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Impact of cell culture media additives on IgG glycosylation produced in Chinese hamster ovary cells

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Abstract

Glycosylation is a key critical quality attribute for monoclonal antibodies and other recombinant proteins because of its impact on effector mechanisms and half-life. In this study, a variety of compounds were evaluated for their ability to modulate glycosylation profiles of recombinant monoclonal antibodies produced in Chinese hamster ovary cells. Compounds were supplemented into the cell culture feed of fed-batch experiments performed with a CHO K1 and a CHO DG44 cell line expressing a recombinant immunoglobulin G1 (IgG1). Experiments were performed in spin tubes or the ambr®15 controlled bioreactor system, and the impact of the compounds at various concentrations was determined by monitoring the glycosylation profile of the IgG and cell culture parameters, such as viable cell density, viability, and titer. Results indicate that the highest impact on mannosylation was achieved through 15 µM kifunensine supplementation leading to an 85.8% increase in high-mannose containing species. Fucosylation was reduced by 76.1% through addition of 800 µM 2-F-peracetyl fucose. An increase of 40.9% in galactosylated species was achieved through the addition of 120 mM galactose in combination with 48 µM manganese and 24 µM uridine. Furthermore, 6.9% increased sialylation was detected through the addition of 30 µM dexamethasone in combination with the same manganese, uridine, and galactose mixture used to increase total galactosylation. Further compounds or combinations of additives were also efficient at achieving a smaller overall glycosylation modulation, required, for instance, during the development of biosimilars. To the best of our knowledge, no evaluation of the efficacy of such a variety of compounds in the same cell culture system has been described. The studied cell culture media additives are efficient modulators of glycosylation and are thus a valuable tool to produce recombinant glycoproteins.

KEYWORDS
additives, ambr®15, cell culture media, IgG glycosylation

1 | INTRODUCTION

The development of therapeutic antibodies designed for the treatment of a variety of diseases is expanding. In the biopharmaceutical industry, therapeutic glycoproteins are mainly produced in mammalian cell lines, in particular, Chinese hamster ovary (CHO) cells (Hossler, Khattak, & Li, 2009; Vijayasankaran et al., 2010). The glycosylation profile of this immunoglobulin G (IgG) represents one of the most important critical product quality attributes (cQAs) because it strongly influences its safety, efficacy, and half-life (Jefferis, 2005).

IgG synthesized in vivo in humans or recombinantly produced in CHO cells are usually glycosylated on the CH_2 domain of the Fc fragment at asparagine 297. Furthermore, IgG Fab glycosylation occurs in approximately 15–30% of human IgG and is also possible in recombinant products if a consensus sequence is present in the Fab region (Jefferis, 2009; Mimura, Ashton, Takahashi, Harvey, & Jefferis, 2007). Depending on the desired mechanism of action and other characteristics, such as antibody half-life, a suitable glycosylation profile needs to be achieved (Hodoniczky, Zheng, & James, 2005; Hossler et al., 2009; Jefferis, 2009; Jiang et al., 2011; Kaneko, Nimmerjahn, & Ravetch, 2006; Liu, 2015; Nimmerjahn & Ravetch, 2007; Raju, 2008). As an example, the absence of core fucose (α-1, 6-linked fucose on the innermost GlcNAc) increases IgG binding affinity to the FcγRIIIα receptor because of decreased steric hindrance and, therefore, results in highly increased antibody-dependent cellular cytotoxicity (ADCC) (Jefferis, 2009; Jiang et al., 2011; Liu, 2015). Furthermore, for approval of biosimilars, the glycosylation profile of the monoclonal antibody (mAb) needs to match with the originator thus making modulation of glycosylation through additives a valuable tool to streamline process development and thus reduce development costs.

During upstream production, the glycosylation profile can be altered by the choice of the cell line, genetic engineering, process conditions, and media and feed formulations. Successful applications of the genetic engineering approach to modulate the glycosylation profile of the therapeutic product include the introduction of human α-2,6-sialyltransferase 1 into CHO (Raymond et al., 2015), overexpression of galactosyltransferase or sialyltransferase (Weikert et al., 1999), and knockout of fucosyltransferase 8 (Liu, 2015). Other successful approaches are nicely reviewed in Wang, Chung, Chough, & Betenbaugh (2018). In addition, process conditions like dissolved oxygen (DO), pH, or temperature are known to influence product quality (Hossler et al., 2009). Reduced DO was reported to decrease galactosylation in IgG1 produced in murine hybridoma cells (Kunkel, Jan, Jamieson, & Butler, 1998). A change in pH from 6.9 to 7.4 was shown to influence the specific growth and mAb production rates as well as the glycosylation profile of IgG3 produced in hybridoma cells (Müthing et al., 2003). In their experiments, the highest galactosylation levels were observed at pH 7.4, whereas maximal sialylation was detected at pH 7.2. Sou et al. evaluated the influence of a temperature shift from 36.5 to 32°C in the late exponential phase of a fed-batch experiment on the mAb Fc-glycan profile. Lowering the temperature to 32°C increased Man5 as well as G0 and G0F glycoforms concurrent with a decrease in galactosylated species (Sou et al., 2015). Finally, a multitude of chemical supplements added in either media or feeds have been described in the literature as effective glycosylation modulators and are summarized in detail in Table 1 (Bischoff, Liscum, & Kornfeld, 1986; Bruhlmann et al., 2017; Chang et al., 2007; Chen & Harcum, 2006; Crowell, Grampp, Rogers, Miller, & Scheinman, 2007; Foddy & Hughes, 1988; Gramer et al., 2011; Gramer, Goochee, Chock, Brousseau, & Sliwkowski, 1995; Green, Adelt, Baenziger, Wilson, & Van Halbeek, 1988; Gross et al., 1986; Gu & Wang, 1998; Hollister, Conradt, & Jarvis, 2003; Hossler et al., 2014; Jing, Qian, & Li, 2010; Jones et al., 2004; Kamińska, Dzięciol, & Kościelak, 1999; Keppler, Horstkorte, Pawlita, Schmidt, & Reutter, 2001; Kuntz, Zhong, Guo, Rose, & Boons, 2009; Li, 2014; McCracken, Kowle, & Ouyang, 2014; Mitchelson, Hughes, Mondia, & Hyde-Deruyscher, (2015); Pande, Rahardjo, Livingston, & Mujacic, 2015; Rillahan et al., 2012; Rodriguez, Spearman, Huzel, & Butler, 2005; Slade, Caspary, Nargund, & Huang, 2016; Tropea et al., 1990; Yin et al., 2017; Zhang et al., 2016).

In this work, we sought to test the efficacy of a wide range of glycan-modulating compounds in two industry-relevant cell lines (CHO K1 and CHO DG44), each producing a unique IgG1, which possess differing sialylation potentials. Glycan-modulating additives were tested in two independent systems used commonly for screening purpose during process development (ambr®15 bioreactors and Spin tubes). The effect of the additives on cellular growth as well as volumetric productivity is presented for each glycosylation category independently (mannosylation, fucosylation, galactosylation, and sialylation). Compounds are ordered depending on their efficacy in modulating absolute glycosylation levels. These data are intended to serve as a starting point to help define a good supplementation strategy to target a specific biosimilar quality profile without sacrificing titer. Although further experimentations and adjustments will remain mandatory to adopt strategies to a specific cell line and product, these data may enable a quick implementation and a reduced development time, finally leading to reduced overall development costs.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell line

Ammonium chloride, castanospermine, cytidine (Cyt), 1-deoxymannojirimycin hydrochloride (deoxymannojirimycin), deoxynojirimycin hydrochloride (deoxynojirimycin), dexamethasone, fetuin from fetal bovine serum, L-fucose (Fuc), D-(+)-mannose (Man), monensin sodium salt (Monensin), mycophenolic acid (MPA), *N*-acetyl-D-mannosamine (ManNAc), D-(+)-raffinose pentahydrate (Raffinose), reactive Red 120, sucrose, D-tagatose, uridine were purchased from Sigma Aldrich (St. Louis, MO). 2-Acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-ß-D-mannopyranose (AC4ManNAc) and tetraacetylated *N*-azidoacetylmannosamine (ManNaz) were purchased from Carbosynth (Compton, UK), mannostatin A HCl from Santa Cruz (Dallas, TX) and copper(II) chloride dihydrate ($CuCl_2$), dimethyl sulfoxide (DMSO), 96% ethanol, D-(+)-galactose (Gal), glycerol, manganese(II) chloride (Mn), *N*-acetyl-2, 3-dehydro-2-deoxyneuraminic acid (DANA) and 2-F-peracetyl fucose (2F-PerAcFuc) were purchased from Merck (Darmstadt, Germany).

CHO K1 and CHO DG44 cell lines producing two different recombinant IgG1 were used in this study.

2.2 | Cell culture media and process conditions

Merck proprietary Cellvento® chemically defined media and feed were used in this work. Fed-batch processes to modulate the mAb

TABLE 1 Summary of small molecules known in the literature to modulate *N*-glycosylation and proposed mechanisms of action

Class	Substance name	*N*-glycan modulation	Proposed mechanism of action	References
Mannosylation	Castanospermine	↑ Glucose-containing high-mannose species	α-Glucosidase inhibitor	Foddy and Hughes (1988), Gross et al. (1986)
	Deoxynojirimycin	↑ Glucose-containing high-mannose species	α-Glucosidase inhibitor	Foddy and Hughes (1988), Gross et al. (1986)
	Kifunensine	↑ High-mannose species	α-Mannosidase I inhibitor	Chang et al. (2007)
	Deoxymannojirimycin	↑ High-mannose species	Golgi α-mannosidase I inhibitor	Bischoff et al. (1986)
	Mannostatin A	↑ High-mannose species	Golgi α-mannosidase II inhibitor	Kuntz et al. (2009), Tropea et al. (1990)
	Tagatose	↑ Man5	Decreased precursor availability of UDP-GlcNAc	Hossler et al. (2014)
	Sucrose	↑ Man5	Decreased precursor availability of UDP-GlcNAc	Hossler et al. (2014)
	Raffinose	↑ Man5	Differences in nucleotide sugar levels were seen and a change in the expression level of glycosylation-dependent genes was identified	Bruhlmann et al. (2017)
	Mannose	↑ High-mannose species	Increased concentration of GDP-Man which results in inhibition of α-mannosidase activity	Slade et al. (2016)
	Monensine	↑ High-mannose species, especially Man5	pH neutralization in Golgi	Pande et al. (2015)
Fucosylation	2-F-peracetyl fucose	↓ Core fucosylation	Fucosyltransferase 8 inhibitor and GDP-mannose 4,6-dehydratase feedback inhibition	Rillahan et al. (2012)
	Reactive red 120 (triazine dye)	↓ Core fucosylation	Fucosyltransferase 8 inhibitor in an enzyme assay	Kaminska et al. (1999)
	Mycophenolic acid	↓ Core fucosylation	Reduced availability of GTP	Zhang et al. (2016)
Galactosylation	Manganese	↑ Galactosylation	Cofactor of galactosyltransferase	Crowell et al. (2007), Gramer et al. (2011)
	Uridine	↑ Galactosylation	Precursor for UDP-Gal	Crowell et al. (2007), Gramer et al. (2011)
	Galactose	↑ Galactosylation	Precursor for UDP-Gal	Crowell et al. (2007), Gramer et al. (2011)
	Ammonia	↓ Galactosylation	Increased intracellular pH causing a decrease in β-1,4-galactosyltransferase activity and mRNA expression	Chen and Harcum (2006), McCracken et al. (2014)

(Continues)

TABLE 1 (Continued)

Class	Substance name	N-glycan modulation	Proposed mechanism of action	References
Sialylation	*N*-Acetylmannosamine	↑ Sialylation	CMP-sialic acid precursor	Gu and Wang (1998)
	Tetraacetylated ManNAc	↑ Sialylation	CMP-sialic acid precursor	Jones et al. (2004)
	N-Azidoacetyl D-mannosamine	↑ Sialylation	CMP-sialic acid precursor	Jones et al. (2004), Keppler et al. (2001)
	1,3,4-O-Bu_3ManNAc	↑ Sialylation	CMP-sialic acid precursor	Yin et al. (2017)
	2,3-Dehydro-2-deoxy-*N*-acetylneuraminic acid	↑ Sialylation	Competitive sialidase inhibitor	Gramer et al. (1995)
	Copper	↑ Sialylation	Competitive sialidase inhibitor	Mitchelson et al. (2015)
	Siastatin B	↑ Sialylation	Competitive sialidase inhibitor	Li (2014)
	Dexamethasone	↑ Sialylation	Increased transfer of sialic acid on terminal galactose, increased α-2,3-sialyltransferase expression, increased β-1,4-galactosyltransferase expression, increased sialidase activity	Jing et al. (2010)
	Fetuin	↑ Sialylation	Highly sialylated protein present in fetal calf serum which might provide NANA as a precursor or might inhibit extracellular sialidase	Green et al. (1988), Hollister et al. (2003)
	Glycerol	↑ Sialylation	Stabilization of glycoprotein sialylation	Rodriguez et al. (2005)

Note. CMP: cytidine monophosphate; mRNA: messenger RNA.

glycosylation profile were performed for the CHO K1 and CHO DG44 cell lines in spin tubes (ST; TubeSpin® Bioreactor 50; TPP, Trasadingen, Switzerland) with vented cap at 37°C, 5% CO_2 and 80% humidity or in the ambr® 15 controlled bioreactor system at 37°C (with possible temperature shift to 33°C), with controlled CO_2, agitation rate, DO, and pH. The maximum working volume was 30 mL, or 10–15 mL for the ST and ambr® 15 vessels, respectively. Further process parameters are summarized in Supporting Information Table 1. Water-soluble glycosylation-modulating compounds were added directly to the cell culture feed. For the water-insoluble compounds 2F-PerAcFuc, monensin, dexamethasone, and ManNaz, concentrated stock solutions were prepared in organic solvents and further diluted in feed. The maximum final concentrations of organic solvents in the cell culture feed were 2.33% v/v of DMSO for 2F-PerAcFuc, 0.25% DMSO for ManNaz, 0.01% methanol for monensin, and 0.08% ethanol for dexamethasone. Raffinose, MPA, glycerol, and $CuCl_2$ were directly supplemented in the medium to test the efficacy of single additions. Feeds containing the glycan-modulating agents were added to the culture at 3% on Day 3 and 6% on Days 5, 7, 10, and 14 for the CHO K1 clone resulting in a total feed volume of 27%. For the DG44 clone, feeds were added at 1.5% on Day 3 and 3 on Days 5, 7, 10, 12, and 14 resulting in 16.5% of volume addition throughout the fed-batch process. Glucose (400 g/L) was fed on demand to up to 6 g/L during the week and up to 10 g/L for the weekend. A summary of all tested compounds and their concentration in feed or the total concentration added in the medium throughout the 14-day process is shown in Supporting Information Table 2.

2.3 | Monitoring of cell growth and titer

Viable cell density (VCD) and viability were measured in biological triplicates with the Vi-CELL™ XR 2.04 (Beckman Coulter, Fullerton, CA). Other cell culture parameters, including titer and ammonia and lactate production, were analyzed in triplicates using colorimetric or turbidometric methods with the CEDEX Bio HT (Roche, Mannheim, Germany) after centrifugation of the sample for 5 min at 4,500 rpm (2,287*g*). Samples for glycosylation analysis were collected on Days 7, 10, 12, and 14 of the fed-batch. The *n* for glycosylation samples refers to biological replicates.

2.4 | Purification and analysis of the glycosylation profile

The antibody was purified from the cell culture supernatant using protein A PhyTips® (PhyNexus Inc, San Jose, CA). Glycosylation patterns were analyzed either by capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) or by ultra performance liquid chromatography coupled to a mass spectrometer (UPLC-MS). The GlykoPrep®-plus Rapid N-Glycan Sample Preparation kit with 8-aminopyrene-1,3,6-trisulfonic acid trisodium (APTS; Prozyme, Hayward, CA) was applied for sample preparation according to the manufacturer's instructions. Briefly, the purified antibody was denatured and immobilized, and the glycans were released from the antibody by digestion with N-Glycanase® followed by labeling with

APTS for 60 min at 50°C. After a cleaning step to remove the remaining APTS, the relative amounts of glycans were determined using the Pharmaceutical Analysis System PA800 Plus (Sciex, Framingham, MA) with a laser-induced fluorescence detector (Ex: 488 nm and Em: 520 nm). Separation was performed in a polyvinyl-alcohol-coated capillary (total length: 50.2 cm and inner diameter: 50 µm) and filled with the carbohydrate separation buffer from the carbohydrate labeling kit (Beckman Coulter, Brea, CA). The capillary surface was first rinsed with separation buffer at 30 psi for 3 min. Inlet and outlet buffer vials were changed every 20 cycles. Samples were introduced by pressure injection at 0.5 psi for 12 s followed by a dipping step for 0.2 min to clean the capillary tips. Separation was finally performed at 20 kV for 20 min with a 0.17-min ramp applying reverse polarity. Peaks were identified according to their individual migration times and integrated according to the following parameters: peak width 0.05, threshold 10,000, and shoulder sensitivity 9,999.

For the UPLC-MS analysis, the GlycoWorks™ RapiFluor-MS™ N-Glycan Kit (Waters, Milford, MA) was used. Briefly, purified IgG were deglycosylated and labeled according to the manufacturer guide. The released and labeled glycans were analyzed by UPLC with an ACQUITY UPLC Glycan BEH Amide Column (300 Å, 1.7 µm, 2.1 × 150 mm^2) coupled to an ACQUITY UPLC® Fluorescence (FLR) Detector (Ex: 265 nm and Em: 425 nm). Glycans were characterized by their mass-to-charge ratio in the mass spectrometer (MS) (Synapt G1 HDMS; Waters) with an electrospray ionisation (ESI) source in positive mode. The scan time was set to 1 min, and the mass range was 100–2,250 Da with the following settings: 2.5 kV capillary, 30 V sample cone, 3 V extraction cone, 100°C source temperature, 350°C desolvation temperature, 50 L/hr cone gas, and 750 L/hr desolvation gas. The acceptable mass error of the system was ±20 ppm. The flow rate of the UPLC was set to 0.5 mL/min with an injection volume of 18 µL and a column temperature of 45°C. Two solvents were used: 50 mM ammonium formate (pH 4.4) and acetonitrile with a gradient of 75 min for the Fab and Fc separated samples, castanospermine, and deoxynojirimycin-treated samples. The gradient was raised stepwise from 20% to 100% ammonium formate/acetonitrile (0 min 20:80, 3 min 27:73, 55 min 43:57, 56.5 min 100:0, 59.5 min 100:0, 63.1 min 20:80, 67.6 min 20:80, and 75 min 20:80). All other samples were measured with a shorter gradient of 55 min (0 min 20:80, 3 min 27:73, 35 min 37:63, 36.5 min 100:0, 39.5 min 100:0, 43.1 min 20:80, 47.6 min 20:80, and 5 min 20:80).

The separation of the Fab and Fc parts was performed using the enzyme Fabricator® (Genovis, Cambridge, MA). The resulting IgG fragments were separated with an HPLC 1100 Series (Agilent Technologies, Waldbronn, Germany) and a Vydac 214MS (HiChrom, Leicestershire, UK; 5 µm C4, 150 × 2.1 mm^2) column at a flow rate of 0.25 ml/min and a column temperature of 55°C. The mobile phase was a water/acetonitrile mixture with 0.1% trifluoroacetic acid, and the gradient program was as follows: 0 min 90:10, 3 min 90:10, 4 min 65:35, 17 min 57:43, 22 min 5:95, 23 min 1:99, 30 min 1:99, 31 min 90:10, and 40 min 90:10. The absorbance was detected with a diode array detector at 214 nm and 280 nm. Fractions were collected between 10–14 min and 15–26 min. The fractions were dried, labeled with the RapiFluor-MS™ N-Glycan Kit and analyzed by UPLC-MS, as described above. Glycosylation data were analyzed as normalized peak area (percentage of peak area from the total sum of all peak areas). Different glycan species were typically clustered as total fucosylation, total high-mannose species, total galactosylation, total sialylation, and total *N*-acetylglucosamine, as shown in Figure 1.

For modulation of antibody sialylation, the total amount of sialic acid per mole of antibody was estimated using the following formula:

$$\frac{\mathrm{Mol}_{\text{sialic acid}}}{\mathrm{Mol}_{\text{antibody}}} = \frac{\sum A_{S1} + \sum A_{S2} \times 2 + \sum A_{S3} \times 3 + \sum A_{S4} \times 4}{100} \times N$$

with A_{sx} representing the normalized area of $x = 1$ mono-, $x = 2$ di-, $x = 3$ tri-, and $x = 4$ tetrasialylated species in percentage and N the number of *N*-glycan sites on the antibody. This calculation assumes that all *N*-glycan sites are 100% occupied (no macro-heterogeneity). For mAb1 produced in the CHO K1 cell line, the number of *N*-glycan sites is two, whereas for the mAb2 (produced in the CHO DG44 cell line) with Fc and Fab glycosylation sites, the number of *N*-glycan sites is four.

The potential effect of the specific productivity (Qp) and the cell growth phase on the glycan distributions after compound

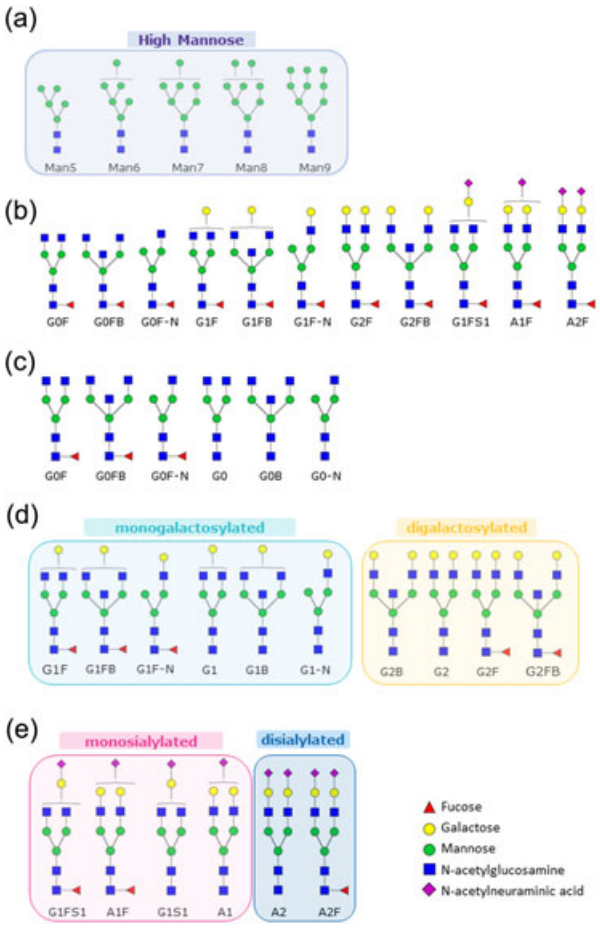


FIGURE 1 Grouping of the different glycoforms according to their terminal sugars into (a) total mannosylation, (b) total fucosylation, (c) total GlcNAc, (d) total galactosylation specifying mono- and digalactosylated species, and (e) total sialylation specifying mono- and disialylated species

supplementation was accounted for by comparing the mass fraction of the mAb glycoform produced relative to the total amount of mAb secreted during the time interval. This was calculated for both the exponential and the stationary phase following the equation published by Fan et al. (2015):

$$\mathrm{fi} = \frac{[\mathrm{mAb}_i](t_2) - [\mathrm{mAb}_i](t_1)}{[\mathrm{mAb}_{tot}](t_2) - [\mathrm{mAb}_{tot}](t_1)}$$

where fi represents the relative amount of each mAb glycoform produced during the considered interval t_2–t_1, mAb_i represents the material balance for glycoform i over the considered interval t_2–t_1 phase, and mAb_{tot} represents the total amount of antibody produced in that period.

2.5 | Statistical analysis

Fed-batch parameters are expressed as mean ± standard error of mean of three biological replicates unless stated otherwise. To compare cell culture parameters for the 2–3 week fed-batch experiments, the integral of the viable cells (IVC) and area under the curve of the product titer until Day 14 was determined by the GraphPad Prism 6 Software for each biological replicate. Furthermore, Qp was calculated for the growth phase and production phase as the linear slope of IVC and titer (Grainger & James, 2013). Statistical analysis using GraphPad Prism 6 was performed by the nonparametric, nonpaired Kruskal–Wallis test for multigroup comparisons with subsequent Dunn's tests or by the Mann–Whitney test for two-group comparison. p Values smaller than 0.05 were considered significant. p Values <0.05, <0.01 and <0.001 are indicated with *, ** and ***, respectively.

3 | RESULTS

3.1 | Glycosylation profiles in controlled versus uncontrolled systems for two mAbs

The glycosylation profiles of IgG1s produced in a CHO K1 (mAb1) (Figure 2a) or a CHO DG44 (mAb2) cell line (Figure 2b) cultured in either ST (uncontrolled system) or in the ambr®15 bioreactor system (controlled system) were compared to understand the impact of the cultivation process on this cQA. It should be noted that mAb1 only contained Fc glycosylation, whereas mAb2 showed additional Fab glycosylation. For mAb1, the average total IgG galactosylation was increased for the controlled ambr®15 system versus uncontrolled ST system (+8.9%, +10.7%, and +11.9% for Days 7, 10, and 12, respectively), confirming the impact of DO and pH regulation on the glycosylation of this IgG. Total GlcNAc (and mannose) was lower in the controlled system, whereas total fucosylation was not impacted by the cultivation conditions.

In contrast, the cultivation mode had no impact on either the galactosylation or sialylation of mAb2, indicating that the glycosylation of IgGs may be more robust to changes in process conditions only in specific cell lines/clones. A time-dependent decrease of total sialylation correlating with an increase in galactosylation was observed for

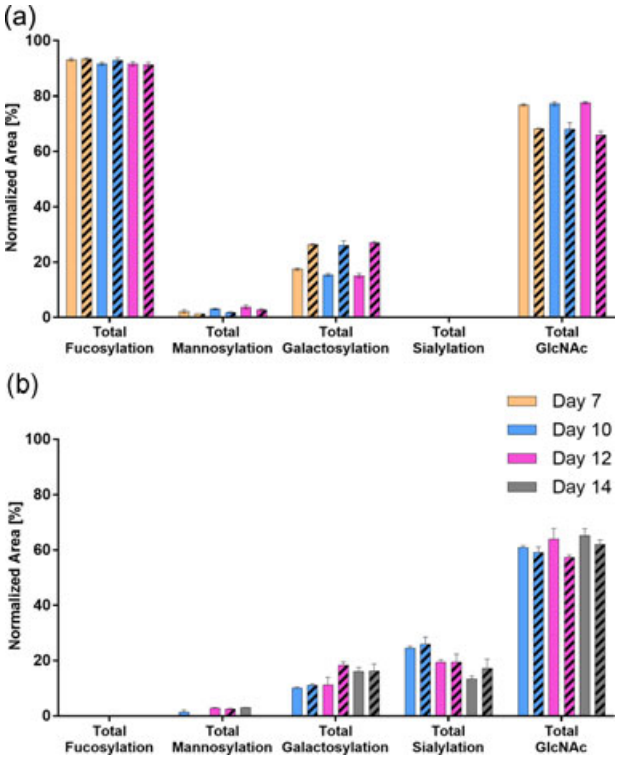


FIGURE 2 Comparison of the IgG glycosylation pattern of the control condition (normal feed) in fed-batch experiments in spin tubes (plain columns) and the ambr®15 system (dashed columns). (a) mAb1 (spin tube: $n = 5$, ambr®15: $n = 2$) (b) mAb2 (spin tube: $n = 3$, ambr®15; $n = 3$). Error bars represent standard error of mean. IgG: immunoglobulin G

mAb2 in both controlled and uncontrolled systems, suggesting either a higher activity of sialidases or an increase in the synthesis of galactosylated glycans during the stationary phase. No statistically significant differences were observed for IVC, viability, titer, or lactate and ammonia production in these experiments (data not shown).

Altogether, these data indicate that glycosylation profiles may or may not be impacted by the cultivation process, depending on the cell line used. In case of mAb1, the galactosylation profile was increased in controlled conditions and may thus limit the efficacy of compounds aiming at increasing these glycoforms.

3.2 | Mannosylation

Compounds intended to increase high-mannose species were evaluated for their impact on glycan species, IVC and titer of mAb1 in the CHO K1 cell line (Figure 3). Addition of 1.3 mM of the glucosidase inhibitor castanospermine in both small-scale systems produced one large unknown peak in the CGE-LIF analysis (50.5% for ST and 52.8% for the ambr®15). Glycosylation analysis of the same samples using UPLC-MS led to the assignment of the unknown peak as Man9Glc1/Man8Glc2 (data not shown). In contrast, a similar amount of the glucosidase inhibitor deoxynojirimycin (1.3 mM) had no effect on mAb1 glycosylation (data not shown).

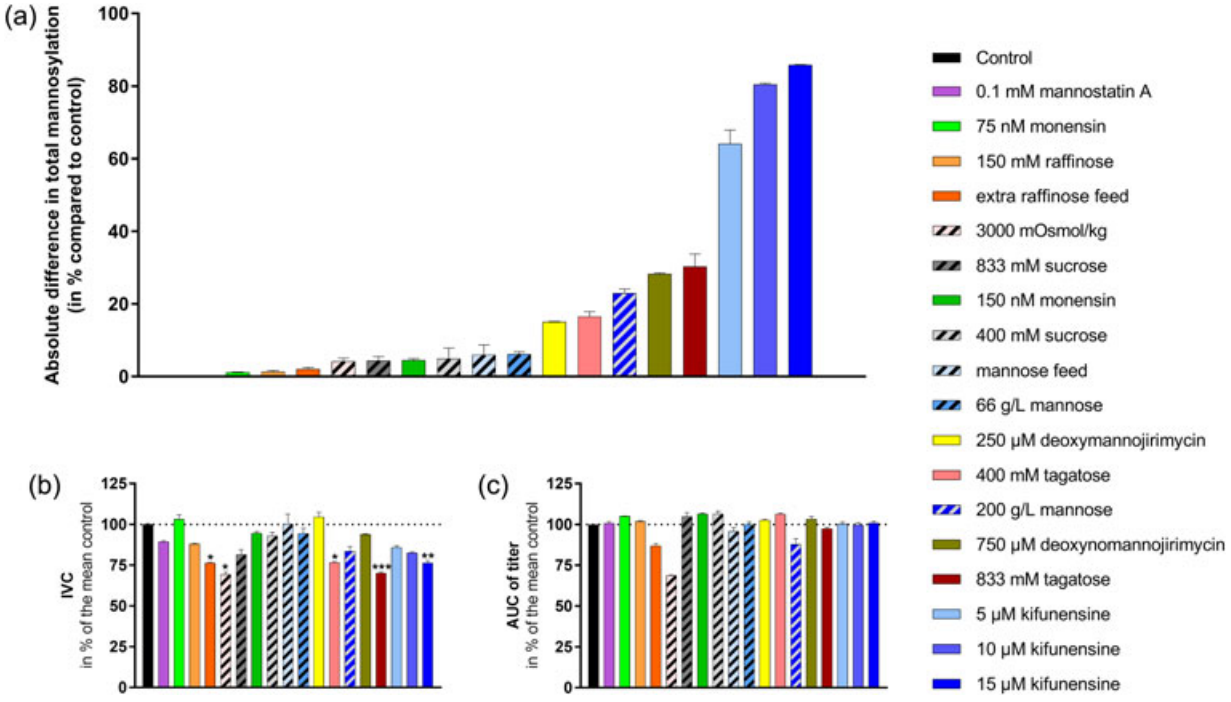


FIGURE 3 (a) Absolute change in total mannosylation (average of mAb1 harvested on Day 10 and Day 12) observed for the different additives in spin tubes (plain columns) and the ambr®15 system (dashed columns) as analyzed by CGE-LIF. (b) Integral of the viable cells (IVC) and (c) titer are shown as normalized area under the curve in percent of the respective control. Error bars represent standard error of the mean (n = 3). CGE-LIF: capillary gel electrophoresis with laser-induced fluorescence

Glycosylation results of the additional compounds to modulate mannosylation are summarized as absolute difference compared with the control condition in Figure 3. The repartition of individual mannosylated species is shown in Supporting Information Table 3, and the impact of the compounds on the other glycosylation groups (total GlcNAc, galactosylation, sialylation, and fucosylation) can be found in Supporting Information Figure 1. Compounds are ordered from lowest to highest according to their capacity to increase mannosylation. Kifunensine, which was applied in ST at a concentration of 15 µM in the feed, showed the greatest ability to increase total mannosylated species (+85.8%), with the largest increase being Man9 (+58.4%). Supplementation with 833 mM tagatose increased total mannosylation by 30.3% in ST with a predominant increase in Man5 (24.0%). 750 µM deoxymannojirimycin caused an absolute increase in total mannosylated species of 28.3% with an overall increase in Man5, Man6, Man7, and Man8. Supplementation with mannose (200 g/L) increased all Man species, with the highest increase being Man5 (+13.0%), most probably because of the inhibition of mannosidase activity in the ER and the Golgi, as suggested by Slade et al. (2016). 150 nM monensin, high osmolality, 833 mM sucrose, and raffinose conditions all increased Man5 (+4.6%, +4.1% +4.1%, and +1.7%, respectively) but no other high-mannose forms. Mannostatin A (0.1 mM in feed) showed no effect on glycosylation.

Generally, if an increase in Man5 is desired, supplementation of tagatose, raffinose, monensin, mannose, or sucrose seems advisable or a high osmolality may be used. Regarding a preferred increase in all high-mannose species (especially Man9), kifunensine may be applied. For an increase of Man5–Man8, supplementation with deoxymannojirimycin would be preferable.

If we consider cellular data and volumetric titer, the IVC was equal or slightly greater than 100% compared with the control for 75 nM monensin, 250 µM deoxymannojirimycin, and mannose feed instead of glucose. Less than 10% reduction in IVC was detected for 400 mM sucrose, 150 nM monensin, 66 g/L mannose, and 750 µM deoxymannojirimycin, whereas >25% decrease in IVC was found for 3,000 mOsmol/kg and 833 mM tagatose. Titer was either not affected or mildly enhanced by 400 mM sucrose, mannostatin A, 150 mM raffinose, 833 mM sucrose, monensine (150 nM and 75 nM), 250 µM deoxymannojirimycin, 400 mM tagatose, 750 µM deoxymannojirimycin, kifunensine (5, 10, and 15 µM), and 66 g/L mannose. Less than 25% reduction in titer was observed for 833 mM tagatose, 200 g/L mannose, and the mannose feed instead of the glucose feed. More than 25% decrease in titer was detected for 3,000 mOsmol/kg. A slight negative influence on IVC, viability, and titer was observed for the supplementation with castanospermine (data not shown).

To understand possible interactions between factors impacting the final glycosylation profile, the mass fraction of mAb glycoform produced relative to the total amount of antibody secreted was calculated for the growth phase (D0–D7) and the stationary phase (D7–D12) and is presented in Supporting Information Table 4. These data indicate that the increase in mannosylation in the conditions with additives is higher in the stationary phase compared with the

growth phase. This is most likely because of the accumulation of the additives in the final medium after successive feed addition. For some compounds like raffinose and sucrose, the increase in mannosylated glycans was only detectable for the fraction of the mAb produced after D7, which may indicate that higher concentrations might be beneficial. If the fi data obtained for all the additives of these groups are correlated to each other and to additional parameters like Qp, correlations between glycoforms and cell culture parameters can be drawn. For this data set, results indicate that overall, mannosylation correlates negatively with both fucosylation and total GlcNAc forms independently of the culture phase (R^2 of −0.99 and −0.97, respectively). In contrast, a weaker correlation of −0.73 was found between mannosylation and galactosylation indicating that other, independent variables impact galactosylation. Finally, no significant correlation was found between any of the glycoforms and Qp, indicating that the effect of the additives on mannosylation and related glycoforms is independent of Qp.

3.3 | Fucosylation

Fucosylation can be decreased by the addition of compounds that specifically reduce fucosylation or by the increase in high-mannose species, which are generally afucosylated. In the following, only results for compounds intended to influence fucosylation without a concurrent increase in mannosylated species are summarized (Figure 4). The fucose derivative 2F-PerAcFuc is a well-known fucosyltransferase inhibitor and was more efficient in reducing fucosylation than Reactive Red 120 or MPA. 2F-PerAcFuc was found to decrease fucosylation in a dose-dependent manner, with the largest decrease being 76.1% at the highest dose tested (800 µM). The mass fraction of fucosylated mAb produced during the exponential and stationary phases (Supporting Information Table 5) indicates that almost 90% of the antibody produced between D7 and D12 is afucosylated, indicating that the accumulation of the additive through multiple feeding time points is beneficial to the final afucosylation level. DMSO (solvent used for solubilization of 2F-PerAcFuc) did not show any effect on antibody fucosylation but resulted in a decreased IVC and increased Qp. Supplementation with the fucosyltransferase inhibitor Reactive Red 120 was not successful in reducing fucosylation even at high concentrations (500 µM), which instead resulted in early cell death on Day 13 (data not shown). MPA was only successful in decreasing fucosylation (3.4%) when added at high concentrations from the beginning of the fed-batch (25 µM). Higher concentrations of 100 and 250 µM had a detrimental effect on viability and cell growth (data not shown). To lower the negative impact on IVC, viability, and titer, a single dose was added at the end of the growth phase on Day 7 but resulted in a similar glycosylation profile as the control. Overall, the additives applied to reduce fucosylation did only show a small impact (<5%) on other glycan species (Supporting Information Figure 2). In summary, to reduce fucosylation and thus increase ADCC, supplementation of the fucose-analog 2F-PerAcFuc seems to be the most efficient method.

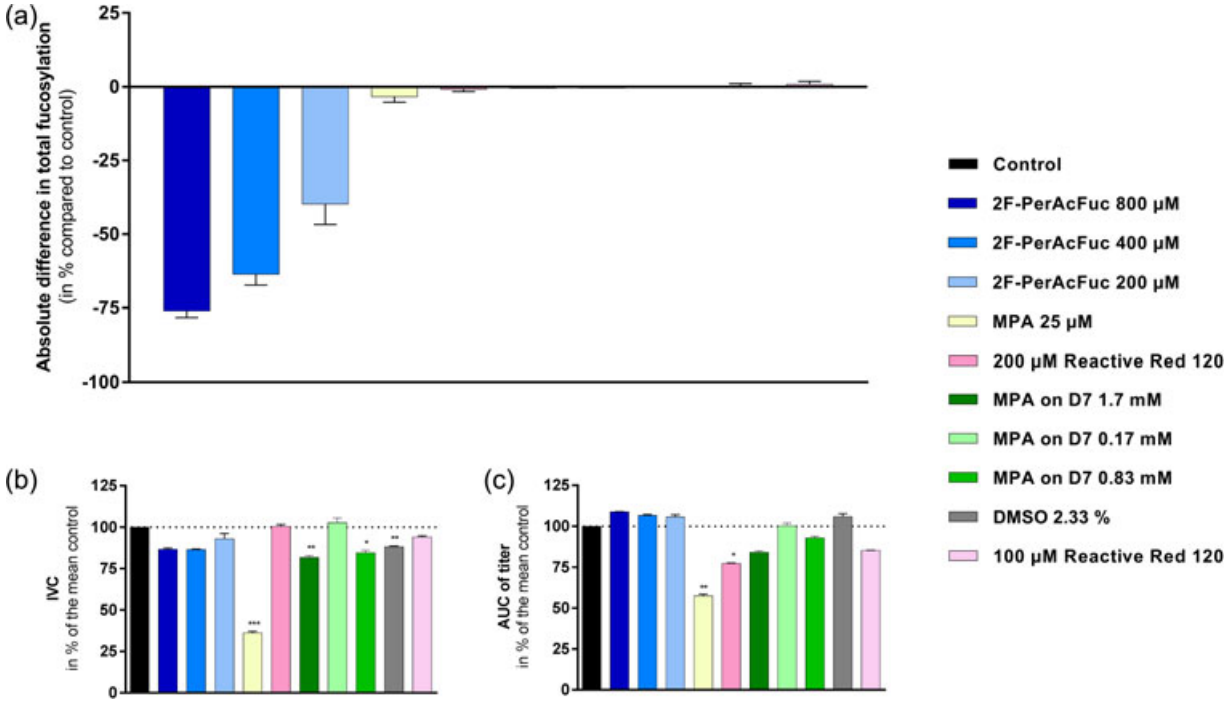


FIGURE 4 (a) Absolute change in total fucosylation (average of mAb1 harvested on Days 10 and 12, except for 25 µM MPA with average of Day 7 and 10) observed for the different additives in spin tubes as analyzed by UPLC-MS ($n = 2$). (b) Integral of the viable cells (IVC) and (c) titer are shown as normalized area under the curve in percent of the respective control. Error bars represent standard error of the mean ($n = 3$). mAb1: monoclonal antibody 1; MPA: mycophenolic acid; UPLC-MS: ultra performance liquid chromatography coupled to a mass spectrometer

3.4 | Galactosylation

Different additives and mixtures of additives were applied to modulate antibody galactosylation. Their impact on the glycan species, IVC, and titer of the mAb1 in the CHO K1 cell line was analyzed (Figure 5). In the control condition, 11.5% ± 2.1% of the IgG were galactosylated on Day 12, with a split between monogalactosylated and digalactosylated species of 10.8% ± 2.2% and 0.7% ± 0.6%, respectively. Combinations of cytidine (Cyt), fucose (Fuc), and uridine, as well as sole addition of manganese chloride (Mn) or a mixture of Mn and galactose (Gal) with or without uridine, increased galactosylation (Figure 5a) without affecting negatively IVC or titer. This indicates that the Mn level already present in the medium was not sufficient to trigger the "on/off response" described as necessary to enable maximum galactosylation (Grainger & James, 2013). Further impact on the glycan profile is shown in Supporting Information Figure 3. The highest increase in galactosylation was obtained with a combination of galactose (120 mM), manganese chloride (48 μM), and uridine (24 mM) (UMG) added in the feed. This condition showed an increased IVC (Figure 5b) and titer (Figure 5c) for mAb1. This clone-dependent effect mediated by uridine might be linked to a low abundance of nucleotide precursors uridine triphosphate and uridine diphosphate (UDP), which are essential to regulate cellular functions, such as proliferation rate and cell cycle. The total cumulative concentration of uridine added to the medium in this study was 6.5 mM and was thus lower than the concentration of 10 mM, shown to act as a potent inhibitor of IVC and mAb production (Grainger & James, 2013). A maximum galactosylation of approximately 50% was observed for the tested conditions, which suggests a saturation of the galactosylation level. The only negative effector of galactosylation was 100 mM ammonium chloride, which decreased total galactosylation by 8.6% (Day 12) when compared with the control, also negatively impacting IVC and titer. Higher concentrations of ammonium chloride (200 mM), which is a toxic cellular waste product, resulted in cell death until Day 10 concurrent with detrimental reduction in titer (data not shown).

In summary, our data demonstrate that galactosylation can be modulated linearly by an appropriate choice of additive or combination of additives, while avoiding to negatively impact growth or viability. However, because of the dynamic nature of a culture in fed-batch, a maximum galactosylation level might be reached over time.

3.5 | Sialylation

Experiments for modulation of antibody sialylation were performed with a CHO DG44 cell line producing mAb2 with higher sialylation levels. For modulation of sialylation, a feed with UMG was used as control feed. This control condition was chosen because galactosylation is critical for binding of sialic acid and increased galactosylation is, therefore, a requirement for high sialylation levels. For mAb2, supplementation of 120 mM galactose, 48 μM manganese chloride, and 24 mM uridine did not only increase overall galactosylation (+17.6% at Day 12 in ST) but also caused an increase in total sialylation by 19.0% (data not shown). To differentiate between the glycosylation structures of the Fab and Fc fragment, the antibody

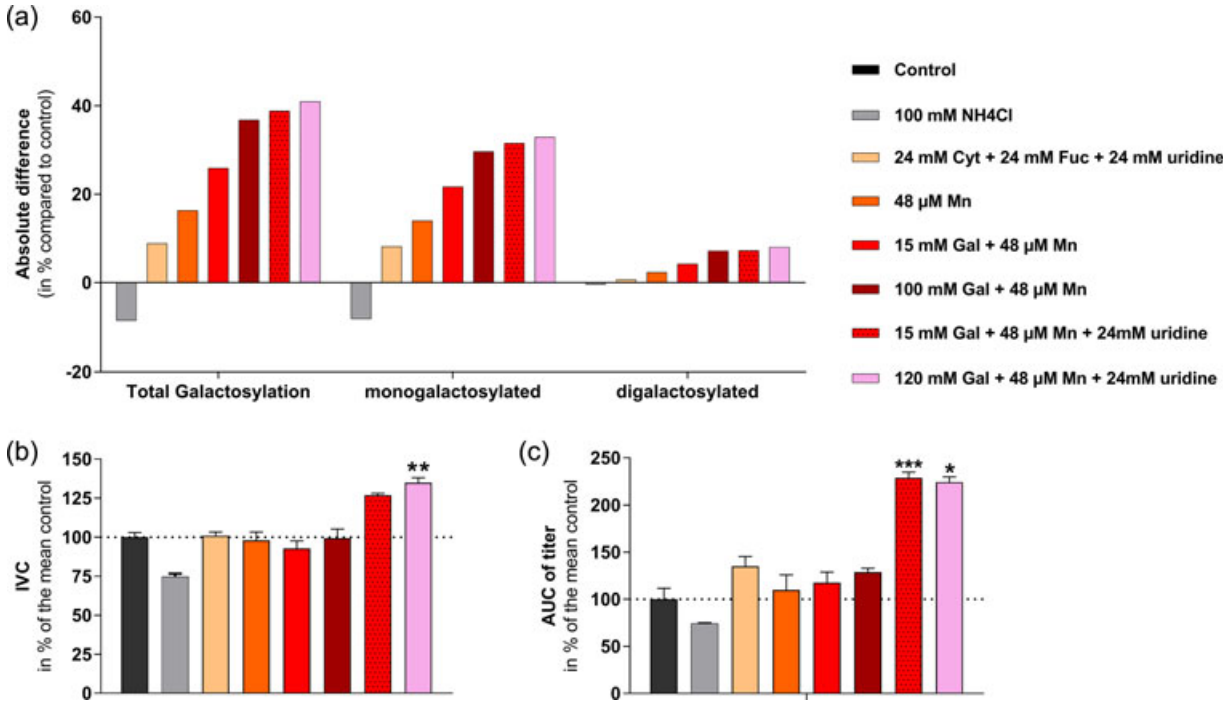
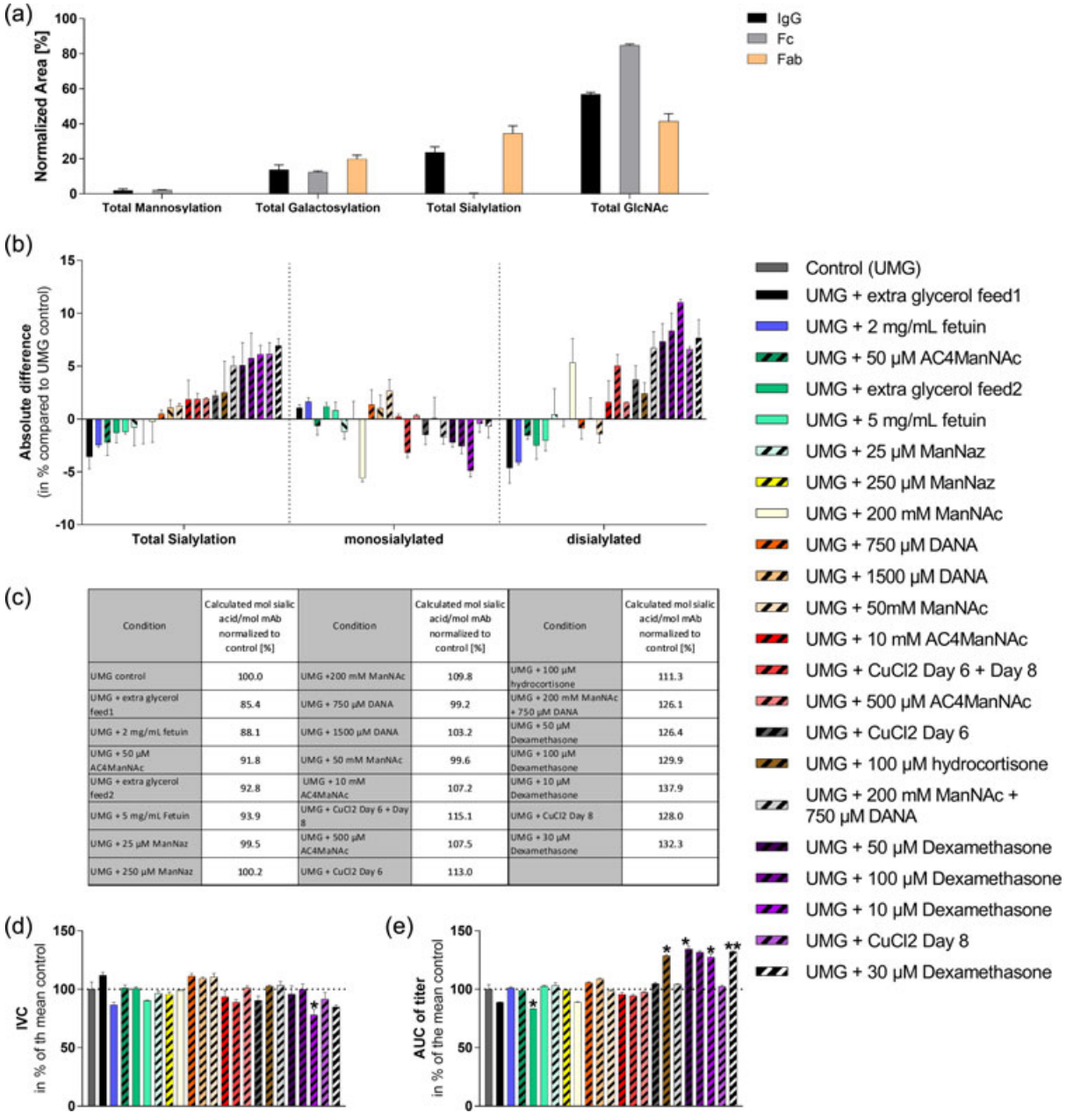


FIGURE 5 (a) Absolute change in total galactosylation of mAb1 observed for the different additives in spin tube experiments as analyzed by CGE-LIF (n = 1). (b) Integral of the viable cells (IVC) and (c) titer are shown as normalized area under the curve in percent of the respective control. Error bars represent standard error of the mean (n = 3). CGE-LIF: capillary gel electrophoresis with laser-induced fluorescence; mAb1: monoclonal antibody 1

was digested with FabRICATOR®, and the generated fragments were separated before glycosylation analysis using high-performance liquid chromatography. As expected from literature (Nigrovic, 2013), our results show that Fc sialylation was almost absent (<0.5%) whereas the Fab part was highly sialylated with 34.5% total sialylation (Figure 6a). Likewise, after the addition of compounds to increase sialylation (750 µM DANA, 50 mM ManNAc, 5 mg/ml fetuin, 0.5 mM $CuCl_2$ on Day 8, and 30 µM dexamethasone), sialylated species were mainly found on the Fab fragment, and a maximum of approximately 2% Fc sialylation was observed (data not shown). Through supplementation with different additives in the cell culture feed, the total relative amount of sialylated species of intact mAb2 was modulated between −3.6% and 6.9% as compared with the control (Figure 6b). When comparing the amount of mono- and disialylated species, a strong decrease in monosialylated species concurrent with a high increase in disialylated species was observed for dexamethasone, hydrocortisone, 200 mM ManNAc, and the combination of ManNAc and DANA, as well as for $CuCl_2$. To provide



(c)

Condition	Calculated mol sialic acid/mol mAb normalized to control [%]	Condition	Calculated mol sialic acid/mol mAb normalized to control [%]	Condition	Calculated mol sialic acid/mol mAb normalized to control [%]
UMG control	100.0	UMG +200 mM ManNAc	109.8	UMG + 100 µM hydrocortisone	111.3
UMG + extra glycerol feed1	85.4	UMG + 750 µM DANA	99.2	UMG + 200 mM ManNAc + 750 µM DANA	126.1
UMG + 2 mg/mL fetuin	88.1	UMG + 1500 µM DANA	103.2	UMG + 50 µM Dexamethasone	126.4
UMG + 50 µM AC4ManNAc	91.8	UMG + 50 mM ManNAc	99.6	UMG + 100 µM Dexamethasone	129.9
UMG + extra glycerol feed2	92.8	UMG + 10 mM AC4MaNAc	107.2	UMG + 10 µM Dexamethasone	137.9
UMG + 5 mg/mL Fetuin	93.9	UMG + CuCl2 Day 6 + Day 8	115.1	UMG + CuCl2 Day 8	128.0
UMG + 25 µM ManNaz	99.5	UMG + 500 µM AC4MaNAc	107.5	UMG + 30 µM Dexamethasone	132.3
UMG + 250 µM ManNaz	100.2	UMG + CuCl2 Day 6	113.0		

FIGURE 6 (a) Glycosylation of the intact IgG (mAb2) as well as the separated Fc and Fab fractions in spin tubes as analyzed by UPLC-MS. (b) Absolute change in total sialylation observed for mAb2 (average Day 12 and Day 14) with different additives as analyzed by UPLC-MS for a spin tube (plain columns) and the ambr®15 system (dashed columns). (c) Calculated mol sialic acid per mol antibody normalized to the UMG control (average of IgG harvested on Days 12 and 14). (d) Integral of the viable cells (IVC) and (e) titer are shown as normalized area under the curve in percent of the respective UMG control. Error bars represent standard error of the mean. IgG: immunoglobulin G; mAb2: monoclonal antibody 2; UPLC-MS: ultra performance liquid chromatography coupled to a mass spectrometer

an overview of the quantitative effect of supplementation on sialylation, the increase in absolute sialic acid amount per mole antibody was calculated in Figure 6c. Furthermore, the impact of those supplements on other glycan structures are visualized Supporting Information Figure 4. The IVC and the AUC of the titer of the fed-batch experiments are shown in Figure 6d,e. It should be highlighted that for the glucocorticoids hydrocortisone and dexamethasone, IVC was similar or lower compared with the control whereas titer was increased for all concentrations.

As for mannosylation and fucosylation, the mass fraction of sialylated glycan compared to the amount of mAb secreted during exponential and stationary phase was calculated (Supporting Information Table 6). For supplementation with DANA alone or the combination DANA/ManNAc, the mass fraction of sialylated glycan was higher during the exponential phase than during the stationary phase. Because DANA is a known sialidase inhibitor, our results suggest that the lower sialylation level observed after D10 is unlikely to be the result of the sialidase activity. For other combinations like $CuCl_2$ and dexamethasone, the increase in the fraction of sialylated mAb was higher in the stationary phase than in the exponential phase. Whereas the increase in sialylation because of dexamethasone treatment can clearly be correlated with a decrease in Qp ($R^2 = -0.84$), the increase seen for $CuCl_2$ was clearly not linked to Qp, suggesting different mechanisms underlying the observed increase in sialylation.

In summary, the additive dexamethasone was the most successful compound for increasing the total amount of sialylated glycans (30 μM dexamethasone) as well as disialylated species (10 μM dexamethasone) concurrent with a positive effect on titer. The application of $CuCl_2$ to modulate sialylation needs to be considered more carefully because of its possible negative impact on cell growth, and application at the beginning of the stationary phase is recommended. Furthermore, the combination of ManNAc and DANA showed a synergistic effect on sialylation increase without affecting VCD or titer, thus highlighting the pertinence of combinations of additives.

4 | DISCUSSION

In this study, a large variety of compounds were found to be successful at modulating the glycosylation profile of recombinant IgG produced in CHO K1 and CHO DG44 cells.

4.1 | Mannosylation

Supplementation of the glucosidase inhibitor castanospermine resulted in a strong increase in high-mannose structures with glucose (>50%). A slight influence of castanospermine on IVC, viability, and titer was also observed. In literature, a strong increase in glucose-containing glycans concomitant with a strong decrease in glycoprotein secretion (up to 40%) was reported in human hepatoma cells due to castanospermine supplementation (Sasak, Ordovas, Elbein, & Berninger, 1985). Sasak et al. suggested that the reduction in glycoprotein secretion was a result of interference in the early glycosylation pathway. Considering the importance of glycosylation for folding and quality control mechanisms, this might indicate that our cell line has less stringent folding requirements and/or quality control mechanisms as compared with human hepatoma cells. The endoplasmic reticulum (ER) quality control process is a specific and complex mechanism to ensure correct folding of the glycoprotein (including proper disulfide bond formation), wherein the glycan structures are used as recognition sequences by various folding proteins and glycosidases in this pathway. These interactions can lead to either refolding or glycoprotein degradation by ER-associated mechanisms (Ferris, Kodali, & Kaufman, 2014; Lederkremer, 2009). After complete deglucosylation of the glycoprotein, the enzyme UDPGlc:glycoprotein glucosyltransferase 1 ensures correct folding and glycoproteins are subsequently allowed to progress to the Golgi. Typically, glycoproteins contain a GlcNAc2Man8 structure for transport into the Golgi and transport may be either specific (receptor-mediated) or unspecific (bulk-flow; Ferris et al., 2014). If the importance of the glycan structure in the ER for folding and degradation is considered, it is interesting to see that such an interference of castanospermine in the early steps of the glycosylation pathway nevertheless allows secretion of IgG-containing glucosylated high-mannose glycoforms. In this context, it is also interesting to note that kifunensine resulted in a 58.4% increase of the Man9 species without a negative influence on titer. Whereas removal of the final glucose residues is needed for the transport of a glycoprotein from the ER to the Golgi apparatus, the titer observed with kifunensine might not be impacted due to the presence of α-mannosidase I activity in the ER or the ER-Golgi intermediate compartment leading to Man8 oligosaccharides (Trombetta, 2003; Tulsiani, Hubbard, Robbins, & Touster, 1982).

4.2 | Fucosylation

In our experiments, the fucose-analog 2F-PerAcFuc was the most efficient at decreasing fucosylation (−76.1%). Rillahan et al. showed that 2F-PerAcFuc is converted to GDP-2F-fucose and accumulates in the cell. This modified nucleotide sugar was reported to possess an inhibitory effect on the fucosyltransferase 8 concomitant with a strong feedback inhibition on the synthesis of the natural GDP-fucose substrate (Rillahan et al., 2012). Addition of a non-fucose analog, MPA, into the cell culture feed also led to decreased fucosylation. Concentrations of 25 μM and higher resulted in a strong negative impact on cell growth, viability and titer whereas the fucosylation of the mAb was only slightly reduced (−3.4%). A single addition at the beginning of the production phase on Day 7 had a smaller negative impact on IVC, viability, and titer but the level of fucosylation was similar to the control. MPA was described to inhibit the synthesis of GDP and therefore reduce fucosylation (Zhang et al., 2016). The extent of the fucosylation decrease caused by MPA was reported to vary for different recombinant CHO cell lines, possibly due to different precursor level distributions. This might explain the absence of fucosylation reduction in our cell line. In addition, the

observed negative effect on VCD, viability, and titer in our experiment highlights that targeting GTP as a common intracellular metabolite might have significant disadvantages. Indeed, inhibited GTP synthesis is known to negatively impact DNA and RNA synthesis and thus cell growth and viability (Catapano, Dayton, Mitchell, & Fernandes, 1995). In addition to other metabolic pathways, the decrease in titer might result from a decreased level of GDP-Man, which is required at the beginning of the *N*-linked glycosylation pathway to initiate glycosylation and ensure correct folding before secretion. In conclusion, our data show that 2F-PerAcFuc is the only efficient modulator of fucosylation for the cell lines tested. Altering the concentration of this compound in cell culture may be a useful method to obtain an intermediary level of fucosylation, but if a total afucosylated glycan is requested, cell line engineering targeting, for example, fucosyltransferase 8 (Liu, 2015) or GDP-mannose 4,6-dehydratase (Kanda et al., 2007) may be a more direct and efficient alternative to increase ADCC.

4.3 | Galactosylation and sialylation

The combination of uridine, manganese, and galactose worked very well to increase antibody galactosylation for mAb1 and mAb2 (+40.9% and +17.6%, respectively). The observed difference in absolute response between clones is very well known in the literature (Davies et al., 2013), and Grainger and James (2013) demonstrated nicely that final galactosylation can be precisely predicted and controlled for each clone with UMG combinations using a statistical design of experiment methodology. Our data also suggest the existence of a "plateau" for the Fc-galactosylation level of IgG at approximately 50–60% as seen in our experiments with mAb1. This is supported by the current literature, in which galactosylation levels of IgG are typically reported to be lower than 60%. For example, Raju and Jordan (2012) reported that three marketed IgGs produced in CHO showed a galactosylation level of 38% or below. Gramer et al. (2011) described that UMG increased antibody galactosylation to 23% (CHO cell line 1) and 29% (CHO cell line 2). This upper limit in the level of achievable galactosylation might be explained by the dynamic variation in the rate of recombinant mAb galactosylation. Indeed, experiments performed using six GS-CHO clones producing a recombinant IgG4 mAb in batch culture indicated that the average galactosylation level increased between the lag phase and the exponential phase, whereas the galactosylation decreased dramatically in the stationary phase, suggesting the existence of a maximum cumulative galactosylation level. In addition, the observed saturation might be because of dynamic behavior occurring in the fed-batch process, where the rate-limiting factor for galactosylation shifts from being the UDP-Gal availability to being the galactosyltransferase availability/activity. In contrast, using in vitro galactosylation techniques, Thomann, Reckermann, Reusch, Prasser, and Tejada (2016) achieved an Fc-galactosylation level of approximately 93–98%. In our lab, in vitro galactosylation experiments with our mAb1 (data not shown) resulted in 84–92% total galactosylated species and, therefore, confirmed the possibility of obtaining high galactosylation levels in vitro. This suggests that the speed of the glycoproteins through the Golgi, and therefore, the time the enzyme can interact with the glycan, might be critical (Fan et al., 2015). In addition, because Fc-glycans are rather protected in the Fc part of the antibody, enzymes might be sterically hindered from interacting with the glycans (Nigrovic, 2013). This may be overcome by protein engineering in the Fc region (Chung et al., 2017; Li, Zhu, Chen, Feng, & Dimitrov, 2017).

Sialylation levels were very low in the Fc part (<2%), and higher amounts of sialylated glycan species were only observed in the Fab part of the mAb2. As previously mentioned, the site-specific *N*-glycan processing of the Fc compared with the Fab part of an antibody is influenced by the accessibility of the Asn residue as well as the linked glycan (Lee, Lin, Fanayan, Packer, & Thaysen-Andersen, 2014) and thus may be the main reason for difference in accessibility of galactosyltransferase and sialyltransferase. Furthermore, a recent study highlighted the impact of the amino acid composition and antibody secondary structure on the likelihood of observing immature or mature glycoforms at a specific site. In particular, γ-branched amino acids residues, such as asparagine, aspartate, or leucine, seemed to occur more frequently in regions containing immature glycans. This indicates that particular amino acid residues may modulate glycan–protein interactions leading to glycan micro-heterogeneity (Suga, Nagae, & Yamaguchi, 2018).

5 | CONCLUSION

Glycosylation is a key critical quality attribute for mAbs and recombinant proteins because of its impact on effector mechanisms and half-life. While glycosylation modulation can be achieved very efficiently through cell line engineering, intellectual property restrictions led the industry to develop alternative approaches using either process parameters or media and feed supplementation. In particular, the addition of specific small molecules is preferred in biosimilar processes, where small modulations may be required to match the cQAs of the originator. The work presented in this study investigated the ability of a wide range of additives to modulate mannosylation, fucosylation, galactosylation, and sialylation in both ST and the controlled ambr®15 bioreactor system. Both systems were used independently, although results of experiments performed in ST should be considered carefully before transferring the process to bioreactors. As pH and DO can significantly alter cQAs, an additional test of any glycosylation-modulating agent in a small-scale bioreactor system is required to ensure a successful scale up and a rapid transfer to manufacturing scale. Our findings showed that the strongest additives to modulate mannosylation and fucosylation were kifunensine and 2F-PerAcFuc, respectively. The combination of manganese, uridine, and galactose lead to an increase in both galactosylation and sialylation, and a specific increase in terminal sialic acid occupancy was achieved with additional supplementation of dexamethasone. Other specific additives or combinations of additives were also efficient at achieving smaller modulations

required during the development of biosimilars. To the best of our knowledge, the testing of such a variety of different compounds in two specific CHO clones in fed-batch experiments has not been described and surely represents a useful toolbox for the biotechnology community.

Although these data are intended to serve as a starting point to help biotechnology scientists define a good supplementation strategy to target a specific biosimilar quality profile without sacrificing titer, further experiments are needed to match the final quality specifications of a biosimilar. For fine-tuning, combinations of compounds at multiple concentrations can be applied in a statistical design of experiment approach, thus allowing a definition of the design space for each glycosylation category and a detailed study of interactions between additives. Further experiments are needed to evaluate the impact of the additives on other cQAs, such as charge variants or aggregates, and to study the effect in processes targeting fusion proteins or bispecific Abs. Although further experimentation and adjustment will remain mandatory to adopt strategies to a specific cell line and drug substance, the data surely enable a quick implementation and a reduced development time, thus leading to reduced overall development costs.

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CONFLICTS OF INTEREST

Janike Ehret, Martina Zimmermann, Thomas Eichhorn, and Aline Zimmer are employees of Merck, Darmstadt, Germany.

AUTHOR CONTRIBUTIONS

J.E. designed and performed the majority of cell culture experiments, performed data analysis, and wrote the manuscript. M.Z. performed the spin tube experiments concerning fucosylation, performed data analysis, and reviewed the manuscript. T.E. performed the glycan analysis using LC-MS. A.Z. designed, supervised the study, and reviewed the data and the manuscript. All authors have approved the final article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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