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Using glyco-engineering to produce therapeutic proteins

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Abstract

Introduction—Glycans are increasingly important in the development of new biopharmaceuticals with optimized efficacy, half-life and antigenicity. Current expression platforms for recombinant glycoprotein therapeutics typically do not produce homogeneous glycans and frequently display non-human glycans which may cause unwanted side effects. To circumvent these issues, glyco-engineering has been applied to different expression systems including mammalian cells, insect cells, yeast and plants.

Areas Covered—This review summarizes recent developments in glyco-engineering focusing mainly on *in vivo* expression systems for recombinant proteins. The highlighted strategies aim at producing glycoproteins with homogeneous N- and O-linked glycans of defined composition.

Expert opinion—Glyco-engineering of expression platforms is increasingly recognized as an important strategy to improve biopharmaceuticals. A better understanding and control of the factors leading to glycan heterogeneity will allow simplified production of recombinant glycoprotein therapeutics with less variation in terms of glycosylation. Further technological advances will have a major impact on manufacturing processes and may provide a completely new class of glycoprotein therapeutics with customized functions.

Keywords

biopharmaceuticals; glycosyltransferases; N-glycosylation; O-glycosylation; recombinant glycoprotein

1 Introduction

Many protein drugs are glycosylated and the attached glycan structures often influence the therapeutic properties. The number and composition of the glycans play an important role for protein folding, solubility and intracellular trafficking. Glycans can shield the protein

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Declaration of interest

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backbone to prevent immunogenic reactions and distinct cellular recognition events depend on the presence on specific glycan structures. Thus glycosylation has a huge impact on the biological activity of glycoproteins and should be carefully controlled during manufacturing to achieve optimized therapeutic efficacy. Dependent on the species, cell-type and physiological status of the production host the glycosylation pattern on recombinant glycoproteins can differ significantly. Glycans on proteins are structurally quite diverse and consist of a set of monosaccharides that are assembled by different linkages. The glycosylation processes in the ER and Golgi generate the majority of rather heterogeneous glycan structures found on recombinant glycoproteins. Due to the recognized importance in therapy, substantial efforts have been made in recent years to overcome glycan heterogeneity and establish *in vivo* and *in vitro* glyco-engineering technologies for efficient production of homogeneous therapeutic glycoproteins. Such recombinant proteins with custom-made glycosylation are essential to understand glycan-mediated functions of glycoprotein therapeutics and represent a new class of next generation drugs with enhanced biological activity.

2 N-glycosylation of proteins

The most prominent and best characterized form of protein glycosylation is the linkage of a glycan to the amide in the side chain of an asparagine (N-glycosylation) on newly synthesized proteins. N-glycosylation of proteins starts in the lumen of the endoplasmic reticulum (ER) by transfer of a conserved preassembled oligosaccharide precursor (Glc₃Man₉GlcNAc₂) (Figure 1a) to the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline) exposed on nascent polypeptide chains. This initial glycan transfer reaction is catalysed by the heteromeric oligosaccharyltransferase (OST) complex and is supposed to precede folding of the protein in the ER [1]. Immediately after the oligosaccharide transfer, the two terminal glucose residues are cleaved off by α -glucosidase I and II and the resulting polypeptide with mono-glucosylated glycan structures (Glc₁Man₉GlcNAc₂) can interact with the ER-resident membrane-bound lectin calnexin or its soluble homolog calreticulin. These lectins support protein folding in a glycan-dependent protein quality control cycle. Secretory glycoproteins that have acquired their native conformation are released from the calnexin/calreticulin cycle and exit the ER to the Golgi apparatus. In the Golgi the ER-derived oligomannosidic N-glycans on maturely folded glycoproteins are subjected to further N-glycan processing which generates the highly diverse complex N-glycans with different functional properties (Figure 1b).

What are the targets for N-glycan-engineering?

2.1 Avoidance of macroheterogeneity

Macroheterogeneity on recombinant glycoproteins arise from variations in glycosylation site occupancy. These differences in glycosylation efficiency are dependent on the manufacturing system (e.g. organism/cell-type-specific) and protein intrinsic features. The presence of the Asn-X-Ser/Thr sequon is necessary but not sufficient for N-glycosylation of mammalian proteins. While all eukaryotic cells have an overall conserved machinery for N-glycosylation and display similar structural requirements for efficient N-glycosylation there

are minor differences in utilization of glycosylation sites between species [2,3]. Consequently, glycosylation sites may be skipped leading to underglycosylation of recombinant proteins or additional sites may be used leading to aberrant glycosylation. Protein intrinsic factors like the surrounding amino acid sequence and secondary structure, the positioning of the consensus sequence within the polypeptide as well as the presence of other protein modifications like disulfide bond formation contribute to N-glycosylation efficiency [4]. Recognition of these glycoprotein-specific features depends on the presence and function of the different OST subunits. Mammalian cells contain two OST complexes that differ in their catalytic subunit as well as in accessory proteins. These OST complexes display partially overlapping functions, but also preferences for certain glycoprotein substrates [3]. Engineering of the OST complex is one possible way to overcome differences related to N-glycosylation site occupancy. Yet, the individual roles of distinct OST catalytic subunits and their accessory proteins are still not fully understood in eukaryotes making rational approaches difficult. Moreover, a recent study has indicated that overexpression of individual subunits is not sufficient to restore N-glycosylation in mutant cells presumably due to the inability to form functional heteromeric OST complexes [5]. However, in protists like *Leishmania major*, OST is composed of a single subunit that can replace the whole *Saccharomyces cerevisiae* OST complex and can be used to overcome underglycosylation of proteins. Overexpression of the single-subunit OST from *L. major* in the methylotrophic yeast *Pichia pastoris* increased the N-glycosylation site occupancy of a monoclonal antibody from 75-85% to greater than 99% [6].

Other engineering attempts to reduce macroheterogeneity are based on mutation of the N-glycosylation consensus sequence, e.g. from Asn-X-Ser to Asn-X-Thr which is more efficiently glycosylated in different eukaryotes [2]. Besides modifying the consensus sequence itself, adjacent amino acids may also be altered to enhance N-glycosylation efficacy. The major drawback of these strategies is an alteration of the primary amino acid sequence of the therapeutic glycoprotein which can have an influence on protein properties and may create unwanted regulatory concerns. Nonetheless, novel N-glycosylation sites have been successfully engineered into recombinant proteins. The most prominent example for this approach is darbpoetin alfa. This hyperglycosylated recombinant erythropoietin (EPO) variant has been engineered to carry five instead of three N-glycosylation sites [7]. The additional two N-glycans increase the total sialic acid content which affects the protein half-life and boost the *in vivo* potency [8]. In another elegant study, introduction of a novel N-glycosylation site into the light chain of a monoclonal antibody against the HIV-1 receptor CD4 substantially improved the virus neutralization activity [9]. Collectively, these examples illustrate the potential for protein engineering towards incorporation of additional glycosylation sites.

2.2 Reduction of microheterogeneity

The commonly used expression systems for recombinant glycoproteins typically produce a mixture of different glycans on the same protein. This means that different glycans are found at the same glycosylation site and that individual sites on the same protein can be furnished with glycans of high structural diversity. The N-glycome of mammalian cells shows a wide range of N-glycan compositions from oligomannosidic to branched complex-type N-glycans

(Figure 2a) [10,11]. Microheterogeneity arises from variations in N-glycan processing which depends on numerous cellular factors including tissue/cell-type-specific expression of processing enzymes, their concentrations and enzyme kinetic parameters, availability of the nucleotide sugar donors and the glycoprotein residence and contact time in the reaction compartment (e.g. Golgi apparatus). In addition, intrinsic protein structural properties can have a strong effect on site-specific N-glycan processing. Systems glycobiology approaches have been used to correlate the transcript expression of glycosylation enzymes and the generation of N-glycan structures on glycoproteins from mouse tissues or cancer cells [10,12]. In mouse tissues a positive correlation was found between transfer of fucose residues and the transcript expression of corresponding fucosyltransferases. On the other hand, for complex N-glycan formation or sialylation this could not be established highlighting a more complicated regulation for these trimming and processing steps [10]. In addition, models have been developed to predict the influence of enzyme expression, subcellular localization, nucleotide sugar levels and culture conditions on glycan processing [13]. While the described models may already be useful to predict the rather homogeneous Fc-glycosylation on a recombinant monoclonal antibody [14], important key assumptions of these models have to be validated by additional experimental data in the future. Moreover, the robustness of current mathematical models needs to be tested in host cells on recombinant glycoproteins which display a more complicated and heterogeneous glycosylation pattern including branching and sialylation of N-glycans [15].

Other factors that contribute to microheterogeneity are the competition of different modifying enzymes for the same oligosaccharide substrate and the presence of endogenous glycoproteins that may interfere with processing or contact time in the Golgi. Although it is commonly accepted that the Golgi processing enzymes are organized sequentially in some kind of assembly line across the individual Golgi cisternae [16], there is typically an overlapping distribution of multiple enzymes. These enzymes may modify the same glycan intermediate structure and block the action of other glycosyltransferases and glycosidases. The addition of the bisecting GlcNAc to complex N-glycans by N-acetylglucosaminyltransferase III prevents, for example, further modifications by other Golgi-resident glycosyltransferases. This substrate competition and inhibition of further processing is the key factor of the GlycoMab technology that was developed by Glycart Biotechnology (acquired by Roche) and is used to produce glyco-engineered monoclonal antibodies with reduced amounts of α 1,6-linked core fucose [17]. The glyco-engineered obinutuzumab, an anti-CD20 monoclonal antibody produced by this platform, was recently approved by the FDA for treatment of patients with previously untreated chronic lymphocytic leukemia [18]. Galactosylation and branching of complex N-glycans are other frequent modifications that use the same substrates (Figure 1b) [15]. Apart from these competing reactions that act in the same biosynthetic compartment, a significant impact of enzymes involved in catabolic processes cannot be excluded. Extracellular α -neuraminidases or β -hexosaminidases can act on terminal sugar residues and remove them from exposed N-glycans after secretion leading to variation in glycan profiles on recombinant glycoproteins [10, 19]. As a consequence, genetic inactivation or inhibition of competing glycosyltransferases and (catabolic) glycoside hydrolases are beneficial strategies to prevent unwanted glycan modifications and reduce the complexity of glycosylation. The

successful generation of recombinant glycoprotein therapeutics with homogeneous glycosylation in yeast or plants [20,21] which have much less complicated N-glycan processing machinery than mammalian cells, demonstrates the capability of glyco-engineering. The generation of defined glycosylation patterns in Chinese Hamster Ovary (CHO) cells with defined defects in glycosylation shows that “simplifying” strategies are also feasible in mammalian cells [22]. Antibodies produced in Lec8 CHO cells which lack UDP-galactose in the Golgi, display mainly homogeneous biantennary N-glycans [23, 24].

The residence time of the secreted recombinant glycoprotein in the Golgi and the contact time with the membrane-anchored glycan modifying enzymes are other factors that may contribute significantly to microheterogeneity. Differences in residence times and transport kinetics through the Golgi have been described for several proteins [25,26] and their influence on glycan modifications is documented [27]. The residence time distribution and the interaction with the glycosylation enzymes are dependent on the mode of intra-Golgi cargo transport (vesicular transport/tubular connections, cisternal maturation, rapid partitioning or mixed models) and is cell-type and organism-specific [28]. Furthermore, recent studies suggest that different protein cargo (e.g. large vs. small cargo) is transported by varying mechanisms and therefore very likely interacts with different Golgi-resident enzymes [29]. For some glycosylation processes it has been demonstrated that skipping of transit through individual Golgi cisternae is an elegant way of regulating glycan modifications [30]. Despite some progress in this field, fundamental experimental data on Golgi kinetics of different recombinant glycoproteins and the impact on glycan processing are not available to rationally engineer transport and trafficking pathways towards more homogeneous glycan structures. In other words, for many cell-based manufacturing systems we need a better understanding of cellular transport processes in order to reduce glycan microheterogeneity related to Golgi residence time or intra-Golgi transport.

Finally, a major contribution to site-specific modifications comes from glycoprotein intrinsic features that are determined by the protein sequence and structure [31]. Certain protein conformations completely prevent or limit the access of the processing enzymes to the glycans by steric hindrance [32]. Monoclonal antibodies like cetuximab with an additional N-glycan in the variable region of the heavy chain are good examples for the site-specific processing on the same molecule. While the Fc N-glycan of cetuximab is less modified, the N-glycan in the variable region is more exposed and displays extensive processing [33,34]. Strategies to engineer the protein intrinsic features without altering the protein function are challenging, but the increasing availability of protein structures together with powerful molecular dynamics simulations will open new possibilities for site-specific glycan engineering in the future [35]. Alternatively, the rational design of glycan-modifying enzymes towards relaxed or novel substrate specificities may allow more efficient processing of partially accessible sites.

2.3 Elimination of non-human and potentially immunogenic sugar residues

The majority of the currently used expression systems for glycoprotein therapeutics produce N-glycans carrying non-human structures that can lead to potential side effects of the drugs. These non-human epitopes may elicit unwanted immune responses that neutralize the

applied drug or even worse cause a hypersensitivity reaction. Recombinant glycoproteins produced in CHO cells carry the non-human sialic acid N-glycolylneuraminic acid (Neu5Gc) [36–38] (Figure 2b). In addition, CHO cells attach N-acetylneuraminic acid (Neu5Ac) in α 2,3-linkage instead of α 2,6-linkage that is mainly found on N-glycans from human glycoproteins because CHO cells lack the corresponding α 2,6-sialyltransferase activity. A number of different approaches have been proposed to eliminate the Neu5Gc incorporation [39]. The most straightforward glyco-engineering strategy appears the genetic knockout of the gene coding for CMP-Neu5Ac hydroxylase (Figure 3c), the enzyme which converts CMP-Neu5Ac into CMP-Neu5Gc. Similarly, the α 2,3-sialyltransferase could be eliminated by genetic modification and ectopically expression of the missing α 2,6-sialyltransferase will lead to humanized N-glycans.

NS0 and SP2/0 mouse myeloma or Baby Hamster Kidney (BHK) cells generate glycoproteins with galactose- α 1,3-galactose (α -Gal epitope) (Figure 2c) that represent antigenic epitopes for humans. The responsible enzyme, α 1,3-galactosyltransferase, is inactive in humans, but present in most non-human mammalian cell lines including CHO [40]. BHK-derived recombinant human factor VIII (rhFVIII) carries 3% of the α -Gal epitope, while it is not detected on rhFVIII produced in human embryonic kidney cells (HEK293) [37]. Significant amounts of the α -Gal epitope are present in the N-glycan from the variable region of cetuximab [33,34] which is produced in SP2/0 mouse myeloma cells and worldwide approved for treatment of different types of cancer. A hypersensitive reaction to the drug in a number of cetuximab-treated patients in the United States has been linked to the presence of the α -Gal epitope [41]. This example highlights the importance of glyco-engineering for the generation of bio-better therapeutic proteins (Figure 3d).

Glycosylation of recombinant proteins derived from human cell lines such as HEK293 can be highly heterogeneous including different biantennary and branched structures with variable terminal sugar residues and thus do not necessarily resemble N-glycosylation of serum derived glycoproteins. Despite the fact that human cell lines deficient in distinct N-glycan processing steps (e.g. GnTI-deficient) have been reported [42] and companies like the German-based Glycotope offer the production of recombinant glycoproteins in glyco-engineered human cell lines [43] an extensive remodelling of the N-glycosylation pathway towards homogeneous complex N-glycans has not been described in human cells. Comparison of several different proteins expressed in CHO and HEK293 cells indicated significant differences in N-glycosylation with reduced sialic acid content in HEK293 cell produced recombinant glycoproteins [44]. Besides reducing heterogeneity, potential targets for glyco-engineering of human cells are elimination of core-fucose (Figure 3b) and knockout of N-acetylglucosaminyltransferase III which generates the bisecting GlcNAc and thus prevents further processing and overexpression of enzymes for branching or sialylation.

The hypermannosylated N-glycan structures of yeast differ significantly from human N-glycans (Figure 2d) and may cause immunogenic reactions that affect the biological activity of drugs [45]. Pioneering work in *P. pastoris* demonstrated that humanized complex N-glycans can be generated in yeast by elimination of deleterious mannosyltransferases (Figure 3e) and controlled expression of mannosidases and N-acetylglucosaminyltransferases from other species [46]. The success of this early work from GlycoFi Inc. (now a division of

Merck) was based on high throughput screening of a combinatorial library of glycosylation enzymes designed for differential subcellular localisation and efficient production of secreted recombinant proteins with defined N-glycans. More recently, similar but more rationally designed approaches were used to eliminate hypermannosylation and engineer *P. pastoris* [47], *Saccharomyces cerevisiae* [48], *Yarrowia lipolytica* [49] and *Hansenula polymorpha* [50] towards the production of humanized N-glycans on recombinant glycoproteins.

Insect cells typically produce truncated (“paucimannosidic”) N-glycans due to the action of a processing β -hexosaminidase [51]. Moreover, a potentially immunogenic core α 1,3-fucose residue is present on recombinant glycoproteins expressed in some insect cell lines [52] (Figure 2e). Different methods have been developed to overcome these shortcomings and glyco-engineered insect cells that produce galactosylated N-glycans on monoclonal antibodies and fucose-deficient N-glycans have been described recently [53,54] (Figure 3f).

Plants typically generate rather simple biantennary complex N-glycans with attached β 1,2-xylose and core α 1,3-fucose residues (Figure 2f) that are not found on N-glycans in mammals and therefore represent potentially immunogenic residues [55]. Genetic knockout as well as knockdown approaches have successfully been used to eliminate β 1,2-xylose and core α 1,3-fucose from recombinant glycoprotein therapeutics produced in different plants [56–59] (Figure 3g). Compared to conventional CHO-produced recombinant monoclonal antibodies the N-glycans from plant-derived IgGs were highly homogeneous and displayed mainly the GlcNAc₂Man₃GlcNAc₂ glycan which is the optimal substrate for further modifications like branching or galactosylation. Notably, the *Nicotiana benthamiana*-based plant expression platform enables also the production of functionally active recombinant IgM variants with defined N-glycosylation [60] and is currently used to manufacture the monoclonal antibody cocktail against Ebola virus [61].

Similar to insect cells, plants contain also β -hexosaminidases that remove GlcNAc residues from some recombinant glycoproteins and expose terminal mannose on truncated N-glycans. While this post-Golgi processing event can be prevented by genetic knockout of the corresponding β -hexosaminidases [62], N-glycans with terminal mannose residues are beneficial structures for recombinant glycoproteins used for enzyme replacement therapy to treat lysosomal storage diseases. Plant-produced recombinant glucocerebrosidase (taliglucerase alfa) has been approved by the FDA in 2013 to treat Gaucher disease. This drug is expressed in genetically modified carrot cells and contains mainly Man₃XylFucGlcNAc₂ N-glycans [63] which are efficiently internalized by mannose receptors in humans. In contrast to other commercially available recombinant glucocerebrosidases, taliglucerase alfa does not require any *in vitro* exoglycosidase digestions or α -mannosidase inhibitors during the production process to generate N-glycans with terminal mannose residues.

2.4 Optimization of pharmacokinetics and biological activity by incorporation or removal of specific monosaccharides

Beneficial properties can be engineered by introduction of additional sugar residues that form novel recognition sites or mask binding sites for receptors involved in clearance. For

instance, optimized pharmacokinetic behaviour is achieved by increasing the sialic acid content of recombinant glycoproteins. Highly sialylated EPO variants have been produced in mammalian cells (darboetin alfa) [7] as well as in non-mammalian systems including yeast [20], insect cells [64] and plants [65]. Moreover, sialylation of antibodies is thought to play an important role for anti-inflammatory properties [66]. However, the potential use of sialylated IgGs in this context is still controversial because the used preparations to perform studies are often heterogeneous in terms of glycosylation [67].

In glyco-engineering approaches towards optimizing activity, unwanted glycosyltransferases are typically inactivated to prevent the transfer of single monosaccharides to glycan structures. Knockout of core α 1,6-fucosyltransferases (FUT8) has been used to generate CHO cells lacking N-glycans with core fucose [68] (Figure 3b). In another approach, the concentration of specific nucleotide sugars can be altered, for example, by inactivation or overexpression of the corresponding nucleotide sugar transporter or metabolic control of nucleotide sugar flux. It has been shown that branching of N-glycans is sensitive towards changes in UDP-GlcNAc concentrations [69] which is used as donor substrate by enzymes that are involved in complex N-glycan formation and initiation of N-glycan branching. Since UDP-GlcNAc levels are dependent on the hexosamine pathway, metabolic control may be used to modify the branching of N-glycans which can lead to the production of therapeutic proteins that have increased amounts of sialic acid and consequently an increased half-life (Figure 3a).

2.5 Other recent N-glycan engineering approaches

Mammalian-type N-glycosylation pathways have been engineered into *E. coli* and N-glycosylation of recombinant glycoproteins has been shown [70]. Such glyco-engineering attempts are very promising, but currently limited by the restricted acceptor site specificity of the used bacterial oligosaccharyltransferase [71]. Sequence-guided protein evolution may help to overcome such bottlenecks [72].

Synthetic and semi-synthetic approaches have been applied to remodel N-glycans on proteins and generate homogeneous glycoproteins with defined glycan structures [73]. *In vitro* approaches include total chemical synthesis of glycoproteins like sialylated EPO [74] or involve cell-based production followed by chemical or enzymatic post-production modifications to obtain homogeneous glycoproteins. Recently, *in vitro* incubation of β 1,4-galactosyltransferase and α 2,6-sialyltransferase together with their corresponding nucleotide sugars (UDP-Gal, CMP-Neu5Ac) was used to increase the disialylated glycan content and homogeneity of commercially available intravenous immunoglobulin (IVIg) [75]. *In vitro* enzymatic processing steps using α -neuraminidase, β -galactosidase, and β -N-acetylglucosaminidase have been applied to generate N-glycans with exposed mannose residues on recombinant glucocerebrosidase (imiglucerase) [76]. In addition to these stepwise modifications of existing N-glycans, enzymatic removal of heterogeneous glycans and *in vitro* enzymatic transfer of a preassembled oligosaccharide has been used to engineer homogeneous disialylated IgG Fc fragments in order to investigate the anti-inflammatory role of sialylation [77,78]. The recombinant glycoproteins are produced in eukaryotic cells or even in genetically engineered bacteria that can perform distinct N-glycosylation

reactions [70]. The cell-type specific heterogeneous N-glycans are enzymatically removed by specific endoglycosidases leaving glycoproteins with a single N-linked GlcNAc (Figure 4a). Enzymatic transglycosylation adds a structurally homogeneous pre-synthesized oligosaccharide to the N-linked GlcNAc resulting in a glycoprotein with defined N-glycans. While it is possible to generate homogeneous glycans on glycoproteins by this chemoenzymatic remodelling strategy, the industrial scale production of semi-synthetic glycoprotein therapeutics remains to be shown.

The Callewaert group recently developed a similar *in vivo* remodelling system for N-glycans in human cells [79] (Figure 4b). In the GlycoDelete strategy, the endoglycosidase-mediated enzymatic removal of N-glycans was engineered into GnTI-deficient HEK293 cells to generate N-GlcNAc modified glycoproteins in the Golgi. Endogenous galactosyltransferase and sialyltransferase can further elongate the N-linked GlcNAc leading to three truncated N-linked glycans. Recombinant antibodies produced in the GlycoDelete system displayed interesting features that need to be further tested to fully assess the potential of this novel glyco-engineering approach.

3 O-glycosylation of proteins

Different types of O-glycosylation (e.g. O-linked fucose, glucose, mannose, xylose or GalNAc) have been described on secretory proteins in mammals [80]. The formation of the highly abundant mucin-type O-glycans is initiated by the polypeptide GalNAc-transferase family. In this common mammalian O-glycosylation pathway, Golgi-resident polypeptide GalNAc-transferases transfer a single GalNAc residue to Ser/Thr sites of fully folded proteins which do not display a clear consensus sequence. Subsequent stepwise elongations are typically carried out by different glycosyltransferases resulting in the formation of highly diverse core O-glycan structures (Figure 5a).

What are the targets for O-glycan-engineering?

Remarkably, the contribution of distinct O-glycans to therapeutic properties (e.g. for erythropoietin which contains a single O-glycan) is still not very well investigated. As a consequence, the specific targets for O-glycan engineering are less obvious than for N-glycan engineering. The development of systems capable of producing defined O-glycans is vital to improve our understanding of O-glycan function for glycoprotein therapeutics. IgA antibodies have, for example, high potential as a new class of drugs to combat infections or kill tumor cells [81]. The engineering of O-glycan residues in the hinge region of IgA1 antibodies towards highly sialylated structures represents an interesting target to optimize the stability and pharmacokinetic behaviour of recombinant IgA1 therapeutics.

Mucin-type O-glycosylation in mammalian cells typically varies in the frequency of site occupancy and display a mixture of different structures [82]. In human cells, the generation of homogeneous mucin-type O-glycans is hampered by the large repertoire of competing glycosyltransferases (Figure 5a and 5b). Insect cells can generate some mucin-type modifications, but produce also a number of structurally diverse non-human O-glycans including the incorporation of fucose and hexuronic acid as well as further substitutions with

phosphocholine or sulfate [83]. In contrast to animal cells, yeast and plants do not contain the typical mucin-type O-glycosylation machinery, which allows the *de novo* synthesis of these mammalian-type O-glycans without any interference from the endogenous glycosyltransferases. However, recombinant EPO derived from *P. pastoris* contained two mannose residues linked to the single O-glycosylation site at Ser126 [84] (Figure 5c). These O-mannose structures differ from mammalian α -dystroglycan-type O-mannosylation and may cause unwanted side effects when present on recombinant glycoproteins. Plants can convert exposed proline residues adjacent to O-glycosylation sites of recombinant proteins into hydroxyproline residues [85], which can be further modified by arabinosyltransferases leading to the presence of small arabinose chains [86] (Figure 5d).

O-glycan engineering

In mammals, control of mucin-type O-glycosylation initiation and elongation are quite complex as a defined consensus sequence for attachment of the first monosaccharide has not been established and the factors that contribute to site-specific O-glycosylation are not well understood [80]. The human polypeptide GalNAc-transferase family consists of 20 members which are differentially expressed and display distinct as well as overlapping peptide acceptor substrate specificities [87]. CHO cells produce mainly core 1 structures that can be engineered for the production of defined and elongated structures [88,89]. Strategies to eliminate O-glycan heterogeneity include expression of the recombinant glycoprotein in non-mammalian hosts that cannot perform mucin-type O-glycosylation [90–92] or systematic elimination of the enzymes that initiate or elongate O-glycans in mammalian cells [35] (Figure 5b).

De novo O-glycan engineering has been achieved in yeast and plants. The mucin-type core 1 O-glycan (T-antigen) was generated on a peptide derived from human mucin in *S. cerevisiae* [90]. The machinery for O-linked GalNAc formation has been successfully expressed in plants [92] and di-sialylated core 1 O-glycans (Figure 5a) have been efficiently generated on *N. benthamiana*-derived human EPO-Fc [91]. A strategy to avoid the non-desired O-mannosylation of yeast, involves genetic engineering with expression of an α -mannosidase. This approach will generate a single O-linked mannose that can either be further elongated with mammalian type modifications such as the generation of α -dystroglycan-type O-glycans [93]. Alternatively, the mannose residues can be removed by *in vitro* digestion of *P. pastoris* produced glycoproteins with recombinant lysosomal mannosidases [94] or the generation of O-linked mannose chains may be prevented by inhibition of the involved protein O-mannosyltransferases (Figure 5c). In a semi-synthetic O-glycan engineering approach a polypeptide GalNAc-transferase was expressed in *E. coli* to initiate mucin-type O-glycosylation on a recombinant protein [95]. The GalNAc-containing proteins were further modified *in vitro* by a glycan-based PEGylation technique to generate proteins with increased half-life. A similar strategy has been used to attach PEG-modified sialic acid to the O-glycan of recombinant blood factor FVIII produced in mammalian cells [96].

4 Glyco-engineered therapeutic glycoproteins

In recent years, the pharmaceutical industry has put considerable effort into the development of novel glycoprotein therapeutics. Table 1 provides an overview of glyco-engineered monoclonal antibodies that are approved or in clinical trials and have been generated using different technologies. Glyco-engineering of cells for IgG1 expression has focused mainly on the elimination of core-fucose from the N-glycan at Asn297 in the Fc region of the heavy chain. Absence of core fucose increases the affinity for Fc γ RIII receptor binding leading to improved antibody-dependent cellular cytotoxicity (ADCC) on natural killer cells [17,97]. A similar glycosylation-dependent mechanism has an impact on antibody-dependent cellular phagocytosis (ADCP) by macrophages [98] and influences the receptor-mediated effector function of virus-neutralizing antibodies [99]. The ZMAPP antibody cocktail for treatment of Ebola virus infections and reversion of the disease [61] contains three plant-produced antibodies that lack core fucose residues. In mice, the fucose-free monoclonal antibody 13F6 which is one of the ZMAPP components displayed clearly enhanced potency against Ebola virus compared to 13F6 variants with core fucose [100]. These examples clearly demonstrate the impact of glycosylation and highlight the potential of glyco-engineered mAbs for different applications (Table 1) in humans. As a consequence, the number of glyco-engineered mAbs approved for different treatments is expected to increase in the near future [43]. Apart from modulating effector functions future glycosylation remodelling strategies will focus also on the engineering of mAbs for enhanced anti-inflammatory properties [66,67]. IgG glycoforms with high amounts of terminal sialic acid can display increased affinity for lectin-type receptors and may represent another emerging field for the development of next-generation antibodies. Apart from the engineered antibody therapeutics, recombinant glycoprotein drugs with enhanced half-life (similar to darboetin alfa), entirely humanized glycosylation (e.g. follicle stimulating hormone) as well as different recombinant products for enzyme replacement therapy (like alpha-galactosidase A) are in the pipelines of companies.

5 Conclusion

Despite the fact that we still do not understand all factors that influence and control glycosylation in eukaryotic cells, major advances in engineering of expression hosts towards homogeneous glycosylation patterns has been achieved in the past. The use of genome editing tools, extensive integration of analytical data and a better understanding of cellular processes will facilitate the development of next-generation expression hosts for custom-made expression of recombinant glycoprotein pharmaceuticals. These glyco-engineering approaches will allow easier production of biosimilar drugs (same homogeneous glycosylation) and lead to the generation of bio-better pharmaceuticals with altered glycosylation and optimized efficacy and safety.

6 Expert opinion

The importance of glycosylation for recombinant glycoprotein biopharmaceuticals is more and more recognized and enormous efforts are currently undertaken in academic groups and industry to provide robust solutions for controlled glycosylation. Recent advances in

engineering of cell-based expression platforms demonstrate that rather uniform and customized N-glycan structures can be made in different systems including mammalian cells (human and non-human), yeast, insect cells and plants. By employing genome editing tools like CRISPR/Cas [35] existing differences and shortcomings of species and cell-types can be eliminated and numerous remodelling tools for the generation of defined N-glycans on specific glycoproteins (e.g. monoclonal antibodies, EPO) are already available in different expression platforms. The ultimate goal will be the establishment of manufacturing platforms for production of recombinant glycoprotein therapeutics with homogeneous custom-made N-glycan structures that are designed for desired activities. Currently, such “on demand” N-glycans include highly sialylated tetraantennary complex N-glycans (Figure 3a), terminally sialylated biantennary N-glycans (Figure 3c, 3d) and fucose-deficient biantennary N-glycans (Figure 3b, 3f and 3g). In addition, the potential to produce homogeneous oligomannosidic structures like Man8GlcNAc2 (Figure 3e) and defined O-linked glycans (Figure 5a) is of interest. The means to produce glycoproteins with defined glycans will drive progress in the understanding of a glycan’s function leading to novel targets for glyco-engineering of glycoprotein therapeutics.

So what are the challenges for the future?

We still do not understand all pathways leading to glycan diversity as seen in most eukaryotic cells and our knowledge of essential enzymes involved in initiation of N- and O-glycosylation (OST complex and polypeptide GalNAc-transferases) is limited. In addition, it is still difficult to produce N-glycans with long terminal elongations like polysialylation which might significantly contribute to half-life extension. Such extensive modifications might depend on so far unknown factors that regulate the retention or contact time in the Golgi. A better characterization of cellular factors like Golgi organisation and cargo transport processes will be essential to overcome some of the current limitations. Moreover, site-specific N-glycosylation and N-glycan processing will be difficult to achieve using available *in vivo* production systems. More research is needed to understand the protein intrinsic factors that influence the relationship of a particular glycan with the polypeptide sequence and structure as well as the contribution of other still unknown aspects of site-specific glycosylation. Together with other experimental techniques, molecular dynamics simulations will be highly suitable to examine carbohydrate-protein interactions and may allow the development of strategies for site-specific remodelling of N-glycans. Structure-guided protein engineering to alter the substrate specificity of glycosylation enzymes like the OST catalytic subunit or glycan processing enzymes may provide additional tools required for site-specific engineering in cell-based production systems [72]. Alternatively, site-specific modifications may be accomplished by advanced chemoenzymatic remodelling or *de novo* synthesis of glycoproteins with precise control of attached glycan structures. In the latter case, it remains to be shown whether such sophisticated and cost-intensive approaches will be of interest for the biopharmaceutical industry or remain the field of specialized laboratories.

Another challenge for the future will be the coordination of N- and O-glycan engineering attempts. So far there are only proof-of-concept studies that have shown the feasibility of extensive remodelling of both glycosylation pathways. Due to the requirement for the

simultaneous engineering of two pathways which act in the same subcellular compartment and utilize the same nucleotide sugar donors, the control over these modifications under manufacturing conditions will be a great challenge. A better understanding of cell biology like cargo transport processes through the Golgi and development of tools to channel nucleotide sugar substrates and processing enzymes will be fundamental for more complex approaches that interfere with different cellular processes. Ultimately, the experimentally obtained parameters need to be incorporated into robust models to accurately predict the N- and O-glycan profiles of recombinant glycoprotein biopharmaceuticals. Given the great potential that glycoprotein therapeutics with defined glycans offers, the biopharmaceutical industry should extend their efforts in glyco-engineering to spur the development of next generation drugs with tailored properties and function.

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
CHO	Chinese Hamster Ovary
ER	endoplasmic reticulum
GnTI	N-acetylglucosaminyltransferase I
OST	oligosaccharyltransferase

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

1. Aeby M. N-linked protein glycosylation in the ER. *Biochim Biophys Acta*. 2013; 1833:2430–7. [PubMed: 23583305]
2. Zielinska DF, Gnad F, Schropp K, et al. Mapping N-glycosylation sites across seven evolutionarily distant species reveals a divergent substrate proteome despite a common core machinery. *Mol Cell*. 2012; 46:542–8. [PubMed: 22633491]
3. Shrima S, Gilmore R. Glycosylation of closely spaced acceptor sites in human glycoproteins. *J Cell Sci*. 2013; 126:5513–23. [PubMed: 24105266]
4. Shrima S, Cherepanova NA, Gilmore R. Cotranslational and posttranslational N-glycosylation of proteins in the endoplasmic reticulum. *Semin Cell Dev Biol*. 2014; doi: 10.1016/j.semcdb.2014.11.005
5. Shrima S, Gilmore R. Reduced expression of the oligosaccharyltransferase exacerbates protein hypoglycosylation in cells lacking the fully assembled oligosaccharide donor. *Glycobiology*. 2015; doi: 10.1093/glycob/cwv018
6. Choi BK, Warburton S, Lin H, et al. Improvement of N-glycan site occupancy of therapeutic glycoproteins produced in *Pichia pastoris*. *Appl Microbiol Biotechnol*. 2012; 95:671–82. [PubMed: 22569635]
7. Elliott S, Lorenzini T, Asher S, et al. Enhancement of therapeutic protein in vivo activities through glycoengineering. *Nat Biotechnol*. 2003; 21:414–21. [PubMed: 12612588]
8. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Br J Cancer*. 2001; 84:3–10. [PubMed: 11308268]

9. Song R, Oren DA, Franco D, et al. Strategic addition of an N-linked glycan to a monoclonal antibody improves its HIV-1-neutralizing activity. *Nat Biotechnol.* 2013; 31:1047–52. [PubMed: 24097413] [● **This elegant study demonstrates the power of protein glyco-engineering.**]
10. Nairn AV, York WS, Harris K, et al. Regulation of glycan structures in animal tissues: transcript profiling of glycan-related genes. *J Biol Chem.* 2008; 283:17298–313. [PubMed: 18411279]
11. North SJ, Huang HH, Sundaram S, et al. Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity. *J Biol Chem.* 2010; 285:5759–75. [PubMed: 19951948]
12. Bennun SV, Yarema KJ, Betenbaugh MJ, et al. Integration of the transcriptome and glycome for identification of glycan cell signatures. *PLoS Comput Biol.* 2013; 9:e1002813. [PubMed: 23326219]
13. Spahn PN, Lewis NE. Systems glycobiology for glycoengineering. *Curr Opin Biotechnol.* 2014; 30:218–24. [PubMed: 25202878] [● **Comprehensive summary of systems glycobiology approaches aiming at prediction of product glycosylation.**]
14. Jedrzejewski PM, del Val IJ, Constantinou A, et al. Towards controlling the glycoform: a model framework linking extracellular metabolites to antibody glycosylation. *Int J Mol Sci.* 2014; 15:4492–522. [PubMed: 24637934]
15. Hossler P, Mulukutla BC, Hu WS. Systems analysis of N-glycan processing in mammalian cells. *PLoS One.* 2007; 2:e713. [PubMed: 17684559]
16. Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol.* 2012; 13:448–62. [PubMed: 22722607]
17. Umaña P, Jean-Mairet J, Moudry R, et al. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol.* 1999; 17:176–80. [PubMed: 10052355] [● **Describes the GlycArt technology for reduction of core fucose on antibodies.**]
18. Goede V, Fischer K, Busch R, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N Engl J Med.* 2014; 370:1101–10. [PubMed: 24401022]
19. Zhang M, Koskie K, Ross JS, et al. Enhancing glycoprotein sialylation by targeted gene silencing in mammalian cells. *Biotechnol Bioeng.* 2010; 105:1094–105. [PubMed: 20014139]
20. Hamilton SR, Davidson RC, Sethuraman N, et al. Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science.* 2006; 313:1441–3. [PubMed: 16960007]
21. Strasser R, Altmann F, Steinkellner H. Controlled glycosylation of plant-produced recombinant proteins. *Curr Opin Biotechnol.* 2014; 30C:95–100.
22. Stanley P. Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. *Mol Cell Biol.* 1989; 9:377–83. [PubMed: 2710109]
23. Wright A, Sato Y, Okada T, et al. In vivo trafficking and catabolism of IgG1 antibodies with Fc associated carbohydrates of differing structure. *Glycobiology.* 2000; 10:1347–55. [PubMed: 11159927]
24. Yoo EM, Yu LJ, Wims LA, et al. Differences in N-glycan structures found on recombinant IgA1 and IgA2 produced in murine myeloma and CHO cell lines. *MAbs.* 2010; 2:320–34. [PubMed: 20431350] [● **Comparison of N-glycan profiles found on IgA subtypes expressed in murine myeloma and CHO cells.**]
25. Patterson G, Hirschberg K, Polishchuk R, et al. Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system. *Cell.* 2008; 133:1055–67. [PubMed: 18555781]
26. Beznoussenko GV, Parashuraman S, Rizzo R, et al. Transport of soluble proteins through the Golgi occurs by diffusion via continuities across cisternae. *Elife.* 2014; 3
27. Nabi IR, Dennis JW. The extent of poly(lactosamine) glycosylation of MDCK LAMP-2 is determined by its Golgi residence time. *Glycobiology.* 1998; 8:947–53. [PubMed: 9675228]
28. Glick BS, Luini A. Models for Golgi traffic: a critical assessment. *Cold Spring Harb Perspect Biol.* 2011; 3:a005215. [PubMed: 21875986]
29. Lavieu G, Dunlop MH, Lerich A, et al. The Golgi ribbon structure facilitates anterograde transport of large cargoes. *Mol Biol Cell.* 2014; 25:3028–36. [PubMed: 25103235]

30. D'Angelo G, Uemura T, Chuang CC, et al. Vesicular and non-vesicular transport feed distinct glycosylation pathways in the Golgi. *Nature*. 2013; 501:116–20. [PubMed: 23913272]
31. Thaysen-Andersen M, Packer NH. Site-specific glycoproteomics confirms that protein structure dictates formation of N-glycan type, core fucosylation and branching. *Glycobiology*. 2012; 22:1440–52. [PubMed: 22798492] [●● This study analyzed N-glycan profiles from 117 published papers to investigate how the individual protein structures affect the N-glycan type, core fucosylation and branching.]
32. Wormald MR, Dwek RA. Glycoproteins: glycan presentation and protein-fold stability. *Structure*. 1999; 7:R155–60. [PubMed: 10425673]
33. Stadlmann J, Pabst M, Kolarich D, et al. Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics*. 2008; 8:2858–71. [PubMed: 18655055]
34. Ayoub D, Jabs W, Resemann A, et al. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. *MAbs*. 2013; 5:699–710. [PubMed: 23924801]
35. Steentoft C, Bennett EP, Schjoldager KT, et al. Precision genome editing: a small revolution for glycobiology. *Glycobiology*. 2014; 24:663–80. [PubMed: 24861053]
36. Ghaderi D, Taylor RE, Padler-Karavani V, et al. Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nat Biotechnol*. 2010; 28:863–7. [PubMed: 20657583] [●● Demonstration of the presence of covalently bound Neu5Gc in cetuximab and generation of immune complexes *in vitro*.]
37. Kannicht C, Ramström M, Kohla G, et al. Characterisation of the post-translational modifications of a novel, human cell line-derived recombinant human factor VIII. *Thromb Res*. 2013; 131:78–88. [PubMed: 23058466]
38. Shahrokh Z, Royle L, Saldova R, et al. Erythropoietin produced in a human cell line (Dynepo) has significant differences in glycosylation compared with erythropoietins produced in CHO cell lines. *Mol Pharm*. 2011; 8:286–96. [PubMed: 21138277]
39. Ghaderi D, Zhang M, Hurtado-Ziola N, et al. Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. *Biotechnol Genet Eng Rev*. 2012; 28:147–75. [PubMed: 22616486]
40. Bosques CJ, Collins BE, Meador JW, et al. Chinese hamster ovary cells can produce galactose- α -1,3-galactose antigens on proteins. *Nat Biotechnol*. 2010; 28:1153–6. [PubMed: 21057479]
41. Chung CH, Mirakhor B, Chan E, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose. *N Engl J Med*. 2008; 358:1109–17. [PubMed: 18337601]
42. Reeves PJ, Callewaert N, Contreras R, et al. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc Natl Acad Sci U S A*. 2002; 99:13419–24. [PubMed: 12370423]
43. Beck A, Reichert JM. Marketing approval of mogamulizumab: a triumph for glyco-engineering. *MAbs*. 2012; 4:419–25. [PubMed: 22699226]
44. Croset A, Delafosse L, Gaudry JP, et al. Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells. *J Biotechnol*. 2012; 161:336–48. [PubMed: 22814405]
45. Chiba Y, Suzuki M, Yoshida S, et al. Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in *Saccharomyces cerevisiae*. *J Biol Chem*. 1998; 273:26298–304. [PubMed: 9756858]
46. Hamilton SR, Bobrowicz P, Bobrowicz B, et al. Production of complex human glycoproteins in yeast. *Science*. 2003; 301:1244–6. [PubMed: 12947202] [●● Using a combinatorial library approach the first production of complex N-glycans in yeast is shown.]
47. Jacobs PP, Geysens S, Vervecken W, et al. Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nat Protoc*. 2009; 4:58–70. [PubMed: 19131957]
48. Parsaie Nasab F, Aebi M, Bernhard G, et al. A combined system for engineering glycosylation efficiency and glycan structure in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2013; 79:997–1007. [PubMed: 23204425]

49. De Pourcq K, Tiels P, Van Hecke A, et al. Engineering *Yarrowia lipolytica* to produce glycoproteins homogeneously modified with the universal Man3GlcNAc2 N-glycan core. *PLoS One*. 2012; 7:e39976. [PubMed: 22768188]
50. Wang H, Song HL, Wang Q, et al. Expression of glycoproteins bearing complex human-like glycans with galactose terminal in *Hansenula polymorpha*. *World J Microbiol Biotechnol*. 2013; 29:447–58. [PubMed: 23136055]
51. Altmann F, Schwihla H, Staudacher E, et al. Insect cells contain an unusual, membrane-bound beta-N-acetylglucosaminidase probably involved in the processing of protein N-glycans. *J Biol Chem*. 1995; 270:17344–9. [PubMed: 7615537]
52. Shi X, Jarvis DL. Protein N-glycosylation in the baculovirus-insect cell system. *Curr Drug Targets*. 2007; 8:1116–25. [PubMed: 17979671]
53. Palmberger D, Wilson IB, Berger I, et al. SweetBac: a new approach for the production of mammalianised glycoproteins in insect cells. *PLoS One*. 2012; 7:e34226. [PubMed: 22485160]
54. Mabashi-Asazuma H, Kuo CW, Khoo KH, et al. A novel baculovirus vector for the production of nonfucosylated recombinant glycoproteins in insect cells. *Glycobiology*. 2014; 24:325–40. [PubMed: 24362443]
55. Altmann F. The role of protein glycosylation in allergy. *Int Arch Allergy Immunol*. 2007; 142:99–115. [PubMed: 17033195]
56. Cox K, Sterling J, Regan J, et al. Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat Biotechnol*. 2006; 24:1591–7. [PubMed: 17128273]
57. Schähns M, Strasser R, Stadlmann J, et al. Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. *Plant Biotechnol J*. 2007; 5:657–63. [PubMed: 17678502]
58. Strasser R, Stadlmann J, Schähns M, et al. Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol J*. 2008; 6:392–402. [PubMed: 18346095]
59. Koprivova A, Stemmer C, Altmann F, et al. Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. *Plant Biotechnol J*. 2004; 2:517–23. [PubMed: 17147624]
60. Loos A, Gruber C, Altmann F, et al. Expression and glycoengineering of functionally active heteromultimeric IgM in plants. *Proc Natl Acad Sci U S A*. 2014; 111:6263–8. [PubMed: 24706782] [●● **The assembly of pentameric and hexameric glyco-engineered forms of IgM is demonstrated using a *Nicotiana benthamiana* plant system.**]
61. Qiu X, Wong G, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*. 2014; 514:47–53. [PubMed: 25171469] [● **ZMapp™ is composed of three "humanized" monoclonal antibodies manufactured in glyco-engineered *Nicotiana benthamiana* plants.**]
62. Castilho A, Windwarder M, Gatteringer P, et al. Proteolytic and N-Glycan Processing of Human α 1-Antitrypsin Expressed in *Nicotiana benthamiana*. *Plant Physiol*. 2014; 166:1839–51. [PubMed: 25355867]
63. Tekoah Y, Tzaban S, Kizhner T, et al. Glycosylation and functionality of recombinant β -glucocerebrosidase from various production systems. *Biosci Rep*. 2013; 33
64. Mabashi-Asazuma H, Shi X, Geisler C, et al. Impact of a human CMP-sialic acid transporter on recombinant glycoprotein sialylation in glycoengineered insect cells. *Glycobiology*. 2013; 23:199–210. [PubMed: 23065352]
65. Castilho A, Neumann L, Gatteringer P, et al. Generation of biologically active multi-sialylated recombinant human EPOFc in plants. *PLoS One*. 2013; 8:e54836. [PubMed: 23372778]
66. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006; 313:670–3. [PubMed: 16888140]
67. Raju TS, Lang SE. Diversity in structure and functions of antibody sialylation in the Fc. *Curr Opin Biotechnol*. 2014; 30:147–52. [PubMed: 25032906]
68. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, et al. 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*. 2004; 87:614–22. [PubMed: 15352059] [● **Provides a description of the Pottelgent® technology.**]

69. Lau KS, Partridge EA, Grigorian A, et al. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell*. 2007; 129:123–34. [PubMed: 17418791]
70. Baker JL, Çelik E, DeLisa MP. Expanding the glycoengineering toolbox: the rise of bacterial N-linked protein glycosylation. *Trends Biotechnol*. 2013; 31:313–23. [PubMed: 23582719]
71. Valderrama-Rincon JD, Fisher AC, Merritt JH, et al. An engineered eukaryotic protein glycosylation pathway in *Escherichia coli*. *Nat Chem Biol*. 2012; 8:434–6. [PubMed: 22446837]
72. Ollis AA, Zhang S, Fisher AC, et al. Engineered oligosaccharyltransferases with greatly relaxed acceptor-site specificity. *Nat Chem Biol*. 2014; 10:816–22. [PubMed: 25129029]
73. Wang LX, Amin MN. Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem Biol*. 2014; 21:51–66. [PubMed: 24439206]
74. Wang P, Dong S, Shieh JH, et al. Erythropoietin derived by chemical synthesis. *Science*. 2013; 342:1357–60. [PubMed: 24337294]
75. Washburn N, Schwab I, Ortiz D, et al. Controlled tetra-Fc sialylation of IVIg results in a drug candidate with consistent enhanced anti-inflammatory activity. *Proc Natl Acad Sci U S A*. 2015; 112:E1297–306. [PubMed: 25733881]
76. Zimran A, Elstein D, Levy-Lahad E, et al. Replacement therapy with imiglucerase for type 1 Gaucher's disease. *Lancet*. 1995; 345:1479–80. [PubMed: 7769903]
77. Huang W, Giddens J, Fan SQ, et al. Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. *J Am Chem Soc*. 2012; 134:12308–18. [PubMed: 22747414]
78. Ahmed AA, Giddens J, Pincetic A, et al. Structural characterization of anti-inflammatory immunoglobulin G Fc proteins. *J Mol Biol*. 2014; 426:3166–79. [PubMed: 25036289]
79. Meuris L, Santens F, Elson G, et al. GlycoDelete engineering of mammalian cells simplifies N-glycosylation of recombinant proteins. *Nat Biotechnol*. 2014; 32:485–9. [PubMed: 24752077]
[●● This study describes a novel approach to reduce the glycan heterogeneity of recombinant proteins by simplification of N-glycan processing steps.]
80. Bennett EP, Mandel U, Clausen H, et al. Control of mucin-type O-glycosylation: A classification of the polypeptide GalNAc-transferase gene family. *Glycobiology*. 2012; 22:736–56. [PubMed: 22183981]
81. Bakema JE, van Egmond M. Immunoglobulin A: A next generation of therapeutic antibodies? *MAbs*. 2011; 3:352–61. [PubMed: 21691145]
82. Taschwer M, Hackl M, Hernández Bort JA, et al. Growth, productivity and protein glycosylation in a CHO EpoFc producer cell line adapted to glutamine-free growth. *J Biotechnol*. 2012; 157:295–303. [PubMed: 22178781]
83. Gaunitz S, Jin C, Nilsson A, et al. Mucin-type proteins produced in the *Trichoplusia ni* and *Spodoptera frugiperda* insect cell lines carry novel O-glycans with phosphocholine and sulfate substitutions. *Glycobiology*. 2013; 23:778–96. [PubMed: 23463814]
84. Gong B, Burnina I, Stadheim TA, et al. Glycosylation characterization of recombinant human erythropoietin produced in glycoengineered *Pichia pastoris* by mass spectrometry. *J Mass Spectrom*. 2013; 48:1308–17. [PubMed: 24338886]
85. Parsons J, Altmann F, Graf M, et al. A gene responsible for prolyl-hydroxylation of moss-produced recombinant human erythropoietin. *Sci Rep*. 2013; 3:3019. [PubMed: 24145658]
86. Karnoup AS, Turkelson V, Anderson WH. O-linked glycosylation in maize-expressed human IgA1. *Glycobiology*. 2005; 15:965–81. [PubMed: 15901675]
87. Kong Y, Joshi HJ, Schjoldager KT, et al. Probing polypeptide GalNAc-transferase isoform substrate specificities by in vitro analysis. *Glycobiology*. 2015; 25:55–65. [PubMed: 25155433]
88. Lindberg L, Liu J, Gaunitz S, Nilsson A, et al. Mucin-type fusion proteins with blood group A or B determinants on defined O-glycan core chains produced in glycoengineered Chinese hamster ovary cells and their use as immunoaffinity matrices. *Glycobiology*. 2013; 23:720–35. [PubMed: 23424082]
89. Liu J, Jin C, Cherian RM, et al. O-glycan repertoires on a mucin-type reporter protein expressed in CHO cell pools transiently transfected with O-glycan core enzyme cDNAs. *J Biotechnol*. 2015; 199:77–89. [PubMed: 25722186] **[● Describes the effect of transiently expressed O-glycan core chain glycosyltransferases on O-glycan biosynthesis pathways in CHO cells.]**

90. Amano K, Chiba Y, Kasahara Y, et al. Engineering of mucin-type human glycoproteins in yeast cells. *Proc Natl Acad Sci U S A*. 2008; 105:3232–7. [PubMed: 18296643]
91. Castilho A, Neumann L, Daskalova S, et al. Engineering of Sialylated Mucin-type O-Glycosylation in Plants. *J Biol Chem*. 2012; 287:36518–26. [PubMed: 22948156] [● **Demonstrates the *de novo* generation of sialylated core 1 O-glycans on recombinant EPO-Fc produced in plants.**]
92. Yang Z, Drew DP, Jørgensen B, et al. Engineering mammalian mucin-type O-glycosylation in plants. *J Biol Chem*. 2012; 287:11911–23. [PubMed: 22334671]
93. Hamilton SR, Cook WJ, Gomathinayagam S, et al. Production of sialylated O-linked glycans in *Pichia pastoris*. *Glycobiology*. 2013; 23:1192–203. [PubMed: 23893788] [● **Generation of α -dystroglycan-type O-glycans in yeast.**]
94. Hopkins D, Gomathinayagam S, Hamilton SR. A practical approach for O-linked mannose removal: the use of recombinant lysosomal mannosidase. *Appl Microbiol Biotechnol*. 2015; 99:3913–27. [PubMed: 25381909]
95. Henderson GE, Isett KD, Gerngross TU. Site-specific modification of recombinant proteins: a novel platform for modifying glycoproteins expressed in *E. coli*. *Bioconjug Chem*. 2011; 22:903–12. [PubMed: 21395336]
96. Stennicke HR, Kjalke M, Karpf DM, et al. A novel B-domain O-glycoPEGylated FVIII (N8-GP) demonstrates full efficacy and prolonged effect in hemophilic mice models. *Blood*. 2013; 121:2108–16. [PubMed: 23335368]
97. Ferrara C, Grau S, Jäger C, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcγRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A*. 2011; 108:12669–74. [PubMed: 21768335] [●● **Insight into the role of specific glycans in receptor-mediated antibody-dependent cytotoxicity using X-ray crystallography.**]
98. Golay J, Da Roit F, Bologna L, et al. Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. *Blood*. 2013; 122:3482–9. [PubMed: 24106207]
99. Forthal DN, Gach JS, Landucci G, et al. Fc-glycosylation influences Fcγ receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. *J Immunol*. 2010; 185:6876–82. [PubMed: 21041724]
100. Zeitlin L, Pettitt J, Scully C, et al. Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. *Proc Natl Acad Sci*. 2011; 108:20690–4. [PubMed: 22143789]

Article highlights box

- Glyco-engineering offers great potential for the generation of glycoprotein therapeutics with reduced side effects and enhanced activity.
- Analysis of recombinant glycoproteins from different mammalian and non-mammalian based expression systems reveals the presence of unwanted glycan modifications that mark targets for glyco-engineering.
- Recent developments in modulation of N-glycosylation and N-glycan processing demonstrate that recombinant glycoproteins with defined homogeneous N-glycans can be efficiently produced in mammalian and non-mammalian expression systems including insect cells, yeast and plants.
- Compared to N-glycosylation, strategies and methods to produce customized O-linked glycans are still in its infancy. Nonetheless, future developments in this area hold great promise for another class of improved glycoprotein biopharmaceuticals.
- The first glyco-engineered monoclonal antibodies have already been approved in the United States and in Japan.
- Further biochemical and molecular understanding of cellular process will be required to optimize current glyco-engineering approaches and develop strategies for so far elusive glycan modifications.

Figure 1.

Schematic presentation of N-glycan processing pathways in mammals. **(a)** N-glycosylation is initiated by OST-catalysed transfer of the lipid-linked preassembled oligosaccharide to Asn with the Asn-X-Ser/Thr consensus sequence. N-glycan processing starts in the endoplasmic reticulum (ER) by removal of glucose and mannose residues and **(b)** continues in the different Golgi cisternae by numerous processing reactions. Only a selection of possible complex N-glycan modifications is shown. OST: oligosaccharyltransferase; GCSI: α -glucosidase I; GCSII: α -glucosidase II; MANI: ER- α -mannosidase I; GMI: Golgi- α -

mannosidase I; GnTI: N-acetylglucosaminyltransferase I; GMII: Golgi- α -mannosidase II; N-acetylglucosaminyltransferase II; FUT8: core α 1,6-fucosyltransferase; GALT1: β 1,4-galactosyltransferase; GnTIV: N-acetylglucosaminyltransferase IV; GnTV: N-acetylglucosaminyltransferase V; ST: α 2,6-sialyltransferase.

Figure 2.

(a) Illustrations of representative N-glycans from human cells/tissues and characteristic structures from (b) CHO cells, (c) mouse myeloma cells, (d) *P. pastoris*, (e) insect cells and (f) plants.

Figure 3.

Strategies for N-glycan engineering. (a) Overexpression of the branching enzymes N-acetylglucosaminyltransferase IV and V (GnTIV/V); (b) knockout of core α 1,6-fucosyltransferase (FUT8); (c) knockout of CMP-Neu5Ac hydroxylase (CMAH); (d) knockout of α 1,3-galactosyltransferase (α -1,3-GalT); (e) knockout of α 1,6-mannosyltransferase (Och1p) in yeast; (f) knockout of core α 1,3-fucosyltransferase (FUT) and β -N-acetylglucosaminidase (β -hex) in insect cells; (g) knockout of core α 1,3-fucosyltransferase (FUT) and β 1,2-xylosyltransferase (XylT) in plants.

Figure 4.

(a) *In vitro* chemoenzymatic synthesis of homogeneous glycoproteins [73]. (b) *In vivo* GlycoDelete system [79]: Recombinant glycoproteins are expressed in mammalian cells lacking N-acetylglucosaminyltransferase I. Man5GlcNAc1 is cleaved off by a specific endo- β -N-acetylglucosaminidase (Endo T) in the Golgi and the resulting N-linked GlcNAc may be further elongated by endogenous galactosyl- (GalT) and sialyltransferases (ST).

Figure 5.

(a) Human mucin-type O-glycan biosynthesis pathway. Only the enzymes involved in the first biosynthesis steps are shown [80]. (b) Strategies for O-glycan engineering in mammalian cells: to produce homogeneous mucin-type O-glycans consisting of O-linked GalNAc without any further extensions (Tn antigen) a knockout of core 1 β 1,3-galactosyltransferase (C1GALT1) or its chaperone COSMC can be performed; knockout of individual polypeptide GalNAc-transferases may completely prevent mucin-type O-glycan formation. (c) In yeast transfer and elongation of O-linked mannose can be avoided by

knockout or inhibition of protein O-mannosyltransferases (PMTs). **(d)** In plants hydroxyproline formation and subsequent transfer of arabinose residues (Hyp) may be abolished by inactivation of the respective prolyl-4-hydroxylases (P4H).

Table 1
Examples of glyco-engineered antibodies that are approved or in clinical studies.

Product	Company and production platform	Glyco-engineered modification	Status
Mogamulizumab (anti-CCR4)	Kyowa Hakko Kirin Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	approved
Obinutuzumab (anti-CD20)	Glycart-Roche GlycoMab technology	core fucose-reduced CHO cells GnTIII overexpression	approved
ZMAPP (anti-Ebola virus)	MAPP Biopharmaceutical	core fucose and β 1,2-xylose-deficient <i>Nicotiana benthamiana</i> RNAi of xylosyl-/fucosyltransferases	in clinical trials
Ublituximab (anti-CD20)	TG Therapeutics LFB Biotechnologies EMABling technology	expression in YB2/0 rat hybridoma cells (low amount of FUT8)	in clinical trials
Roledumab (anti-RhD)	LFB Biotechnologies EMABling technology	expression in YB2/0 rat hybridoma cells (low amount of FUT8)	in clinical trials
SEA-CD40 (anti-CD40)	Seattle Genetics SEA technology	inhibition of core fucose incorporation by addition of modified sugars to the culture medium	in clinical trials
Benralizumab (anti-IL-5R α)	Kyowa Hakko Kirin MedImmune Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
MEDI-551 (anti-CD19)	Kyowa Hakko Kirin MedImmune Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
BIW-8962 (anti-GM2)	Kyowa Hakko Kirin Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
KHK2804 (anti-tumor specific antigen)	Kyowa Hakko Kirin Teva Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
KHK2823 (anti-CD123)	Kyowa Hakko Kirin Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
KHK2898 (anti-CD98)	Kyowa Hakko Kirin Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
KHK4083 (immunomodulator)	Kyowa Hakko Kirin Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
Ecromeximab (anti-GD3)	Kyowa Hakko Kirin Life Science Pharmaceuticals Pottelgent technology	core fucose-deficient CHO cells FUT8 knockout	in clinical trials
Lumretuzumab (anti-HER3)	Glycart-Roche GlycoMab technology	core fucose-reduced CHO cells GnTIII overexpression	in clinical trials
PankoMab (anti-TA-MUC1)	Glycotope GlycoExpress technology	human cell lines low or no core fucose	in clinical trials
TrasGEX (anti-HER2)	Glycotope GlycoExpress technology	human cell lines low or no core fucose	in clinical trials
CetuGEX (anti-EGFR)	Glycotope GlycoExpress technology	human cell lines low or no core fucose	in clinical trials

CCR4, CC chemokine receptor 4; EGFR, epidermal growth factor receptor; FUT8, core α 1,6-fucosyltransferase; GnTIII, N-acetylglucosaminyltransferase III; HER, human epidermal growth factor; IL-5R α , interleukin 5 receptor alpha; MUC1, mucin 1; RhD, rhesus D antigen.