GDP-6-deoxy-d-lyxo-hexos-4-ulose-4-reductase.

Figure 4. Enzymatic glycan remodeling of intact IgG-Fc domain with synthetic glycan oxazolines. Abbreviations: GlcNAc, *N*-acetylglucosamine; HM, high mannose; Man, mannose.

Figure 5.

(a) Endoglycosidase-catalyzed glycoside hydrolysis via a substrate-assisted mechanism. (b) Typical glycosynthase-catalyzed glycosylation using sugar oxazoline as activated substrate without product hydrolysis.

Figure 6. Enzymatic glycan remodeling of intact IgG antibodies using Endo-S and Endo-S mutants. Abbreviations: Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Man, mannose.

Figure 7. Enzymatic glycan remodeling of intact IgG antibodies using Endo-S2, Endo-F3, and their mutants. Abbreviations: Fuc, fucose; GlcNAc, *N*-acetylglucosamine.

Figure 8.

(a) Glycoengineering by chemical oxidation of core fucose. (b) Glycoengineering by chemical oxidation of sialic acid under mild conditions. The oxidized antibody can be conjugated with cell-toxic MMAE (drug) with oxime linkage. Abbreviations: $\alpha 2,6\text{-SiaT}$, $\alpha 2,6\text{-sialyltransferase}$; $\beta 1,4\text{-GalT}$, $\beta 1,4\text{-galactosyltransferase}$; CMP, cytidine monophosphate; CMP-Sia, cytidine 5'-monophosphatesialic acid; Gal, galactose; MMAE, monomethyl auristatin E; Sia, sialic acid; UDP, uridine diphosphate; UDP-Gal, uridine 5'-diphosphate-galactose.

Figure 9.

(a) Enzymatic antibody engineering using C9 azido-sialic acid as substrate. A modified sialic acid substrate was applied and facilitated the copper-free click reaction for antibody–drug conjugates with high drug/antibody ratio. (b) GalT mutant promoted antibody glycoengineering for antibody–drug conjugate production. Abbreviations: CMP, cytidine monophosphate; CMP-Sia, cytidine 5′-monophosphate-sialic acid; CMP-9-N₃Sia, cytidine 5′-monophosphate-9-azido-sialic acid; Gal, galactose; GalT, galactosyltransferase; Sia, sialic acid; SiaT, sialyltransferase; UDP, uridine diphosphate; UDP-Gal, uridine 5′-diphosphate-galactose.

Figure 10.

(a) Chemoenzymatic remodeling for antibody–drug conjugates using endoglycosidase-catalyzed deglycosylation and glycosynthase-catalyzed glycosylation followed by click chemistry. (b) Chemoenzymatic remodeling for antibody–drug conjugates using endoglycosidase-catalyzed deglycosylation and galactosyltransferase-catalyzed tagging followed by click chemistry. Abbreviations: DM1-SMCC, $N^{2'}$ -deacetyl- $N^{2'}$ -[3-[[1-[[4-[[2,5-dioxo-1-pyrrolidinyl]oxy]carbonyl]cyclohexyl]methyl]-2,5-dioxo-3-pyrrolidinyl]thio]-1-oxopropyl]-maytansine; GalT Y289L, galactosyltransferase-Y289L mutant; MMAE, monomethyl auristatin E; MMAF, monomethyl auristatin F; UDP-C2-GalNAz, uridine 5'-diphosphate-N-azidoacetyl galactosamine.