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Applying quality by design to glycoprotein therapeutics: experimental and computational efforts of process control

Therapeutic glycoproteins represent one of the most important classes of products in the pharmaceutical industry, accounting for 77 high-value drugs out of 642 pharmaceuticals approved by the European Medicines Agency. Their therapeutic efficacy, serum half-life and immunogenicity depend on glycosylation, a complex and prominent post-translational event, which in turn is influenced by manufacturing process conditions. For this reason, protein glycosylation is a critical quality attribute for these drugs. Herein, we review the impact of glycosylation on product function, and the role of manufacturing conditions on the resulting glycoform distribution. We further present promising developments in terms of alternative, genetically engineered hosts, as well as advances in process operation that influence the glycan profile of the recombinant product. Finally, we review work on dynamic mathematical modeling for protein glycosylation that allows researchers to evaluate genetic engineering and process operation strategies *in silico*, with the aim of guiding experimentation. We demonstrate that such model-based approaches, when substantiated by experimental evidence, can support the quality by design initiative and expedite process development.

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Quality by design (QbD) is a conceptual framework that was proposed as a generic approach for manufacturing in order to try and build end-product quality into the process [1–3]. It has been widely adopted in various manufacturing industries, but until recently, the complexity of biopharmaceutical manufacturing processes has meant that most pharmaceutical companies have relied on a traditional Quality by Testing approach. Within the past decade, however, the three major pharmaceutical regulatory bodies (the US FDA, European Medicines Agency and Pharmaceutical and Medical Devices Agency of Japan) are encouraging implementation of the QbD approach for the development of all new drugs in the pipeline [3–6]. To this effect, several guidance documents have been issued [1,7,8].

QbD is an information-driven process that uses all available knowledge on the drug product to define an acceptable range of manufacturing conditions that will preserve product safety and efficacy. This includes, but is not limited to, information on the drug mode of action, manufacturing process, and potential sources of variability in the end product. More specifically, the QbD guidelines require the manufacturer to identify the critical quality attributes (CQAs) of the product and the process inputs that affect these, define the quality target product profile, select an appropriate manufacturing process to produce the quality target product profile, and define a process control strategy in order to maintain the process within the predefined limits.

A CQA is defined as 'a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality' [1]. Identification

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of CQAs relies on in-depth knowledge of how the drug properties influence its safety and efficacy, and, therefore, requires a detailed physicochemical and biological characterization of the molecule. However, knowledge for the assessment of quality attribute criticality may also be drawn from relevant data and experience from similar molecules and literature references [3]. CQAs of biologics have been thoroughly reviewed in [9] and include drug characteristics that impact the pharmacokinetics/pharmacodynamics profile, immunogenicity, and toxicity. For example, protein aggregation, which can occur at any stage of the manufacturing process due to the primary amino acid sequence, protein tertiary structure, concentration in the process conditions or final formulation, is a common protein-related CQA that affects both efficacy and immunogenicity [10,11]. Protein fragmentation is another structural CQA that can impact biological activity, serum half-life and immunogenicity due to the generation of novel epitopes [12,13], while deamidation within a complementarity-determining antibody region can also impact on biological activity [14]. Finally, glycation, a post-translational modification that involves the chemical addition of a monosaccharide on the side chain of a lysine residue in the presence of reducing sugars [15], also has an adverse effect on biological activity [16].

In addition to the product-related CQAs (e.g., inherent aggregation propensity or the number of surface-exposed lysine residues that could potentially be glycated), the choice of host cell line and bioprocessing conditions, as well as the raw materials, can introduce impurities or contaminants rendering the product unsuitable for *in vivo* use. Due to the potential for severe adverse reactions by patients, sufficient clearance of the major contaminants during the manufacturing process must be demonstrated to regulatory authorities for approval to be gained. For example, host cell proteins or DNA can contaminate the product even after downstream processing. Host cell protein release due to cell lysis at late stages of culture can be immunogenic [17], especially when microbial production systems are employed [18]. Host cell DNA is a potential carcinogen due to the potential for integration into the patient's genome. Therefore, its concentration in the final formulation is tightly controlled to be less than 10 ng in a typical dose [19]. The most significant impurities introduced by raw materials or during bioprocessing are viruses and microbial cells and their products, such as endotoxins, which are highly toxic to humans [20].

For therapeutic glycoproteins, there is an additional CQA, arising from differences in the number and type of carbohydrate structures that are attached during protein secretion by the cell. Differences in glycan attachment (macroheterogeneity) and composition as well as configuration (microheterogeneity) can have large impacts on product safety and efficacy. Batch-to-batch variation in terms of glycomic profile is, therefore, undesirable. N-linked glycosylation (attachment of glycans onto an asparagine residue located within a particular consensus sequence) occurs in 40% of all approved therapeutic proteins, including eight of the top ten selling biologics in 2010 [21,22]. As we are reaching the theoretical maximum for specific productivity thanks to genetic engineering strategies [23], increased emphasis is placed on improving biotherapeutic efficacy, including the modification of the glycoform to enhance efficacy, control the mode of action and improve half-life [24]. N-linked glycosylation has an equally important role to play in product safety as certain N-linked oligosaccharides have triggered undesired side-effects in patients [25–27], thus posing safety concerns [28]. N-linked glycosylation is, therefore, a CQA. Although a well-defined product may have consistent protein backbones, its glycomic profile can include more than a hundred detectable isoforms [29]. Narrowing and targeting the glycan profile to more closely match the most efficacious structure is expected to improve overall efficacy and safety.

This review examines the impact of protein glycosylation on glycoprotein function as a therapeutic *in vivo*, using three licensed products as examples: monoclonal antibodies, erythropoietin (EPO) and IFN-γ. We analyze where glycoform variability can arise and summarize experimental findings linking process conditions to altered glycomic profile. We finally present experimental and computational efforts towards narrowing this profile to the desired structure.

Examples of glycoform impact on product function

» Monoclonal antibodies

Antibodies are used by the immune system to recognize foreign matter and target it for elimination. These proteins form symmetric structures comprising two identical heavy chains and two identical light chains covalently bonded by disulphide bridges. The four chains are spatially arranged in a Y-shaped structure as shown in **Figure 1**. Parts of the heavy and light chains contain hypervariable segments also referred to as complementarity-determining regions, which form the antigen binding site. This is also known as the F_{ab} region. Most monoclonal antibodies on the market recognize a target epitope on a diseased cell and act to 'tag' that cell for removal by the immune system. The crystallizable (F_c) region is responsible for effector functions and has a constant amino acid sequence within the same isotype and organism. For antibodies of the IgG isotype, which are currently the only isotype contained in antibody-based licensed therapeutics [21], there are three fundamental modes of

biological action, which have been explored in mAb biotherapeutics: complement-dependent cellular cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC) and immune response modulation. In complement-dependent cellular cytotoxicity, the antibody complementarity-determining regions binds to a target cell and recruits the C1q component of the complement cascade through binding to the Fc region. This results in the formation of the membrane attack complex, which disrupts the target cell's membrane and leads to its lysis. In ADCC, the antibody Fab binds to a target cell, and through the Fc, binds to Fcγ receptors on the surface of natural killer cells and neutrophils. Through this antibody-mediated interaction, the effector cells release cytokines and cytotoxic granules, which attack the target cell and trigger apoptosis. Lastly antibodies can modulate immune response through blockage of FcR activation, as reviewed in greater detail by Hogarth *et al.* [30].

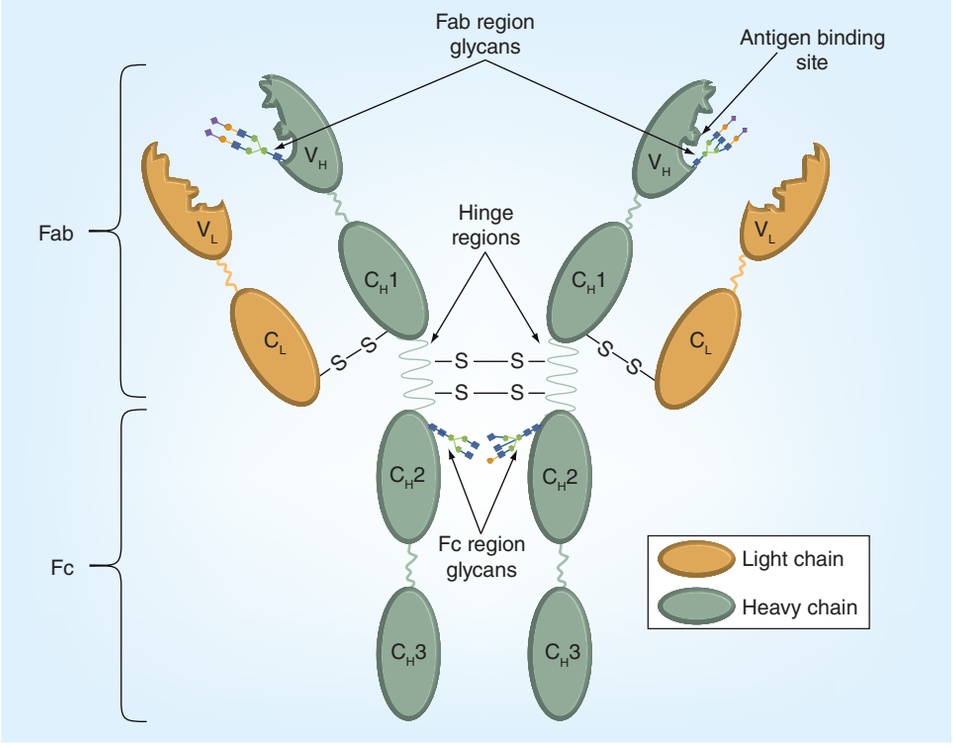

Figure 1. Immunoglobulin gamma (IgG). Heavy chains are depicted in green, light chains in orange, disulphide bonds in black.

The glycan moiety on the Cγ2 domain affects the mechanism by which antibodies act *in vivo*. Specifically, N-linked glycans directly impact the tertiary structure of the Fc [31], which modulates the affinity of Fcγ receptors for the IgG Fc [32,33]. The diversity of the oligosaccharide structures found on the Fc region is limited, in terms of complexity, to complex bi-antennary structures due to steric hindrance by the Cγ2 domains, which are in close proximity. However, significant variation in glycoform can arise due to the host cell line or the production process. The origin of the production cell line can determine the presence or absence of specific carbohydrate residues, such as bisecting GlcNAc residues or core fucosylation. Studies have shown that bisecting GlcNAc residues increase ADCC response [34], while absence of core fucose can increase ADCC up to 50-fold [35,36]. Such features have been embedded into the development of next generation antibodies, of which mogamulizumab was the first to be approved in Japan and with a number that are currently in clinical trials [37].

Conversely, structures terminating in galactose (a common terminal monosaccharide on the Fc of human polyclonal antibodies [38]) substantially increase the affinity of the IgG Fc for the C1q protein. It is, therefore, unsurprising that their absence results in decreased complement activation as reported for Rituxan [32] and Alemtuzumab [32,39] and reviewed in [33]. Recently, Raju and Jordan reported that the relative proportions of terminal galactose residues vary significantly among commercially available full-length antibody products and attributed the variation to the cell culture conditions and cell line by which they were produced [40]. Despite these variations, there were no reported adverse effects or safety issues. However, in light of earlier findings, there is an obvious need to study the effect of galactosylation on the mechanisms of action of these products, predominantly the impact on effector pathways.

The proportion of antibody with sialic acid-terminating glycan moieties can also strongly influence the efficacy of antibodies, via modulation of anti-inflammatory activity, despite the fact that only a small proportion of human IgGs are naturally sialylated [41] due to spatial constraints in the Cγ2 domain [42]. While sialic acid can be found in either a α2,3- or α2,6-linkage to the galactose moieties of IgGs, only the α2,6-linkage has been reported in human intravenous immunoglobin [43]. In the study presented in [43], removal of IgGs containing α2,6-linked sialic acid on their Fc glycan reduced anti-inflammatory activity, while a tenfold enrichment in sialic acid content induced anti-inflammatory response even with a tenfold reduced dose. In contrast, IgG with α2,3-linked

sialic acid-terminating structures failed to induce an anti-inflammatory response in mice, thus demonstrating the specificity of *in vivo* bioactivity, not only due to microheterogeneity but also due to conformational differences in the bond structure. In addition to modulating the *in vivo* mechanisms, glycan moieties can also have an effect on the serum half-life and liver clearance rates of biotherapeutic antibodies. It has also been reported that high mannose structures (oligosaccharides with five mannose residues or more) will increase plasma clearance and, thus, decrease *in vivo* half-life with a significant negative impact on drug efficacy [44].

In parallel to the Fc region, the variable region (Fab) can also contain glycans, which could potentially be manipulated in order to enhance their function. Approximately 20–30% of antibodies are N-linked glycosylated in the Fab region (either the heavy or/and the light chain) [45,46], and although the role of these glycans still has not been fully elucidated they may influence antigen-binding affinity, specificity of the interactions, and the solubility and stability of the molecule (in turn limiting aggregation) [47,48]. Mouse studies with a humanized IgG containing a variety of glycan moieties on a glycosylation site on the heavy chain variable region have shown the ability of the variable region carbohydrates to modulate half-life *in vivo* [49]. In this study, sialic acid- and galactose-terminating glycan structures had very limited effect on clearance, but exposed GlcNAc residues showed slightly faster clearance rates, possibly due to interactions with Man/GlcNAc receptors that facilitated increased clearance. Interestingly, the half-life was also dependent on the tissue where the antibodies accumulated after clearance, thus potentially providing future avenues for improvement in targeting of mAb biotherapeutics if these results can be exploited.

Unfortunately, Fab glycosylation has also been associated with negative outcomes. Although adverse effects in commercial mAbs have been limited to cetuximab, Wright *et al.* have shown that glycans in the Fab region can also have an adverse impact with respect to antigen affinity [50]. The range of glycans in the Fab region is less restricted than on the Fc region because many parts of the variable region show greater surface exposure leading to triantennary structures and a much wider glycoform distribution [45,49]. Triantennary structures have also been found to exist in the cetuximab Fab region, as well as a greater abundance of sialylated structures than in the Fc region. However, this leads to a greatly increased capacity for the formation of the α1,3-galactose terminal structure, a murine glycan motif that has generated an anaphylactic response in patients treated with cetuximab [25]. Since the consensus sequence for N-linked glycosylation can, in theory, occur anywhere within the variable region, different glycoforms are possible at different positions due to changing accessibility [50]. One study demonstrated that shifting the tripeptide sequence in the variable region could produce glycan structures ranging all the way from a high-mannose (under-processed) structure to a complex structure. This suggests that while the glycan precursor is added co-translationally to the unfolded polypeptide, the local conformation affects the accessibility of the glycan site for further processing. It follows from this that changing the position of glycosylation within the variable region can impact binding affinity for the antigen, either enhancing it or abrogating it altogether. Therefore, glycan engineering of antibody variable regions is of relevance with respect to drug safety, but also as another potential strategy for influencing drug properties.

» More complex glycoproteins

IFN-β is a human cytokine produced by the immune system in response to viral infections or other adventitious agents [51]. IFN-β is marketed as a biotherapeutic for the treatment of multiple sclerosis. Human IFN-β contains a conserved Asn-80 N-linked glycosylation site, whose site occupancy varies depending on the production system for the therapeutic. In total, there are three IFN-β products: AVONEX®, Rebif® and Betaseron®. Both AVONEX and Rebif are produced in Chinese hamster ovary (CHO) cells and are, therefore, fully glycosylated products, while Betaseron is produced in *Escherichia coli* resulting in an α-glycosylated biologic [52]. In a comparison of AVONEX and Betaseron, it was shown that the glycosylated form had a tenfold increased antiviral activity compared with the aglycosylated form [53]. Even upon enzymatic deglycosylation, the AVONEX product still showed a threefold larger antiviral activity than the Betaseron, suggesting that the glycan structure also plays an important role in the folding of this molecule [54].

Another important glycoprotein therapeutic is EPO which is used to counteract anemia associated with disease, such as chronic renal failure, cancer and HIV infection [55–58]. EPO can be glycosylated on up to four different sites (three N-linked glycan attachment sites and one *O*-linked site at amino acids 24, 38, 83 and 126, respectively [59,60]), and when fully glycosylated the carbohydrates constitute up to 40% of the total molecular weight of EPO and cover most of the surface of the molecule [61] due to the presence of large, predominantly fucosylated and tetra-antennary complex glycans at the N-linked glycosylation sites [62]. This degree of enzymatic processing suggests surface exposure of the sites and a lack of steric hindrance. The glycan structures of EPO play an important role in the biological activity, for example, it has been shown that higher

glycan antennarity leads to an increase in *in vivo* activity [63]. It was found that while EPO receptor affinity decreases with an increase in sialic acid motives, the *in vivo* activity still increases overall due to a greatly increased plasma half-life [61,64,65]. A study conducted using the desialylated form of EPO showed that the hormone was cleared with a plasma half-life of 2 min due to increased clearance by asialoglycoprotein receptors in the liver [65,66] and, thus, displaying virtually no *in vivo* activity. This observation explains why structures with higher antennarity are linked to increased plasma half-life as more antennae lead to a greater capacity for sialylation. Interestingly, for naturally occurring EPO (i.e., in the human body) all three N-linked glycan sites must be occupied to attain maximum biological activity *in vivo*, while the O-linked glycan structure does not appear to be required [67]. For recombinant human EPO, the N-linked glycans are necessary for product secretion by the host cell line and to ensure the solubility of the drug molecule [67,68].

The impact of manufacturing conditions on glycoform distribution

The majority of the 77 glycoprotein therapeutics licensed in the EU [69], are produced in mammalian cell culture systems, primarily in order to produce proteins with human-like glycan structures in order to ensure reduced immunogenicity and higher *in vivo* stability and efficacy [33,70,71]. Industrial recombinant production systems include mammalian cells (65) and transgenic animals (2), while several drugs are isolated directly from the blood plasma of healthy donors (10) [69]. CHO cells are the dominant cell line for the production of recombinant glycoproteins, followed by hybridoma cells. Baby hamster kidney (BHK) cells along with HT-1080 cells, a human sarcoma cell line, are also used in commercial production. Increasingly, there is interest in the use of humanized *Pichia pastoris* strains [72,73] as a production platform, with one product, Kalbitor®, having gained FDA approval so far.

Mammalian cell culture systems produce a heterogeneous mixture of glycan structures. These structures are generally classified into high-mannose, hybrid and complex types, as shown in Figure 2, with certain types leading to decreased therapeutic efficacy or increased immunogenicity. Product half-life and activity are, therefore, reduced from their theoretical maximum, meaning higher doses are usually required for efficacy. Variation in glycoforms is derived from the degree of enzymatic processing (driven by inherently stochastic encounters between the enzymes and the therapeutic proteins) and the availability of metabolic precursors (nucleotide sugar donors; NSDs). These NSDs, which, depending on the organism, include UDP-GlcNAc, UDP-Gal, GDP-Fuc CMP-NeuAc and CMP-NeuGc, as well as UDP-GalNAc, are metabolic products whose concentrations are influenced by the availability of nutrients. The metabolic pathways for their biosynthesis in mouse and rat cells are known as presented in Figure 3.

Numerous studies have indicated that bioprocess conditions impact not only the glycan structures produced in cell culture systems, but also the heterogeneity of the mixture. Table 1 summarizes the process factors that have been shown to alter the glycomic profile either directly or via altering cell growth (also presented in [3]). These include the CO_2 concentration in the culture medium, culture modes, growth phase and temperature, among other factors that can influence the production rates of NSDs, the quantity and localization of glycosyltransferases, and the secretion rate of the therapeutic protein itself. These effects could initially be seen as potential sources of heterogeneity. If the underlying mechanisms were to be fully understood quantitatively,

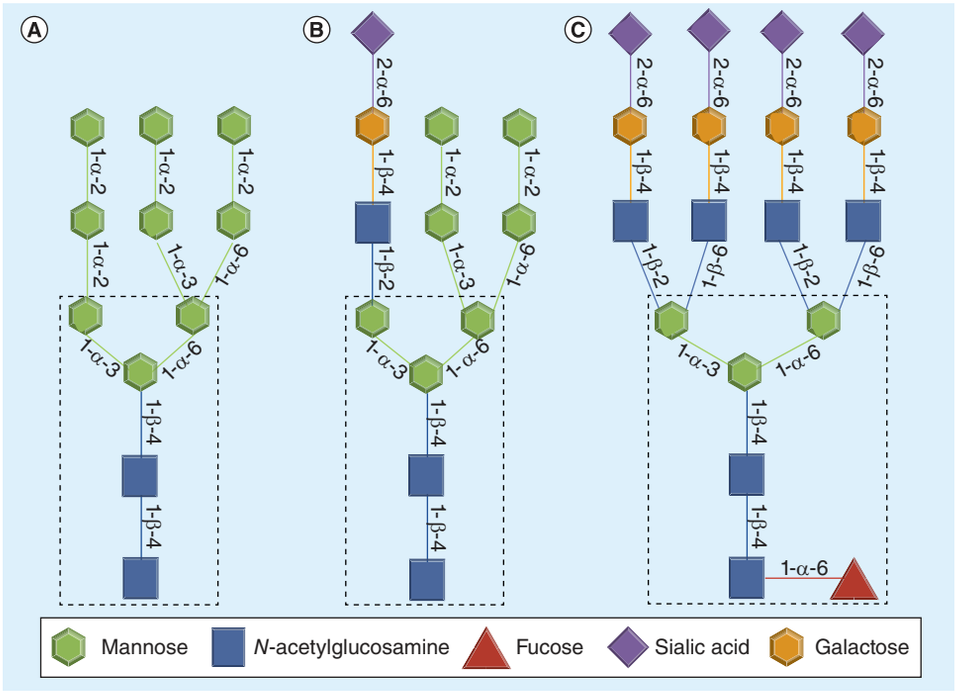

Figure 2. Classification of N-linked oligosaccharides. (A) represents a high-mannose, **(B)** a hybrid and **(C)** a complex tetra-antennary oligosaccharide. Highlighted by a box is the Man3GlcNAc2 core structure present in all N-linked glycans.

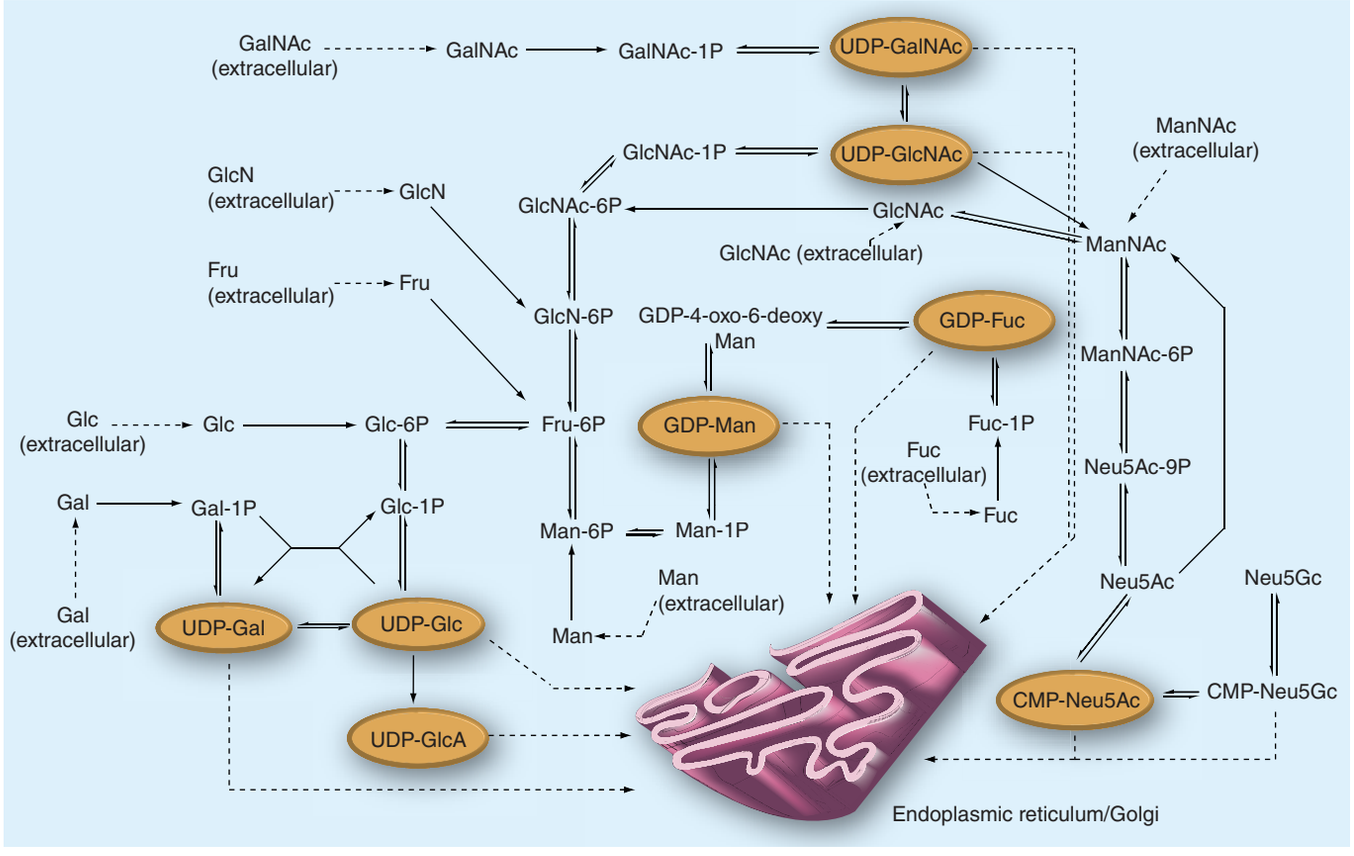

Figure 3. Metabolic pathway of mammalian nucleotide sugar metabolism.

they could serve as variables for the modulation and control of glycosylation-associated quality attributes of therapeutic proteins. However, when designing process operating strategies for this purpose, attention must be paid to a number of inhibitory mechanisms, which naturally regulate the metabolic network depicted in Figure 3.

» Nutrient availability & macroheterogeneity

Macroheterogeneity can be affected by folding rate as it has been demonstrated that folding and glycosylation do in fact compete with each other to some extent in the endoplasmic reticulum [74]. IFN-γ has been identified by Kochanowski *et al.* to be of interest in the study of metabolic control and related changes in glycosylation macroheterogeneity [75]. This glycoprotein has two glycosylation sites with varied occupancy, and as such there are three possible heterogeneous glycoforms of non-, mono- and doubly glycosylated structures where each glycoform has been reported to be secreted by CHO cells [76]. Hayter *et al.* have previously found that nutrient limitation; as well as changes in pH caused by changes in ammonia concentration, can lead to a reduction in IFN-γ glycosylation [77,78]. The Kochanowski *et al.* study found a maximum proportion of 70% of doubly glycosylated IFN-γ, which showed a decline as early as the late stage of the exponential growth phase. The reduction was not attributed to limitations in glucose or glutamine but to limitation in UTP availability, which led to reduced UDP-Glc concentrations in one of the media employed. UDP-Glc is required for the synthesis of the dolichol-bound glycan precursor molecule in the endoplasmic reticulum. Additionally, a build-up in ammonia concentration was detected at the late exponential phase, which is known to affect IFN-γ glycosylation. However, more pronounced changes were observed with respect to adenylate energy charge (AEC) levels at later stages of the cell culture. The AEC, an indicator of the metabolic and physiological status of a cell, is defined as (ATP + 0.5ADP)/(AMP + ADP + ATP) and is approximately 0.9 in cells growing exponentially [79]. The authors came to the conclusion that a strong relationship between doublyglycosylated IFN-γ and its metabolic energy level exists. This was further supported by batch-fed experiments where dynamic feeding maintained AEC levels and the doublyglycosylated IFN content remained steady. Although only investigated with IFN-γ, it would be interesting to examine if this trend is also observable for other glycoproteins.

Table 1. Summary of reported effects of bioprocessing conditions on therapeutic protein glycosylation.

Process condition	Product	Cell line	Effect	Proposed cause(s)	Ref.
Nutrient availability	Monoclonal antibody	Human hybridoma	Change in microheterogeneity when glucose is substituted by fructose, mannose and galactose	Differential efficiency with which the cells consume the different carbon sources	[105]
	IFN-γ	CHO	Reduced site occupancy on at glucose <0.3 mM	Low biosynthesis of UTP and glucosamine 6-phosphate	[106,107]
	IFN-γ	CHO	Reduced sialylation and increased high mannose glycans at 0.35–0.7 mM glucose and 0.1 mM glutamine	Decline of UDP-GlcNAc synthesis	[108]
Dissolved O_2	EPO-Fc	CHO	No effect between 10–90% DOT, reduced sialylation at 100% DOT	Mechanism not defined	[109]
	Human follicle stimulating hormone	CHO	Maximum sialylation at 100% DOT	Mechanism not defined	[110]
	Interleukin-2 glycovariant	BHK-21	No effect at 0% DOT	Mechanism not defined	[111]
	IgG1 mAb	Murine hybridoma CC9C10	Increased galactosylation with increasing DOT	UDP-Gal increases with DOT due to increased flux through glycolysis	[112]
Ammonia and pH	Mouse placental lactogen-I	Murine	Reduced site occupancy on at 9 mM NH_4^+	Mechanism not defined	[113]
	TNF-IgG	CHO	40% reduction in galactosylation and sialylation	High pH inhibits GalT and SiaT activities. It also causes GnTI, ManII, GalT and SiaT to mislocalize in Golgi	[114–116]
	IL-2	BHK-21	Increased branching in at 15 mM NH_4^+	Increased concentration of UDP-GlcNAc and UDP-GalNAc reduces the concentration of UDP-Gal	[86]
	EPO	CHO	Reduced sialylation of at 10 mM NH_4^+	Increased concentration of UDP-GlcNAc decreases transport of CMP-Neu5Ac into Golgi	[92,117]
	Mouse placental lactogen-I	Murine	28% reduction in sialylation at pH between 6.8 and 7.8	High pH inhibits GalT and SiaT activities. It also causes GnTI, ManII, GalT and SiaT to mislocalize in Golgi	[113]
	IgG3	Murine hybridoma	Highest levels of galactosylation at pH = 7.4, lowest levels of sialylation at pH = 6.9	High pH inhibits GalT and SiaT activities. It also causes GnTI, ManII, GalT and SiaT to mislocalize in Golgi	[118]
Viability, growth phase and temperature	EPO	CHO	Reduced branching at 32°C <T <37°C	Lower temperatures decrease UDP-GlcNAc and UDP-GalNAc concentration	[119]
Serum and lipid supplements	IFN-γ	CHO	Increased branching with lipid supplementation	Mechanism not understood because serum composition is unknown	[120]
	IgG1	CHO	Increased sialylation with serum-free medium	Mechanism not understood because serum composition is unknown	[121]

BHK: Baby hamster kidney; CHO: Chinese hamster ovary.

Table 1. Summary of reported effects of bioprocessing conditions on therapeutic protein glycosylation (cont.).

Process condition	Product	Cell line	Effect	Proposed cause(s)	Ref.
Serum and lipid supplements	IL-2	BHK-21	Increased fucosylation and increased sialylation with serum-free medium	Mechanism not understood because serum composition is unknown	[111]
	Monoclonal antibody	Murine hybridoma	Broader glycan distribution and increased galactosylation with BSA supplementation	Mechanism not understood because serum composition is unknown	[122]
pCO_2 and osmolality	IgG2	SP2/0	Unaffected up to 250 mmHg pCO_2, increased galactosylation at 320 mOsm/kg and high pCO_2 25% reduction in galactosylation at 195 mmHg pCO_2 and 435 mOsm/kg	Osmotic stress increases pH that inhibits GalT and SiaT activities and causes them to mislocalize	[123]
Stirring speed	Tissue plasminogen activator	CHO	72% reduction in site occupancy at low stirring speed (40 rpm)	Shear stress increases protein synthesis, which reduces endoplasmic reticulum retention time	[124]
Culture modes	IFN-γ	CHO	Reduced low site occupancy at low dilution rates between 0.5 and 0.8 day^{-1}	Concentration of metabolites inhibits GalT and SiaT activity and mislocalizes these compounds	[107]

BHK: Baby hamster kidney; CHO: Chinese hamster ovary.

Strategies to control glycomic profile

There have been several experimental studies reported that have attempted to control the glycomic profile of recombinant glycoprotein therapeutics. These involved genetic engineering attempts, feeding precursors to the nucleotide sugar donor synthesis pathway, as well as supplementing the culture with essential amino acids. These efforts and their outcomes are described below and are summarized in **Table 2**.

» Genetic engineering strategies

Genetic modification is one strategy to improve the characteristics of cell lines and could be used in efforts to reduce glycan heterogeneity by, for example, overexpressing or knocking out genes encoding for glycosyltransferase enzymes. Such attempts aim to produce glycoproteins with tailored structures in order to induce specific biological functions or to avoid glycan structures that are potentially immunogenic to patients.

One study aimed to introduce bisecting GlcNAc and increase the frequency of triantennary structures by overexpressing the *N*-acetylglucosylaminlytransferase III and V (GnT III and V) in CHO-DUKX cells. Although the frequency of the desired glycan structures did increase, these two glycosyltransferases introduced a metabolic burden that decreased cell growth rate [80]. Another study overexpressed human β1,4 galactosyltransferase and α2,3 sialyltransferase genes in order to increase the frequency of terminal galactosylation and sialylation (and reduce the level of terminal GlcNAc). In this case, more than 90% of IgG Fc-oligosaccharides expressed in this cell line were sialylated [81]. Knockdown of the α1,6 fucosyltransferase by RNA silencing resulted in a cell line that produced mAbs with a higher frequency of afucosylated glycans (60%). When tested in *in vitro* assays, the ADCC activity of these mAbs was approximately 100-fold higher [82].

These examples highlight the power of genetic engineering as a potential approach to modify glycan structures under the QbD strategy. In many cases, optimization is still required to minimize possible side-effects, but this is expected to become easier as our biological understanding of the glycosylation process increases.

An additional strategy is to engineer the product itself in order to change the number and location of glycan attachment sites (glycoengineering). This has been demonstrated to increase the product quality, by increasing protein *in vivo* half-life, which means dosing rates and frequency of administration can be decreased. For example, a glyco-engineered darbepoetin (EPO-like product) [61] that contains two additional N-linked glycosylation sites introduced by site-directed mutagenesis was created because of the obvious impact of N-linked glycans on the half-life of native EPO. The resulting drug, (marketed commercially as Aranesp®) showed threefold lower plasma clearance rates and, consequently, increased *in vivo* potency [83].

» Feeding strategies & microheterogeneity

Feeding strategies have considered adding glycosylation reaction inhibitors to achieve desired glycoforms. More specifically, nonreactive fucose analogues have been

Table 2. Summary of the reviewed glycomic profile control strategies.

Strategy	Cell line	Glycoprotein	Culture system	Outcome	Remarks	Ref.
Overexpression of glycosyltransferases						
Overexpression of GnTIII (EC 2.4.1.144) and GnTV (EC 2.4.1.155)	CHO	Cellular glycoproteins	Six-well plates; batch	Higher fraction of GnTIII and GnTV reaction products	Greatly inhibited growth was observed at high enzyme expression levels	[125]
Overexpression of GT (EC 2.4.1.38) and ST (EC 2.4.99.6)	CHO	TNFR-IgG and TNK-tPA	3 l stirred-tank bioreactor; batch	>90% of available glycan branches were sialylated for both TNK-tPA and TNFR-IgG	TNFR-IgG contains three glycans per Fab region in addition to the conserved Asn297 glycan	[82]
Knockdown of glycosyltransferases						
Knockdown of FUT8 (EC 2.4.1.68) and GMD (EC 4.2.1.47)	CHO	IgG1	1 l spinner bioreactor; batch	No detectable amounts of core-fucosylated glycans		[83]
Feeding of analogues						
Feeding of fucose analogues	CHO	IgG	n/a	Reduced core-fucosylated glycans	Patent filed 2009	[126]
Feeding of glucosamine						
Feeding of glucosamine (10 mM)	BHK-21	IL-2 mutant polypeptide	2.5 l perfusion bioreactor; continuous	Threefold increase in UDP-HexNAc levels and increased glycan antennarity		[86]
Feeding of glucosamine (12.5 mM) + uridine (2 mM)	BHK-21	IL-2 mutant polypeptide	2.5 l perfusion bioreactor; continuous	26-fold increase in UDP-HexNAc levels and increased glycan antennarity	Reduced cell growth	[87]
Feeding of glucosamine (10 mM) + uridine (2 mM)	CHO and NS0	TIMP-1	1 l spinner flask (0.4 l working volume); batch	18-fold increase in UDP-HexNAc (NS0) and 60-fold increase in UDP-HexNAc (CHO) levels	Cell growth and yield were affected negatively	[88]
Feeding of glucosamine (10 mM)	NS0	IgG1 (Fc only)	0.5 l spinner flask (0.2 l working volume); batch	17-fold increase in UDP-HexNAc levels and a 63% reduction in UDP-Hex levels	Reduced galactosylation of glycans	[94]
Feeding of glucosamine (10 mM) and glucosamine (10 mM) + uridine (5 mM)	CHO	IFN-γ	1 l flask (0.3 l working volume); batch	Five to sevenfold and 12–17-fold increase in UDP-HexNAc levels for GlcN and GlcN + Urd feed. The latter also showed a 15-fold increase in CMP-SA		[89]
Feeding of glucosamine (20 mM)	HB4C5	mAb-C5 (Fab only)	n/a	Higher fraction of tri- and tetra-antennary structures	Improved antibody affinity and highest yield with 0.5 mM glucose	[95]
Overexpression of glycosyltransferases						
Feeding of N-acetylmannosamine (20 mM)	NS0	IgG1 (Fc only)	0.5 l spinner flask (0.2 l working volume); batch	13-fold increase in CMP-SA levels	No increase in sialylation of glycans	[94]
Feeding of *N*-acetylmannosamine (20 mM) and *N*-acetylmannosamine (20 mM) + cytidine (10 mM)	CHO	IFN-γ	1 l flask (0.3 l working volume); batch	30–120-fold increase in CMP-SA levels for ManNAc and ManNAc + Cyt feed, respectively	26–52% increased yield	[89]

BHK: Baby hamster kidney; CHO: Chinese hamster ovary.

Table 2. Summary of the reviewed glycomic profile control strategies (cont.).

Strategy	Cell line	Glycoprotein	Culture System	Outcome	Remarks	Ref.
Overexpression of glycosyltransferases						
Feeding of acetylated *N*-acetylmannosamine	HeLa, CHO, COS-7 and SF-9	n/a	24-well plate; batch	100–900-fold increased CMP-SA levels	Greatly reduced cell growth	[92]
Feeding of galactose						
Feeding of galactose (10 mM)	NS0	IgG1 (Fc only)	0.5 l spinner flask (0.2 l working volume); batch	No increase in UDP-Hex levels but a shift of the UDP-Gal fraction from 20 to 94%	The lack of UDP-Hex level increase is attributed to a UDP-HexNAc 'sink', where uridine is trapped	[94]
Feeding of galactose (10 mM) and galactose (10 mM) + uridine (5 mM)	CHO	IFN-γ	1 l flask (0.3 l working volume); batch	20–22-fold increase in UDP-Gal level in both cases. The Urd supplemented cultured showed fivefold and eightfold increase in UDP-Glc and CMP-SA levels, respectively	40% decrease in cell growth attributed to glucosamine and glucose uptake competition	[89]
Feeding of glutamine						
Feeding of glutamine (4 and 8 mM)	CHO	HCG	0.5 l flask (0.15 l working volume); batch	Overall decreased sialylation, fucosylation and antennarity of glycans	Small decrease in yield	[93]
Feeding of glutamine (2–8 mM)	CHO	HCG	2 l bioreactor; continuous	Overall decreased sialylation, fucosylation, antennarity of glycans as well as UDP-GlcNAc levels	Varying glutamine concentrations can lead cells into multiple steady states	[93]

BHK: Baby hamster kidney; CHO: Chinese hamster ovary.

added to mammalian culture medium to avoid core fucosylation of antibody Fc glycans [84]. Similarly, Siadak *et al.* added mannosidase inhibitors to prevent Fc oligosaccharides from reaching more processed states [85]. In terms of precursor feeding, Gawlitzek *et al.* investigated the effect of 10 mM glucosamine feeding, which is known to be converted into GlcNAc and GalNAc as part of sugar metabolism in mammalian cells, on the level of UDP-HexNAc (UDP-GalNAc and UDP-GlcNAc) [86]. The result was a threefold increase of UDP-HexNAc levels in BHK cells and an increase in glycan antennarity, where the glycan structure was fully exposed with no steric hindrance. Grammatikos *et al.* took the investigation a step further by increasing the glucosamine feed to 12.5 mM and also adding 2 mM of uridine, the precursor of the nucleotide UTP, in order to prevent depletion [87]. The results showed a 26-fold increase in UDP-HexNAc levels in BHK cells, but surprisingly a slightly smaller increase in antennarity than in the previous study. Baker *et al.* extended this study further by comparing the impact of glucosamine (10 mM) and uridine (2 mM) feeding on NS0 and CHO nucleotide sugar pools and glycoform [88]. The UDP-HexNAc levels in this study were elevated 18-fold in NS0 and 60-fold in CHO cells. While CMP-Sialic acid and UDP-Hex (UDP-Gal and UDP-Glc) levels also showed an increase (albeit more moderate), both cell growth and protein yield were negatively affected in both cell lines. Wong *et al.* observed the same behavior when feeding CHO cells with glucosamine as well as uridine and reported a 40% decrease in cell growth, which was attributed to competitive inhibition in the uptake of glucosamine and glucose [89].

It has been suggested that the reduced growth was a result of UTP being consumed in the synthesis of UDP-HexNAc and, thus, depletion of the nucleotide. Valley *et al.* also speculated this behavior may be the result of altered O-glycosylation motifs of cytoplasmic proteins [90]. As part of the investigation by Wong *et al.* the effect of 20 mM *N*-acetylmannose (ManNAc) feeding on sialic acid pools was observed. Sialic acids are produced from UDP-GlcNAc through a pathway that is subject to product inhibition at the conversion step from GlcNAc to ManNAc. This step is bypassed in this feeding strategy, resulting in greater accumulation of sialic acids. A 30-fold increase in sialic acid levels was observed for CHO cells. However, as well as not inhibiting cell growth, ManNAc supplementation did also not lead to significantly increased sialylation of glycan moieties, despite an increase in the NeuAc fraction over

NeuGc. This phenomenon can be explained by the limitation of CMP-NeuAc/CMP-NeuGc transport into the Golgi by inhibition through UDP-HexNAc, which has been observed by Pels Rijcken *et al.* [91].

Again, this has been confirmed and extended further by Wong *et al.* who supplemented the culture with cytidine (10 mM) resulting in a 120-fold increase in CMP-SA levels [89]. The authors of the study concluded that while each combination of sugar with and without nucleotide medium supplementation can have a limited effect on glycosylation, a combination of supplements can increase overall nucleotide sugar concentrations and lead to increased glycosylation flux and, therefore, lead to more complete glycosylation. Another approach towards increasing sialic acid pools has been taken by Jones *et al.* who substituted ManNAc for its acetylated form Ac4ManNAc [92]. Although much more efficient at supporting sialic acid production and yielding 100- to 900-fold increases in intracellular sialic acid levels, the presence of Ac4ManNAc has been identified to reduce cell growth rate. Moreover, as discussed above, an increase in sialic acid pools alone does not increase sialylation significantly.

In a different strategy, a metabolic flux analysis in CHO cells was carried out by Burleigh *et al.* to investigate the effects of glutamine concentrations in batch and continuous culture [93]. Glutamine plays a crucial role in the metabolic synthesis of nucleotide sugars as it provides the amino group for the conversion of fructose-6-P to glucosamine-6-P, which is necessary for the synthesis of GlcNAc and, thus, forms the cornerstone for further *in vivo* processing. Not surprisingly, it was shown that lack of glutamine supplementation during batch cell culture led to overall decreased sialylation, fucosylation and antennarity of glycans, while cell product secretion only decreased by a small amount. Although glutamine is synthesized in low quantities by CHO cells, lack of supplementation slows initial growth rate, and glucose uptake rate as shown in the Burleigh *et al.* study [93]. In continuous culture, however, no difference in growth rate was observed when no glutamine was supplemented, although a decreased UDP-GlcNAc pool was noted as well as a decrease in glucose consumption. Again, the effects of this were overall decreased glycan antennarity, sialylation and fucosylation. However, one of the key findings of the study was that the presence of varying glutamine concentrations can lead cells into multiple steady states under otherwise unchanged culture conditions, which may guide cells into key metabolic states that could improve the attributes of biotherapeutics.

While the above discuss the effect of feeding strategies of fully exposed glycan moieties with no steric hindrance, Hills *et al.* investigated the effect of feeding strategies specifically for a monoclonal IgG antibody with Fc glycosylation produced in NS0 cells [94]. The glycoform of control samples showed predominantly fucosylated biantennary structures varying in β1,4-linked galactose content where no sialylation was detected. The α1,6-mannose arm was preferentially glycosylated compared with the α1,3-mannose arm at a ratio of 4:1. The galactose content decreased with cell culture time from 0.3 galactose molecules per complex glycan to 0.2 molecules after 168 h of culture time. This has been argued to be not only an effect of varying glycosylation with respect to changes in nutrient availability, but also due to extracellular digestion of N-glycans.

In order to increase galactosylation of glycan moieties, galactose feeding at 10 mM has been employed with no increase in total UDP-Hex levels but a shift of the UDP-Gal fraction from 20 up to 94%. The lack of increase in total UDP-Hex concentration has been argued to be due to the UDP-HexNAc 'sink' where the uridine is trapped. The effect on β1,4-galactosylation was minor with an increase of only 6%. As part of the same study, glucosamine was supplemented to the culture medium at 10 mM concentration leading to a 17-fold increase in cellular UDP-HexNAc content and a 63% reduction in mean UDP-Hex levels. A 56% reduction in galactosylation was observed. Finally, ManNAc addition to the culture medium at a concentration of 20 mM did not achieve sialylation despite a 13-fold increase in cellular CMP-sialic acid content. This result is not surprising as sialylation is limited by enzyme accessibility at the Asn297 position rather than CMP-SA levels.

Even before the above investigation, Tachibana *et al.* examined the effect of feeding strategies and the resulting glycosylation patterns at the Fab region on antibody affinity [95]. The initial finding showed that when 20 mM glucose was substituted for 20 mM glucosamine, antibody affinity increased despite 40% lower antibody productivity. Interestingly, a 0.5 mM addition of glucose to the 20 mM glucosamine yielded the highest, although marginally increased, antibody production from a range of glucose and glucosamine concentrations. Antibody light chain structures secreted in a range of glucose, glucosamine and combined monosaccharide media were analyzed through electrophoresis, where populations of 27, 28, 29 and 32 kDa were observed. 32 kDa chains were mainly observed with high glucose and/or GlcNAc concentrations and were attributed to tri- or tetra-antennary glycan attachments, while 29kDa light chains were believed to show complex asialylated bi-antennary glycan moieties with bisecting GlcNAc structures. The study showed that the light chains of 29 kDa displayed greatest antigen affinity and were produced where GlcNAc was supple-

mented to the culture medium. Unfortunately, no further data was gathered about the glycoforms obtained, but this still demonstrates the potential of optimizing the monosaccharide composition of media with respect to antibody efficacy.

» Mathematical modeling efforts

QbD is heavily reliant on the ability to understand and eventually predict the effects of changes to the process on the final product. This can be facilitated through mathematical modeling. Early mathematical models of the glycosylation process focused on single aspects of the biochemistry. In order to understand the impact of diffusion on the level of sialylation [96], a model was constructed that assumed an isotropic compartment with respect to both substrate and enzyme concentration. This effort concluded that diffusion is not a rate-limiting factor for sialyltransferase-catalyzed reactions, indicating that the bottleneck in sialylation lies elsewhere. Other work investigated macroheterogeneity of glycoproteins [97] by focusing on the attachment of the glycan precursor to nascent protein in the endoplasmic reticulum. Site occupancy is very relevant for complex glycoproteins with surface sites such as EPO. The authors study how site occupancy may depend on protein synthesis rate, oligosaccharyldolichol availability, and mRNA elongation rate, although no comparison with experimental findings was available.

The first computational model of microheterogeneity was produced in order to guide cell line engineering of glycosyltransferase expression [98]. In order to limit the number of parameters, the effort focused on the 33 most relevant species that comprise the central reaction network of interest, including mannosidases and GlcNAc-transferases (GnTs), and termination upon the addition of the first galactose residue. The concentrations of species were calculated based on total enzyme concentrations and localization, the kinetic constants of the reactions, and the space–time yield of the glycoprotein. The Golgi apparatus is approximated as four continuously stirred tank reactors (CSTRs) in series (the *cis*-, medial, *trans*-Golgi cisternae and the *trans*-Golgi network each as a separate reactor), which biologically equates to the vesicular transport model. The focus of the study was the glycosyltransferase GnTIII, which catalyzes the transfer of a bisecting GlcNAc to an agalactosylated glycan that prevents further processing by other GnTs and, thus, caps antennarity. Using the model, the authors examined the effect of overexpression of GnTIII *in silico*, which confirmed their prediction that antennarity was reduced and hybrid glycan content increased. This study provided an important first insight into the power of mathematical modeling as an approach towards glycan engineering.

To obtain further detailed information on microheterogeneity, the above reaction network was extended to include more glycosyltransferases, increasing the number of potential structures from 33 to 7565 arising from 22,871 reactions [99]. This allowed for the modeling of structures that were core fucosylated, galactosylated and sialylated. Experimentally determined enzyme dissociation constants and competitive product inhibition constants for each glycosyltransferase were used and the model was fitted with experimental data from a study of glycan heterogeneity in recombinant human thrombopoietin [100]. Enzyme concentrations were altered to give the closest fit to experimental data on an average thrombopoietin glycan site. Enzyme concentrations were varied because these are likely to be cell-line dependent, while kinetic constants are available in the literature. Golgi-resident enzyme concentrations (that are difficult to measure experimentally) were changed to match data and resulted in improved model simulation results.

Further work focused on improving the predictive capability of microheterogeneity models by varying reaction-related variables and incorporating bisubstrate reaction kinetic mechanisms into the mathematical description [101]. Previous models assumed the vesicular transport regime and modeled this as four CSTRs in series, one for each of the Golgi compartments. New biological evidence suggested that each compartment undergoes a maturation process to transform from early cisternae to late cisternae (Golgi maturation model). In an idealized case this can be mathematically described as a single plug flow reactor (PFR) with varying enzyme concentrations. However, due to constraints in *in vivo* measurements of enzyme concentrations in the Golgi and, thus, limited availability of experimentally observed enzyme distributions, four PFRs, each with constant enzyme concentrations, were employed to approximate changes in the enzyme concentration along the length of the Golgi apparatus. The results generally showed less deviation from the experimental data than those obtained with the CSTR-in-series model, particularly with respect to the ability to predict underprocessed structures. It was further shown that modifications of the enzyme concentrations for the PFR-in-series model could lead to the most targeted glycoform, thus, demonstrating enzyme localization to be a very potent approach in glycan engineering.

Recent work has focused on the ability to accurately model the structures on the IgG Fc [102]. This model also uses the cisternal maturation approach,

but expands the kinetic expressions to differentiate between the different types of bisubstrate reactions, choosing the most appropriate one based on knowledge of the specific glycosyltransferase mechanism. The Golgi apparatus is modeled as a single ideal PFR (no axial dispersion) of constant diameter, constant flow and no mass transfer limitations. Enzyme recycling along the biological reactor length leads to localization of the glycosyltransferase to the part of the PFR corresponding to each cisterna. The enzyme spatial distributions were approximated by an optimization-based method, where the unknowns of the three parameter normal functions were found by solving the minimum amount of total enzyme necessary to achieve terminal oligosaccharide processing, including 50% sialylation. A similar treatment was used to find the distribution of NSD transport proteins, where concentrations were estimated such that NSD consumption by glycosyltransferase reaction was met by NSD transport rates. **Figure 4** compares the computational results of the Krambeck and Bettenbaugh, Hossler *et al.* and Jimenez del Val *et al.* models with experimental data [103] for commercial vials of licensed monoclonal antibodies Herceptin® **(Figure 4A)** and rituximab **(Figure 4B)**. All three models are capable of reproducing experimental findings and identifying the most abundant species. The models' predictive capability is further tested by comparison with the experimental results of a glycosyltransferase gene silencing. Specifically, parameter values for the three values were fitted against data for the parental cell line **(Figure 4C)** [83] and then the fucose transferase concentration was set equal to zero to mimic the effect of gene silencing. **Figure 4D** shows that the mod-

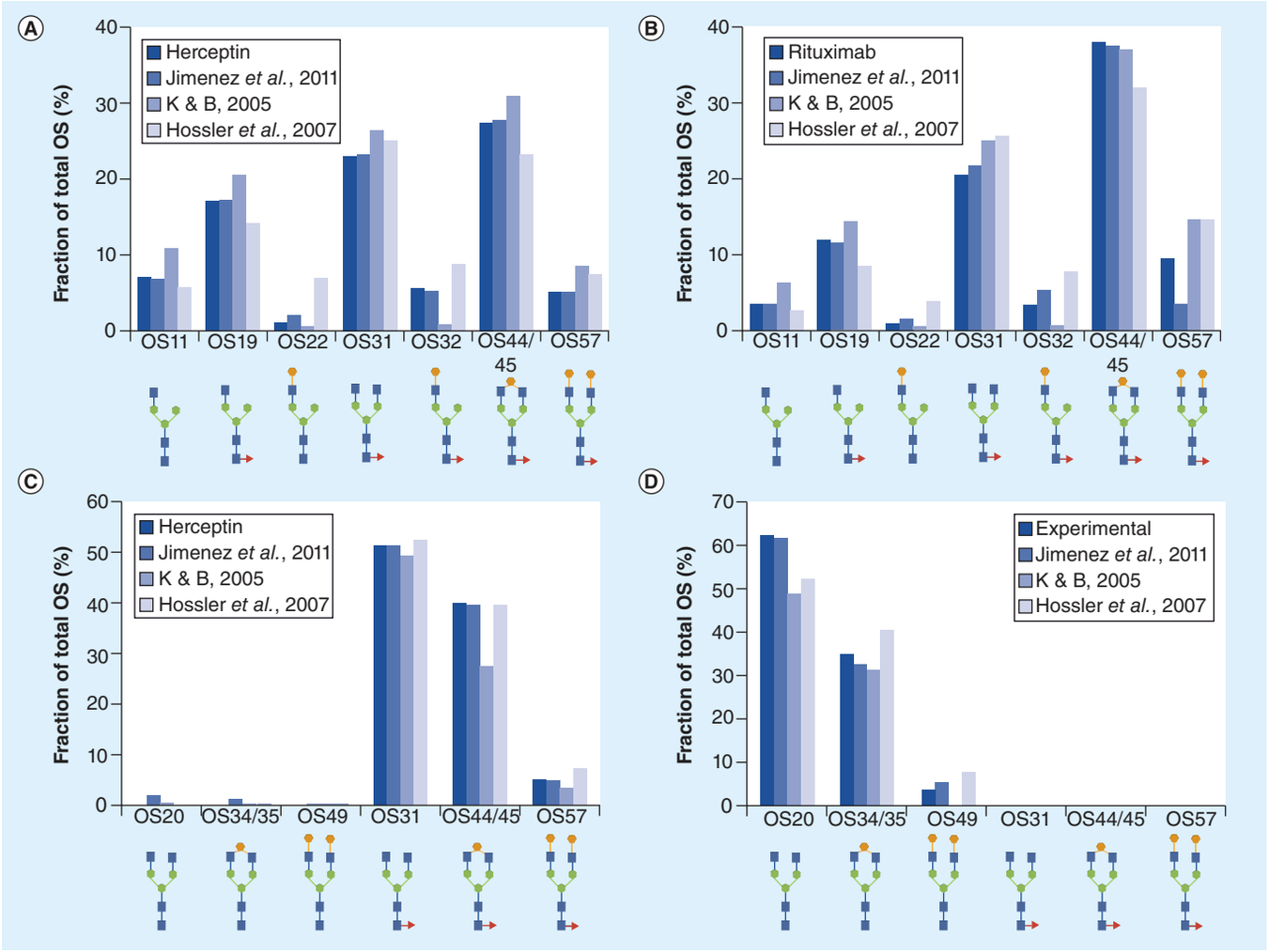

Figure 4. Calculated versus experimental oligosaccharide profiles [103]. Profiles based on the Jimenez del Val *et al.* model [127], Krambeck and Betenbaugh model [99] and the Hossler *et al.* model [101] for **(A)** Herceptin® and **(B)** Rituxan®. Comparison of reported oligosaccharide profiles [83] with the calculated profiles **(C)** before and **(D)** after FucT gene silencing.
K & B: Krambeck and Betenbaugh; OS: Overall survival.

els successfully predicted the shift in most abundant species observed experimentally.

Future perspective

QbD aims to build product quality into the manufacturing process by building on knowledge of how process conditions affect the end result. QbD is expected to decrease process reject rates, lower compliance costs and lead to the faster release of a biotech product, in an industry where 90% of the cash-to-cash cycle for a single batch is comprised of quality release times [104]. Adopting this approach requires a large volume of data; however, the benefits far outweigh the effort. Given the available knowledge of the biology of glycosylation, effect of process conditions, and feeding strategies, a first goal for QbD application to glycoprotein production could be to narrow the glycomic profile. The computational tools reviewed above are a considerable first step towards the organization of knowledge into quantitative tools that can help guide genetic engineering or feeding strategies, and ultimately form the basis for the rational design of manufacturing conditions. The next logical step is, therefore, to extend the mathematical models to allow for changes in the extracellular environment and, in turn, metabolic activities (particularly the precursor synthesis depicted in **Figure 3**). Modeling can then become an enabling tool that integrates process-level information to intracellular metabolism and protein processing steps and the final glycomic profile. Such a tool would, of course, require robust analytical information to verify its predictions for different cell lines, product types and process conditions. Modeling offers the additional capability of examining possible stochastic phenomena that may affect protein glycosylation in the endoplasmic reticulum and Golgi apparatus. In short, an integrated computational/experimental approach will generate a rich and systematized knowledge base relating manufacturing conditions to glycoprotein product characteristics, which may lead to more robust cell/process optimization or, eventually, online process control, potentially leading to the development of novel and efficient platforms for the manufacture of biotherapeutics. A key incentive for its implementation is the promise of considerable reduction in approval times associated with the QbD initiative, which is expected to reduce costs of product and process development considerably.

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Executive summary

Background

- The quality by design framework aims to build product quality into the design process.
- Protein glycosylation is an important critical quality attribute for glycoprotein therapeutics due to the impact of differences in carbohydrate structures on efficacy, stability and immunogenicity.

Examples of glycoform impact on product function

- While the glycans on the Fc of monoclonal antibodies can have an impact on the biotherapeutic in vivo mechanism and half-life, the more complex Fab region glycans can also impact drug targeting, affinity to antigen or lead to an anaphylactic response.
- Comparison of glycosylated and aglycosylated therapeutic forms of IFN-β show that the glycans affect drug stability.
- The three N-linked glycans of erythropoietin improve *in vivo* activity greatly.

The impact of manufacturing conditions on glycoform distribution

- Glycoform distribution is much impacted on by the availability of intracellular nucleotide sugar donor species as well as the glycosyltransferases of the Golgi, which, in turn, can be affected by process conditions.

Strategies to control glycomic profile

- Genetic engineering strategies focus on the modification of glycosyltransferase expression or addition of new glycan sites.
- Feeding strategies involving sugars, nucleotides or their precursors alter the availability of nucleotide sugar donors, which has been shown to be a potent method of driving the glycoform.
- Computational efforts to describe macro- and micro-heterogeneity of the glycoform show an ever improving fit with experimental data.

Future perspective

- The combination of computational and experimental work can facilitate quality by design implementation and reduce time and cost of development.

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