Comprehensive manipulation of glycosylation profiles across development scales

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ABSTRACT
The extent and pattern of glycosylation on therapeutic antibodies can influence their circulatory half-life, engagement of effector functions, and immunogenicity, with direct consequences to efficacy and patient safety. Hence, controlling glycosylation patterns is central to any drug development program, yet poses a formidable challenge to the bio-manufacturing industry. Process changes, which can affect glycosylation patterns, range from manufacturing at different scales or sites, to switching production process mode, all the way to using alternative host cell lines. In the emerging space of biosimilars development, often times all of these aspects apply. Gaining a deep understanding of the direction and extent to which glycosylation quality attributes can be modulated is key for efficient fine-tuning of glycan profiles in a stage appropriate manner, but establishment of such platform knowledge is time consuming and resource intensive. Here we report an inexpensive and highly adaptable screening system for comprehensive modulation of glycans on antibodies expressed in CHO cells. We characterize 10 media additives in univariable studies and in combination, using a design of experiments approach to map the design space for tuning glycosylation profile attributes. We introduce a robust workflow that does not require automation, yet enables rapid process optimization. We demonstrate scalability across deep wells, shake flasks, AMBR-15 cell culture system, and 2 L single-use bioreactors. Further, we show that it is broadly applicable to different molecules and host cell lineages. This universal approach permits fine-tuned modulation of glycan product quality, reduces development costs, and enables agile implementation of process changes throughout the product lifecycle.

Introduction
Posttranslational modification is a central mechanism for the regulation of mammalian protein function. In particular, N-linked glycosylation can profoundly influence biochemical and physiological properties of antibodies. The bio-manufacturing industry has used mammalian expression systems, such as Chinese hamster ovary (CHO) or NS0 cells, for the production of monoclonal antibody (mAb) therapeutics to recreate human-like posttranslational glycosylation patterns, but the complexities of controlling glycan structures continue to pose substantial challenges, including the selection of an appropriate glycosylation product quality profile suitable for a given pharmaceutical antibody. In particular, maintaining a consistent product quality profile across cell age, between different master or working cell banks, and when producing mAbs in different types of vessels, at different manufacturing sites, or at different scales remains challenging. Such difficulties may occur during process transfers, scale-up or scale-out, or after changes in raw material sourcing, in particular when executing against aggressive timelines, and they underscore the importance of maintaining consistent product quality over the entire product lifecycle. In order to enable agile implementation of process changes at later developmental stages, it is crucial to build a solid understanding of the underlying design space and its limitations. Importantly, such an adaptable platform for glycan product quality modulation should be effectively implemented early in development. Analogous considerations apply for the growing field of biosimilar development, where the product quality of a reference medicinal product must be matched within tight specification limits.

Antibody N-linked glycosylation encompasses a multi-step biochemical process in different compartments of the secretory pathway, resulting in a varied population of predominantly biantennary glycoforms in the final product pool. This is particularly important because different glycoforms have been demonstrated to mediate effector functions, influence circulatory half-life, or elicit immunogenicity, all of which can affect drug efficacy or patient safety. The presence or absence of fucose on the first core GlcNAc dramatically impacts antibody-dependent cell-mediated cytotoxicity (ADCC). Similarly, the level of terminal galactosylation affects complement-dependent cytotoxicity (CDC). Antibodies with high mannose-containing glycosylation structures (Man5 and higher) are cleared from the
bloodstream at a faster rate than those with mature, complex type oligosaccharides, which has direct implications for drug efficacy. Therefore, the product quality profile should be defined and maintained early on. Potential liabilities, especially those that may impinge on the mechanism of action, should be identified at the beginning of the product life cycle, and effectively remedied in order to obviate complications at later stages of development. The ability to react quickly and with precision to undesired changes in product quality necessitates an exquisite understanding of the direction and extent to which the profile can be modulated.

Numerous media additives have been used to modulate glycosylation structures (reviewed in ref. 8). Similarly, a variety of selective inhibitors have been exploited, blocking either glycosidase, mannosidase, or sialyl- or fucosyl-transferase activities. More recent studies have used design-of-experiment (DoE) approaches in combination with statistical tools to characterize combinatorial effects of multiple additives, including fine-tuned control of galactosylation as a function of uridine, manganese and galactose supplementation, using response surface modeling.

Here, we report an inexpensive and highly adaptable screening approach for comprehensive modulation of N-linked glycans using a 24-well deep well plate culturing system. We characterized 10 of the most commonly used media additives in variable studies and demonstrate their effect on aspects of the glycosylation profile in a dose-dependent manner. We then conducted a comprehensive resolution IV fractional factorial screening DoE (32 experiments in 4 blocks, randomized setup) to identify main factors and two-factor interactions, and mapped the design space for tuning glycosylation profile attributes. We applied the resulting model for targeted modulation of oligosaccharide quality attributes, and demonstrate that the effects observed in 24-well deep well plates can be replicated in 30 mL shake flasks, in an AMBR-15 cell culture system, and at 2 L single-use bioreactor scale. Finally, we compared the effects of feed additives on different cell lineages, and different mAbs. Taken together, we introduce a highly adaptable and robust workflow that does not require automation, yet enables rapid process optimization across different development scales. This universal approach permits exquisite control over glycan product quality modulation, reduces development costs, and enables agile implementation of process changes throughout the product lifecycle.

**Results**

We selected a CHO-K1 derived cell line expressing a humanized monoclonal IgG1 based on the following criteria. The cell line should be platform relevant, with maximum viable cell densities ranging between 1.0–2.0 x 10^7 cells/mL, and multi-gram per liter volumetric productivity over a standard 14-day fed-batch platform process, to ensure that enough material for analysis of N-linked glycans was generated even at small scale. The product quality profile should be within a range that allowed for both increases and decreases to be reliably measured in every aspect of the glycosylation profile: the content of G0 should be above 10% to assess effective decreases in afucosylation, the content of Man5 species should be above 15%, and the galactosylation profile should feature G1 or G1F species of at least 10% combined, as well as G2 and G2F species of above 2% combined. We evaluated several independent clones (Supplemental Figure 1), and based on the aforementioned criteria, we chose clone 2D9 for the majority of our experiments.

We conducted three independent fed-batch experiments under control conditions, each with duplicate wells, to assess inherent variability of the glycosylation profile in 24-well deep well plates. On average, control cultures showed 13.0% (± 0.3) G0 species, 46.9% (± 0.8) GOF, 3.7% (± 0.3) G1, 0.2% (± 0.1) G1F, 13.0% (± 0.7) G1F, 4.3% (± 0.3) G1F, 0.6% (± 0.1) G2, 1.5% (± 0.1) G2F, and 17% (± 1.5) Man5 species (Figure 1A). Pictograms in Figure 1A illustrate the biochemical composition of each species. In addition, we tested three well-characterized additives known to modulate the glycosylation profile for proof-of-concept in the 24-well deep well culture system. We used concentrations that were within the ranges previously shown to be effective by others. Some of the observed effects were quite dramatic when expressed as percent difference from control. For example, 12.5 mM glucosamine feeding led to a reduction of G1 species by 85% (± 10.7), a 50% (± 1.8) diminishment in G1F, and a 62% (± 1.0) decrease in G1F. Similarly, addition of 1.0 mM copper reduced Man5 species by 40% (± 2.3) (Figure 1B). Of note, the standard error of the mean is meaningfully greater for species, which are less abundant in the control profile, most notably G2 and G2F species. While administration of 5.0 mM uridine causes a 101% (± 25) increase in G2, as well as a 69% (± 9.0) augmentation of G2F, the overall deflection from the original glycosylation profile is moderate because both these species are of low abundance under control conditions.

To assess how the relative differences from control translate into actual changes of the overall glycosylation profile, we expressed the data as weighted percent change by multiplying the percentage of the observed difference with the fraction of total area for a given aspect of the profile (Figure 1C). Note that the weighted percent changes for any feed additive will be net zero across the entire glycosylation profile, within rounding errors. For example, feeding of 1.0 mM copper reduced Man5 area by 6.8%, but increased G0 by 0.7%, GOF by 5.5%, and G1F by 0.5%. Through this analysis, it becomes apparent that glucosamine and uridine affect galactosylation in opposite directions and to varying degrees across individual species.

We next investigated if the observed effects on the glycosylation profile followed a dose-response relationship, and whether or not we could observe a linear response across a range of doses. In addition to copper, glucosamine and uridine, we included cytidine, galactose, fucose, manganese, N-acetyl-mannosamine (ManNAc), glycerol, and N-acetyl-neuraminic acid (NANA) as feed additives, all of which have been described previously as modulators of glycosylation patterns or cell culture performance. We tested each of these...
additives in at least three different concentrations and compared their effects on all aspects of the glycosylation profile by graphing the weighted percent change in a nonagonal radar plot format (Figure 2). The center of the concentric nonagons represents a 10% decrease compared to control, and each ring layer signifies a 5% change step. The red nonagon represents control values (0% change). Hence, data points proximal to the center (inside of the red nonagon) signify a reduction from control; those in the periphery (outside of the red nonagon) indicate an increase over control values (Figure 2A). In addition to the weighted percent changes represented in the radar plots, dose-response relationships for those glycosylation profile aspects most affected are shown as raw values in bar graphs for a given additive. For example, addition of cytidine increased G1F species in a dose-dependent manner over a concentration range from 1 mM to 15 mM (15.25% ± 0.09 at 1 mM, 17.2% ± 0.1 at 5 mM, 20.4% ± 0.4 at 10 mM, and 21.9% ± 0.25 at 15 mM). Similarly, G1F’ species showed a dose-dependent increase, whereas both G0 and Man5 species were diminished with increasing cytidine (Figure 2B).

In contrast to the broad effects of cytidine, administration of galactose elicited more specific effects in the tested concentration range. As expected, feeding galactose led to a dose-dependent decrease in G0F species (52.14% ± 0.09 at 1 mM, 51.3% ± 0.4 at 10 mM, and 50.0% ± 1.5 at 30 mM), and a concomitant increase in G1F (Figure 2C). The effects were relatively minor as is apparent from the deflections of the overall profile in the radar plot, and, at least with respect to G1F, they reached saturation above 10 mM galactose. Similarly, addition of copper only affected two of nine attributes, G0F and Man5, both in a dose-dependent fashion.
Interestingly, already the lowest dose of 0.2 mM showed marked reduction of Man5 (7.36% ± 0.06, which represents a 34% reduction from control). Gradually increasing the copper concentration further lowered Man5 species (6.91% ± 0.09 at 0.5 mM, 5.32% ± 0.04 at 1.0 mM, 3.92% ± 0.74 at 1.5 mM, and 3.18% ± 0.03 at 2.0 mM, Figure 2D).

The largest deflection from control profile observed across all experiments was a 14.6% increase in G0F after feeding of 20.0 mM glucosamine (Figure 2E). Glucosamine had widespread effects over the glycosylation profile, increasing G0F and Man5 species in a dose-dependent manner over a concentration range from 2.0 mM to 20.0 mM, while simultaneously reducing G0, G1, G1F, and G1F' (Figure 2E), in agreement with our earlier observations from the single concentration proof-of-concept study (Figure 1).

Uridine was the most powerful modulator of G1F species, effecting a 9.8% increase over control at the highest tested concentration of 10.0 mM. The effects of uridine were widespread, with G1, G1F, G1F', and G2F species being increased, and G0, G0F, and Man5 being diminished, all in a dose-
dependent manner (Figure 2F). Similarly, addition of fucose also reduced Man5 species and increased G1F, although the observed effects are smaller than those from uridine feeding. Fucose administration shifted the balance of fucosylated and afucosylated species toward increased fucosylation, and led to a dose-dependent increase of G1F, G1F', and G2F species, predominantly at the expense of Man5 and, to a lesser degree, G0 species (Figure 2G). Application of manganese reduced Man5 species and led to an increase in G1F, and G1F', which saturated at concentrations above 1.0 µM (Figure 2H). The most selective of all tested additives was ManNAc, which affected solely G0F in a dose-dependent manner (53.16% ± 0.09 at 2 mM, 55.73% ± 0.09 at 10 mM, and 58.8% ± 0.48 at 20 mM, Figure 2I). We observed a minor decrease of Man5 species, but the effect saturated at concentrations above 10 mM. Lastly, both glycerol and NANA did not notably affect any of the nine profile aspects when tested at concentrations spanning at least one order of magnitude (0.1% (v/v) – 2.0% (v/v) for glycerol; 0.1 mM – 1.0 mM for NANA, Figure 2J and 2K, respectively).

In summary, we observed numerous changes from control with varying selectivity and magnitude for most of the tested additives, and most observed effects showed a linear dose-response relationship across a concentration range spanning at least one order of magnitude (see Supplemental Figure 2). Note, that the increase in G1F in response to galactose feeding reached saturation beyond 10 mM. Similarly, the effects of manganese on G1F and G1F’ were saturated beyond 1.0 µM (Figure 2C and H, respectively).

In a next step, we investigated how combinations of these additives influenced the glycosylation profile. We designed a resolution IV $2^{10-5}$ fractional factorial DoE, selecting concentrations from within the characterized range. The upper range of concentration for each additive was chosen at a level where the observed effects had not yet reached saturation, to ensure we would be able to detect interactions. We elected to include glycerol and NANA in the DoE conditions to test if their effects become significant in the context of two-factor interactions with other additives, and to evaluate their potential benefits on cell culture parameters. The concentration levels for each additive are listed in Table 1.

The DoE setup was randomized, with 32 experimental conditions spread over four 24-well deep well plates. For each experiment, two pseudo-replicates were included, and their responses averaged for analysis. In addition, we included a fifth plate with univariable conditions of all tested additives. The pattern for addition of the 10 additives in each experiment is depicted in Table 2. Further, we included two extra conditions without any feed additives on each of the five plates for internal control. For the duration of the fed-batch experiment, we monitored viable cell count and culture viability on day (D) 7, D9, D11, and D14. After the experiment was concluded on D14, cell culture fluid was harvested, purified by protein A capture chromatography, and glycosylation patterns were analyzed by the LabChip GXII Touch system.

We combined the resulting data from cell culture performance and glycosylation profile analysis and built a predictive model that encompassed main effects and two-factor interactions, using JMP software. Although not required for validity of the statistical model, which depends on normality of the residuals, we also evaluated normality of the raw data to understand the model characteristics better. We found that for several analytical variables the data set showed a normal distribution, with p-values > 0.05 in the Shapiro-Wilk W test. For example, total afucosylation was normally distributed over the observed range from 10–20%, with a mild deflection of the histogram plot toward higher percentages (Figure 3A). We assessed homogeneity of variance after modeling by plotting the residuals against the predicted values, with no apparent patterns emerging, consistent with the model encompassing all relevant factors for prediction of afucosylated species (Figure 3B). Similarly, we saw no patterns when plotting the residuals vs. the row numbers, demonstrating a lack of bias in the experimental setup (Figure 3C).

In marked contrast, we observed highly non-normal distribution in some other variables, foremost cell culture viability. Three main groups were observed with average viabilities at D14 in the range of 85, 45, and 10%, respectively (Figure 3D). This stark pattern was also observed when plotting the residuals vs. the predicted values, with notable deflections of residuals into the negative for the lower range of predicted viabilities, and into the positive for the higher range (Figure 3E). This pattern is consistent with the pronounced grouping observed in Figure 3D. However, when plotting the residuals over the row numbers, it becomes apparent that the observed clustering is not due to positional effects within the experimental setup. Rather, the differences in viability are linked through the shared combination of additives in the affected

| Additive   | Low | High | Unit  |
|-----------|-----|------|-------|
| Galactose  | 0   | 20.0 | mM    |
| Glucosamine| 0   | 12.5 | mM    |
| ManNAc    | 0   | 40.0 | mM    |
| Uridine   | 0   | 5.0  | mM    |
| Cytidine  | 0   | 10.0 | mM    |
| Fucose    | 0   | 40.0 | mM    |
| Manganese | 0   | 2.0  | µM    |
| Copper    | 0   | 1.0  | mM    |
| NANA      | 0   | 0.5  | mM    |
| Glycerol  | 0   | 1.0  | % v/v |
We used the resulting data set from the DoE study to build a statistical model, encompassing main effects and two-factor interactions. We evaluated the residuals by normal probability plot to assess validity of the model (data not shown). The predictive ability of the model fit was evaluated using $R^2$ and Press RMSE values, as the latter indicates the predicted standard deviation. For example, the $R^2$ value for prediction of G0 species was 0.982, with a Press RMSE of 1.205, indicating good precision around the predicted values for G0. The excellent fit of the model to the data can be seen in the actual vs. predicted plot in Figure 3G. Similarly, the model had good ability to explain variation in other aspects of the glycosylation profile, as shown by the $R^2$ and Press values in Table 3.

We next asked which feed additives or combinations had significant effects on both cell culture performance and glycosylation profile. Traditionally, such data can be visualized with a half normal plot, where model terms associated with a normal distribution of the data set are aligned on a sloping line across quantiles. Terms, which significantly affect the parameter of interest, will deflect from the line. For example, G0 species was significantly modulated by numerous additives, including glucosamine, fucose, cytidine, uridine, ManNAc, and manganese (Figure 3H).

We devised a more effective way to visualize both main effects and two-factor interactions by using multivariate analysis software tools. First, we examined parameters of cell culture performance, including viability and integrated viable cell density (IVCD) at D7, D9, D11, and D14 of the fed-batch experiment (Figure 4). The use of multivariate representation

| Aspect | $R^2$ | Press | Press RMSE |
|--------|-------|-------|------------|
| G0     | 0.982 | 66.81 | 1.205      |
| G0F    | 0.941 | 918.57| 4.468      |
| G1     | 0.964 | 79.57 | 1.315      |
| G1F    | 0.958 | 99.14 | 1.468      |
| G1F'   | 0.988 | 64.22 | 1.182      |
| G1F''  | 0.986 | 11.32 | 0.496      |
| G2     | 0.956 | 48.50 | 1.027      |
| G2F    | 0.945 | 16.95 | 0.607      |
| Man5   | 0.821 | 1303.44| 5.323      |
allowed us to depict four different aspects of the data set in a single graph: 1) We encoded the type of effect in the symbol shape: diamonds represent main effects, Ying-yang icons signify two-factor interactions, and hatched squares denote non-aliased interactions (Figure 4A); 2) We used a different color for each model term, and combined two of these colors to represent two-factor interactions of the corresponding model terms; 3) The amplitude and direction of each effect is immediately apparent by the location of the symbol with reference to the y-axis (Figure 4B); and 4) The size of the symbol encodes the t-ratio (the effect estimate divided by the standard error of that particular estimate), to indicate robustness of the effect. All statistically significant effects are depicted.

(B) Estimates impacting IVCD throughout the duration of the experiment. Note, that beneficial effects of uridine are relatively constant, whereas the detrimental effects of cytidine are getting greater during the experiment. Only main effects are significant for IVCD. (C) Estimates impacting culture viability. Note that several positive effects are observed throughout the entire experiment, including main effects and two-factor interactions. Negative effects are seen mid-experiment, but not toward the later days.

Through this type of visualization, it becomes apparent that uridine has moderately beneficial effects on IVCD, which remain relatively constant throughout the experiment (Figure 4B, pink diamonds), whereas cytidine strongly and negatively influences IVCD, with the effect amplitude progressively increasing over the duration of the fed-batch culture (Figure 4B, green diamonds). In addition, we observed smaller and less robust effects for glucosamine and fucose, with the latter reaching significance only on D14. Similarly, a variety of main effects and two-factor interactions modulate culture viability throughout the experiment (Figure 4C). For example, ManNAc leads to an increase in viability, with benefits increasing as the experiment progressed (orange diamonds in Figure 4C). On the other hand, the combination of cytidine with fucose (green-grey hatched square) provided a small but constant burden to culture viability. Of note, glycerol showed the greatest increase of viability at D7 (7.06%), and a sustained effect on D9 (5.43%). Similarly, the interaction of galactose with NANA showed a comparable pattern of increasing viability benefit (brown-yellow Ying-yang symbols, 4.27% on D7, 5.32% on D9, 7.58% on D11, and 9.63% on D14). Since these two additives did not show significant effects on the glycosylation profile when tested in univariable
experiments (Figure 2J and 2K, respectively), including them in the DoE study has revealed their benefits on cell culture performance.

We next analyzed the data for the model terms of glycosylation profile aspects (Figure 5A), and grouped them based on the estimate effect size (Figure 5B-E) to maximize resolution in the plot. As expected, the least abundant species, G2 and G2F, showed the smallest deflections of the overall glycosylation profile, with effects ranging from $+0.45\%$ (G2F in response to galactose feeding), to $-0.28\%$ (G2F, glucosamine, Figure 5B). Similarly, the most abundant species, G0F, showed the greatest effect estimates, ranging from $+2.7\%$ (glucosamine) to $-3.5\%$ (galactose, Figure 5E). G0 species was significantly modulated by numerous additives, including glucosamine ($-1.2\%$), fucose ($-0.22\%$), cytidine ($-1.06\%$), uridine ($-0.59\%$), ManNAc ($-0.42\%$), and manganese ($-0.25\%$, Figure 5D, left most group). To illustrate, the same terms are shown in the half normal plot for G0 (see Figure 3H), but their absolute effect size, relative influence, and robustness are not immediately apparent from the traditional depiction. As expected we observed that, in the majority of cases, the greatest effect estimates were from main effects, with one exception: the interaction of galactose with NANA had the greatest effect on elevating Man5 species (Figure 5C), and showed a positive effect estimate on D14 viability on par with ManNAc ($9.6\%$ increase vs. $9.4\%$, respectively; see Figure 4C).

Globally, we were successful in manipulating all aspects of the glycosylation profile with the array of additives we chose. All aspects were influenced by more than one additive, or combinations of additives. Similarly, every additive changed multiple profile aspects, and to varying degrees. Over the entire data set, we observed a wide span of absolute changes for several glycosylation aspects. For example,
the control condition had 46.1% G0F, and the lowest and highest measured G0F area were 40.3% vs. 66.9%, respectively. Likewise, values spanned from 5.4% to 18.2% for Man5 (control: 13.9%). We found total afucosylation to range from 10.3% to 20.0%, with the control value being 17.9%. For total galactosylation, the data set spanned from 11.0% up to 40.1%, with control values of 26.9%.

Taken together, the univariable data and the predictive DoE model enable fine-tuning of a given glycosylation profile. However, successful targeted manipulation will only be possible within a certain range for each glycosylation aspect. The combined data set can be utilized to define such ranges for matching a hypothetical target product quality profile.

We next asked if we could observe the same relative effects when applying glycosylation tuning additives to different cell lines. To this end, we designed several targeted experiments using different combinations of multiple additives, and evaluated their effects on three CHO-K1 derived cell lines that have diverged from a common ancestral lineage by several single-cell cloning events, and express different IgG1 mAbs (cell lines 2D9, 404, and 386). We observed that for several additive combinations, the results were in good agreement between cell lines. For example, experiment 30 (a combination of glucosamine, ManNAc, manganese, and copper) showed very similar deflections in all aspects of the glycosylation profile across all cell lines, both in valence and magnitude (Figure 6A). By contrast, experiment 01 (a different combination containing uridine, fucose, manganese, and copper) showed pronounced differences between the three cell lines: While the combination led to a general rise in galactosylation, the magnitude of the increases in G1 and G1F are quite different between cell lines (Figure 6B). Similarly, the decreases in G0 and G0F species are not consistent across cell lines. Most strikingly, we observed completely opposite readouts for Man5 across the three cell lines. While 2D9 showed a robust decrease in Man5, we saw nearly no reduction at all in cell line 404, and even observed an increase in cell line 386 (Figure 6B).

To understand which component or interaction of components might be driving these differences, we performed a series of experiments testing additives in univariable and two-factor combinations to dissect the relationship between media additives and effect variability. Among other combinations, we tested 20.0 mM galactose, 7.5 mM uridine, and 12.5 mM glucosamine feeding in univariable, and compared the weighted percent change on the glycosylation profile aspects for all three cell lines (Figure 7). Feeding of galactose introduced similar changes across all three cell lines, with marked gains in G1, G1F, G1F', and G2F species, at the expense of G0F and Man5 species (Figure 7A). The three cell lines showed subtle differences in effect magnitude, most prominently in the changes to G1, G1F, and G0F modulation. As expected, feeding of glucosamine elicited changes opposite of those seen after galactose feeding. The modulations were similar across all cell lines, with minor variations in effect magnitude (Figure 7B). By contrast, we observed pronounced differences in the responses to uridine feeding (Figure 7C). The increases in G1F, G1F' and G2F observed for 2D9 did not recapitulate in either of the other cell lines. Cell line 386 showed a greater decrease in G0F than 2D9 and 404. The

Figure 6. A) Weighted percent changes for a targeted glycosylation profile tuning experiment, across three different cell lineages expressing different mAbs. Experiment 30 contains glucosamine, ManNAc, manganese, and copper. Note that both valence and magnitude of the effects are largely consistent among the three cell lines. (B) Weighted percent changes for another targeted glycosylation profile tuning experiment. Experiment 01 contains uridine, fucose, manganese and copper. Note that for most profile attributes deflections from control are consistent between cell lines and mAbs in valence but not necessarily in magnitude. Of note, the deflection of Man5 is starkly different between cell lines.
modulation of Man5 was grossly different for the three cell lines: While we observed a robust decrease in Man5 species after uridine feeding for 2D9, we saw no significant effect for 404. Cell line 386 even showed a strong increase. These results are consistent with our observations from combinatorial experiment 01, which contains uridine (Figure 6B).

Taken together, this data demonstrates that commonalities exist among different cell lineages, but that even closely related cell lines can show surprising readouts, depending on the additives applied. Such idiosyncrasies are likely innate to the host cell and may already vary significantly within a host cell pool. These observations underscore the need to characterize the effects of glycosylation modulating agents on any given cell line in univariable mode, and demonstrate the limits of our ability to generalize, even across relatively closely related CHO lineages.

We next evaluated the efficacy of glycosylation tuning across different development scales. We leveraged the well-characterized experiment 30, and applied the additive combination to 2D9 cells grown in 24-well deep well plates, 250 mL shake flasks, AMBR-15 cell culture system, and 2 L single-use bioreactors, alongside osmolality-matched control conditions that did not receive any media additives. We observed very good consistency in the glycosylation profile of control conditions across at least three independent experiments for each scale, as can be seen from the error bars in Figure 8A. Interestingly, we saw a somewhat smaller percentage of G1F species and greater variability in 24-well deep well format (13.0% ± 0.7), compared to shake flasks (15.6% ± 0.1), 2 L single-use bioreactors (16.4% ± 0.1), and AMBR-15 system (15.9% ± 0.2). Likewise, we noticed an increase in Man5 species in 24-well deep wells (17.0% ± 1.5), when compared to shake flasks (12.4% ± 0.2), 2 L bioreactors (12.2% ± 0.4), and AMBR-15 system (11.7% ± 0.3). This discrepancy may be attributable to greater evaporation at small scale, which could lead to increased osmolality, and potentially influence the degree of total afucosylated species.

Figure 7. A) Weighted percent changes from control profile for 20 mM galactose feeding on three different cell lineages producing different mAbs (2D9, 404, and 386). Note that for all profile attributes deflections from control are consistent between cell lines and mAbs, with respect to their valence. Differences in effect magnitudes exist between cell lines. (B) Weighted percent changes from control profile for 12.5 mM glucosamine feeding. Note that for all profile attributes deflections from control are consistent between cell lines and mAbs, with respect to their valence. Differences in effect magnitudes exist between cell lines. (C) Weighted percent changes from control profile for 7.5 mM uridine. Note that uridine has dramatically different effects on G1F, G1F', G0 and Man5 species across cell lines.
Conversely, we observed a mild offset in G0 and G0F species in the AMBR-15 system (Figure 8A), shifted toward slightly higher fucosylation on non-galactosylated species. Of note, the targeted modulation of experiment 30 was relatively consistent across scales. We observed a slightly more pronounced increase in G0F in the shake flask model and AMBR-15 system, and a concomitant greater decrease in G1F, compared to the other scales (Figure 8B). Similarly, the relative decrease in Man5 species was slightly less pronounced in shake flasks and AMBR-15 system, when compared to 24-well deep wells and 2 L bioreactors.

**Discussion**

Antibodies and their derivatives have emerged as a leading class of therapeutic proteins. A broad body of literature shows that different glycoforms can mediate cellular effector functions, like ADCC\(^ {3-6}\) (reviewed in refs. 1, 7, and 8), and CDC\(^ {3,6,35}\) (reviewed in refs. 10, 11). In addition, the glycosylation profile can influence circulatory half-life.\(^ {12,13}\)

Therefore, product quality liabilities should be identified early on in the product life cycle, and effectively remedied in order to obviate complications at later stages of development. Here, we present a comprehensive platform approach for the successful manipulation and fine-tuning of glycosylation profiles across different development scales, encompassing thorough characterization of media additives, multivariate analysis, and an assessment of universality and scalability through exploration of different cell lineages, expressed molecules, and development scales. We chose a model IgG1 antibody with a glycosylation profile that was amenable to exploring the maximum achievable extent of modulation. The findings from this approach are directly applicable to any clinical candidate mAb with more favorable glycosylation product quality attributes.

**Univariable findings**

Numerous media additives have been used to modulate glycosylation structures, including galactose, glucosamine,\(^ {15,19}\) N-acetyl glucosamine (GlucNAc), uridine, mannose, N-acetyl neuraminic acid.
acid (NeuNAc), N-acetylmannosamine (ManNAc), ammonia, manganese, dolichol phosphate, cytidine, and glycerol. Similarly, a variety of enzyme inhibitors has been exploited.

In our study, we tested 10 commonly used media additives and characterized their effect on the glycosylation profile of different IgG1 molecules. We found that feeding of 12.5 mM glucosamine led to dramatic decreases in galactosylated species, in line with observations by others. For example, Hills and colleagues saw a 57% drop in galactosylation in GS-NS0 cells in response to 10 mM glucosamine. The same concentration has also been reported to reduce the degree of sialylation, (reviewed in ref. 1), in line with the notion that the addition of sialic acid residues occurs on terminally galactosylated glycoforms (reviewed in refs. 1,10, and 11). The suppression of galactosylated species in response to glucosamine feeding is widely believed to stem from competition for UTP in the formation of UDP-Gal, which hampers generation of UDP-GlcNAc, which produces the galactose moiety from other carbon sources, likely from inhibition of glucose uptake.

Supplementation with galactose increased levels of galactosylated species, as expected. However, compared to the drastic decreases seen after glucosamine feeding, the increases are modest, in line with findings from others. Uridine supplementation increased all galactosylated glycoforms, and decreased G0, G0F, and Man5 species. In our hands, uridine was a stronger modulator of G1F species than galactose, indicating that the synthesis of UDP-galactose was limited by uridine, and that the cells could produce the galactose moiety from other carbon sources, likely from glucose via UDP-galactose 4-epimerase. Supplementation of molecular precursors, while greatly raising intracellular nucleotide sugar levels, has not resulted in significant increases of the desired glycoform. Hills and coworkers reported a 5-fold increase in UDP-galactose levels in response to feeding with galactose, but only a 6% rise in actual galactosylation. We observed only modest increases in galactosylation after galactose feeding, and no increases in sialylation in response to either NANA or ManNAc. Similarly, Hills and coworkers reported that even though cytidine-monophosphate-sialic acid (CMP-SA) levels were increased 44-fold after feeding of 20 mM ManNAc, no increase in sialylation followed. These findings are in line with observations from non-mAb biologics, such as tissue inhibitor of metalloproteinase 1, and interferon gamma, (reviewed in ref. 11).

We had several interesting observations of the effects of glycosylation tuning media additives on cell culture performance. We observed a significant negative impact of cytidine on culture growth throughout the fed batch experiment. Wong and colleagues reported a 20–40% growth decrease for cytidine in combination with ManNAc. Interestingly, the same study observed benefits to cell culture performance in response to galactose feeding and identified the combination of uridine with glucosamine as growth inhibiting. In our experiments, we saw mild but substantial benefits for IVCD in response to uridine feeding. Unexpectedly, we observed substantial improvements to culture viability in response to both ManNAc, and the interaction of galactose with NANA. Since NANA and ManNAc can be interconverted through cellular metabolism, the fact that we see benefits from both of them suggests a common underlying mechanism of action. Similarly, we observed improvements to cell viability from glycerol, both as a main effect and in combination with glucosamine and galactose. These observations are novel, and contrast with other reports, which have seen improved specific productivity, but also growth inhibition above 1.0% of glycerol, and enhanced sialylation levels and greater product yield due to reduced aggregation.

Combinatorial findings
Greater increases in galactosylation have been reported as a result of combinatorial supplementation with manganese, galactose, and uridine. Where increased availability of UDP-galactose shows synergistic effects with allosteric modulation of galactosylating enzymes by manganese, (reviewed in ref. 36). Our study looked at more additives, but was designed as a screening study, revealing main effects and two-factor interactions, which can be characterized more thoroughly in follow-up work. Grainger and colleagues used a response surface DoE to build a predictive model for galactosylation, by feeding uridine, galactose, and manganese, and achieved more than 100% increase in galactosylation through synergistic effects. Using an approach similar to ours, Bruehlmann and colleagues assessed 17 feed additives including enzyme inhibitors, in five parallel DoE groups. While this approach allowed rapid interrogation of a greater number of variables, its ability to detect two-factor interactions was limited to compounds that were initially grouped together. This setup is particularly suited for novel additives, which may prove toxic to the culture, and could otherwise affect the entire experiment. Others have successfully used DOE studies to optimize both yield and a varied panel of product quality attributes across different cell lines and media, or to develop control strategies for glycosylation by combining a DOE approach with pursuant controllability analysis.

Scalability
A key finding of our study is the fact that both the valence and amplitude for product quality modulation can be highly consistent across scales. We observed very similar deflections for all aspects of the glycosylation profile across four different development scales, spanning three orders of magnitude in volume. Such ability to predict product quality changes across scales is a prerequisite for the successful development of a scale down model, and has been achieved by others, for example, between micro scales and small scale, and between small and large scales. Of note, we observed that different cell lines may show significant differences in modulation of the glycosylation profile in response to certain
additives. Although unlikely, at this time we cannot rule out that this variability might be tied to the scale at which these experiments were conducted.

Conclusion

We tested 10 commonly used media additives in univariate and in a combinatorial DoE to assess their effect on the glycosylation profile of IgG1 mAbs. We assessed these effects across multiple cell lines and development scales, effectively outlining a platform development workflow that allows rapid characterization, and manipulation of the glycosylation profile of any candidate IgG1 slated for development. The collection, interpretation, and use of multivariate data is key to successful fine-tuning of product quality attributes, especially when multiple inter-dependencies exist between modulators and output variables. The screening approach presented here allows for rapid identification of factors, which influence aspects of the glycosylation profile of interest. These results can be followed up with tailored response surface DoE studies where a smaller number of variables are characterized in greater detail to fine-tune aspects of the glycosylation profile. Similarly, this approach can easily be adapted to characterize other, non-glycosylation product quality attributes.

Materials and methods

Deep well cell culture system

CHO cell cultures were scaled-up in shake flasks and seeded at 7 × 10⁵ cells/mL density in 24-well deep well plates for a 14-day fed-batch assay using proprietary basal and feed media formulations. Cultures were maintained at 36.5 °C, shaken at 440 RPM, and incubated under 5% CO₂. Cells were seeded at a 3.0 mL starting volume, and viable cell density and viability were measured every 48 h using a CellaVista automated cell imager (Synentec, Germany). Cultures received 210 µL of feed media every 48 h starting on the third day of culture (D3) and until including D13. All additives were from Sigma-Aldrich (St. Louis, MO) and were dissolved in the feed. Additive concentrations referenced throughout the manuscript represent final concentrations at the end of the fed-batch culture. Three independent repeat experiments were conducted with duplicate wells for each condition and quadruplicate repeats for controls. Control wells received plain feed media without additives and were sampled every 48 h for metabolites using a Cedex Bio HT Analyzer (Roche, Germany).

The assay was concluded on D14 when 2.0 mL of each culture was harvested by centrifugation at 1,500x g, and incubated at 70 °C for 2 h or until dry. Samples were then transferred to the proprietary dye and incubated at 55 °C for 2 h or until dry. Since glycans do not have a chromophore on their own, glycans were labeled with a fluorophore for visualization. Through reductive amination, the labeled glycans were separated electrophoretically by size on the Labchip and subsequently detected by laser-induced fluorescence.

Protein A purification

The mAb protein material was purified using MabSelect SuRe Protein A resin (GE Life Sciences) in the 0.6 mL Opus RoboColumns (Repligen, Germany) on the JANUS BioTx workstation (Perkin Elmer, Hopkinton, MA). Starting from complex material such as harvested cell culture fluid or mAb in media, the high specificity of Protein A binding the Fc region of antibodies allows the removal of ~98% of impurities. Recovered eluate concentration was determined using UV absorbance at 280 nm on the Perkin Elmer Envision Plate reader (Perkin Elmer).

Glycan quantitation by capillary electrophoresis

High throughput analysis of all samples was performed on the LabChip GXII Touch (Perkin Elmer) with the GXII Glycan Release and Labeling Kit (Perkin Elmer, Part # 760,523), Glycan LabChip Reagent Kit (Perkin Elmer, Part # 760,525), and High-Resolution Chip (Perkin Elmer, Part # 760,524) as described by the manufacturer. The purified eluate was diluted between 1.25–7.5 mg/mL in proprietary buffer in a 96-well PCR plate (BioRad Laboratories Waltham, MA). Protein was denatured with SDS-βME and incubated at 70 °C for 10 minutes. Then samples were diluted in N-glycosidase F (PNGase) and incubated at 37 °C for 1.5 hours. The PNGase step removes the N-linked glycan, specific cleaving of the N-acetyl glucosamine-asparagine linkage at the Asn298 site. Samples were then transferred to the proprietary dye and incubated at 55 °C for 2 h or until dry. Since glycans do not have a chromophore on their own, glycans were labeled with a fluorophore for visualization. Through reductive amination, the labeled glycans were separated electrophoretically by size on the Labchip and subsequently detected by laser-induced fluorescence.
controlled through either addition of 0.4 M sodium carbonate or injection of CO₂. Cells were cultured at 36.5 °C for 14 days and counted daily on a Cedex HiRes cell analyzer (Roche, Germany) using Accumax® (Sigma, St. Louis, MO) dispersion reagent. Bioreactors were controlled by TruBio controllers (Finesse, Santa Clara, CA), through DeltaV software (Emerson Electric, St. Louis, MO). Metabolites and IgG concentration were measured on a CedexBioHT analyzer (Roche, Germany). Feed media with or without glycosylation modulating additives was supplied through continuous feeding at a rate of 3.5% of final culture volume per day, starting at D3.

**Abbreviations**

AMBR automated miniature bioreactor  
CoA coenzyme A  
CHO Chinese hamster ovary  
DoE design of experiments  
GlCNac N-acetyl glucosamine  
IgCD integrated viable cell density  
ManNac N-acetyl mannosamine  
NANA N-acetyl neuraminic acid  
PCR polymerase chain reaction  
PNGase N-glycosidase  
RMSE root-mean-square error  
PMP rotations per minute  
SDS sodium dodecyl sulfate  
UDP uridine diphosphate  
UDP-Gal uridine diphosphate galactose  
UV ultraviolet

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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