

Effects of Culture Conditions on *N*-Glycolylneuraminic Acid (Neu5Gc) Content of a Recombinant Fusion Protein Produced in CHO Cells

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ABSTRACT: CHO cells express glycoproteins containing both the *N*-acetylneuraminic acid (Neu5Ac) and minor amounts of the *N*-glycolylneuraminic acid (Neu5Gc) forms of sialic acid. As Neu5Gc is not expressed in humans and can be recognized as a foreign epitope, there is the potential for immunogenicity issues for glycoprotein therapeutics. During process development of a glycosylated fusion protein expressed by CHO cells, a number of culture conditions were identified that affected the Neu5Gc content of the recombinant glycoprotein. Sodium butyrate (SB), a well-known additive reported to enhance recombinant protein productivity in specific cases, minimally affected product titers here, but did decrease Neu5Gc levels by 50–62%. A shift in culture temperature to a lower value after the exponential growth phase was used to extend the culture period. It was found that the Neu5Gc levels were 59% lower when the temperature shift occurred later near the stationary phase of the culture compared to an early-temperature shift, near the end of the exponential growth phase. Studies on the effects of pCO₂ with this product showed that the Neu5Gc levels were 46% lower at high pCO₂ conditions (140 mmHg) compared to moderate pCO₂ levels (20–80 mmHg). Finally, a comparison of sodium carbonate versus sodium hydroxide as the base used for pH control resulted in a reproducible 33% decrease in Neu5Gc in bioreactors using sodium hydroxide. These results are of practical importance as SB is a commonly tested additive, and the other factors affecting Neu5Gc can conveniently be used to reduce or control Neu5Gc in processes for the manufacture of glycoprotein therapeutics.

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Introduction

Approximately 70% of therapeutic proteins and biologics candidates are glycoproteins (Sethuraman and Stadheim, 2006), which are proteins modified post-translationally by the attachment of oligosaccharide chains. Sialic acid is typically the desired terminal sugar on both N- and O-linked oligosaccharide chains. Proper glycosylation and sialylation is a major consideration for the production of glycoprotein therapeutics as it can affect protein stability, susceptibility to proteases, bioactivity, pharmacokinetics, protein clearance in the circulation system, as well as immunogenicity (Hossler et al., 2009; Sethuraman and Stadheim, 2006). Immunogenicity issues may arise if any non-human epitopes are present such as galactose α -1,3-galactose (α -Gal) (Chung et al., 2008), or the sialic acid derivative *N*-glycolylneuraminic acid (Neu5Gc) (Noguchi et al., 1995; Padler-Karavani et al., 2008).

There are more than 40 naturally occurring forms of sialic acid (Lamari and Karamanos, 2002), and Neu5Ac (*N*-acetylneuraminic acid) and Neu5Gc are the two most common forms. Neu5Gc is produced in the cytosol by the enzymatic conversion of CMP-Neu5Ac to CMP-Neu5Gc by CMP-Neu5Ac hydroxylase (CMAH) (Malykh et al., 1998; Muchmore et al., 1989; Shaw et al., 1992). Sialylation by Neu5Gc is present in most animal species (Malykh et al., 1998). A significant exception is humans, which cannot produce Neu5Gc due to a genetic mutation in the human CMAH gene (Irie et al., 1998), yet traces of Neu5Gc have been detected in human serum. Neu5Gc has been reported to be expressed in certain human tumors and the presence of Neu5Gc in humans has also been attributed with consumption of red meats and milk (Tangvoranuntakul et al., 2003). Despite the source, it is well known that humans can elicit immune responses to Neu5Gc containing molecules, or Hanganutziu-Deicher antigens (Noguchi et al., 1995; Padler-Karavani et al., 2008; Tangvoranuntakul et al., 2003). Anti-Neu5Gc antibodies have been detected

in 85% of the human population (Zhu and Hurst, 2002). In addition, high Neu5Gc levels on a chimeric CT4-IgG fusion protein were associated with more rapid clearance profiles in vivo (Flesher et al., 1995).

The selection of the cell type used for the expression of protein therapeutics is extremely important as the ratios of Neu5Gc and Neu5Ac can vary according to species and tissue type (Malykh et al., 1998). It is equally important to understand how cell culture conditions can affect Neu5Gc expression since most cell lines used in the production of human therapeutics are not typically of human origin. There are numerous studies and reviews describing the effects of environmental factors such as nutrient and waste product concentrations, temperature, pH, and osmolality among others on recombinant protein expression and glycosylation (Butler, 2006; Hossler et al., 2009). In contrast, there are very few literature reports describing the impact of culture conditions on glycoprotein Neu5Gc levels. Neu5Gc levels have been modulated using antisense-RNA techniques (Chenu et al., 2003), and there are reports of Neu5Gc being affected by environmental conditions such as high pCO₂ (Kimura and Miller, 1997), manipulation of nucleotide-sugar levels by feeding *N*-acetylmannosamine (Baker et al. 2001), and by HEPES buffer and high pH (Muthing et al., 2003).

Here we report the results from several experiments exploring bioreactor conditions to control Neu5Gc content of a recombinant fusion glycoprotein expressed in CHO cells. The culture conditions include the addition of sodium butyrate (SB), the timing of the temperature shift in the bioreactor, culture carbon dioxide concentrations, and the type of base used for bioreactor pH control. These results provide practical methods for controlling glycoprotein Neu5Gc content in manufacturing through environmental parameters. Understanding factors that affect Neu5Gc in manufacturing can contribute to a Quality by Design (QbD) methodology for protein therapeutic development and is important when assessing the equivalency of different cell culture parameters.

Materials and Methods

Cell Line

The stable CHO cell line expressing the recombinant fusion glycoprotein B1 used in this study was originally subcloned from DG44 parental cells and cultured in a protein-free proprietary growth and feed media formulations (Xing et al., 2008; Zhu et al., 2005). Glycoprotein B1 is a heavily glycosylated protein of which oligosaccharides account for approximately 15% of the protein mass.

Bioreactor Operation

Bioreactor experiments were performed in 1-L (DASGIP, Julich, Germany) and 7-L (Sartorius, Goettingen, Germany) bioreactors with working volumes of 1 and 4 L, respectively.

The bioreactors were operated in fed-batch mode at 37°C and then shifted to a lower temperature as viable cell density (VCD) reached a predetermined value, or as specified in the individual experiment. The pH and dissolved oxygen were maintained at 7.00 ± 0.10 and 50% air saturation, respectively. The bioreactor pH was controlled by CO₂ gas and base addition using either 1 N sodium hydroxide or 1 M sodium carbonate. VCD and viability were measured using a Cedex automated cell counter (Innovatis, Bielefeld, Germany). Culture samples were analyzed offline using a BioProfile 400 Analyzer to monitor pH, pCO₂, pO₂, glucose, glutamine, lactate, and ammonium (Nova Biomedical, Waltham, MA). Osmolality was measured using a Vapro 5520 osmometer (Wescor, Logan, UT). In experiments testing sodium butyrate (SAFC, St. Louis, MO), SB was added to a final concentration of 1 mM the day following temperature shift.

Titer Assay

Titer was determined by high performance liquid chromatography (HPLC) using a Protein-A affinity chromatography column. The eluted protein was detected by absorbance at 280 nm and quantified using reference standards. Titters are reported as normalized values, which are the actual values divided by an arbitrary value. The same divisor was used to normalize all titer data in the studies reported here.

Sialic Acid Content Assay

Sialic acid levels are reported as a ratio of moles sialic acid per mole of recombinant protein. Sialic acid (Neu5Ac and Neu5Gc) was released from Protein-A purified samples by mild acid hydrolysis. The derivatives were then separated using an RHM monosaccharide column (Phenomenex, Torrance, CA) and sialic acids were monitored by absorbance at 206 nm. The peak areas were quantified by linear regression of a standard curve. Neu5Gc levels reported in this study were all within the range of the standard curve used for quantification of Neu5Gc. The Protein-A purified protein concentrations were determined by absorbance at 280 nm. Both Neu5Ac and Neu5Gc contents are reported as normalized molar ratio, which are the moles Neu5Ac or Neu5Gc per mole of recombinant protein divided by an arbitrary value. The same divisor was used to normalize all Neu5Ac, Neu5Gc, as well as the total sialic acid data.

Results

Effect of Sodium Butyrate Addition

SB is a cell culture additive known to upregulate gene expression and used to increase specific productivity of secreted proteins (Hendrick et al., 2001; Jeon and Lee, 2007; Lamotte et al., 1999; Sung et al., 2004); however, the effect of SB increasing productivity can be variable (Jeon and Lee, 2007). SB was evaluated in controlled 1-L bioreactors based

upon results from screening experiments in shake flasks that showed minimal titer benefits, but significantly reduced glycoprotein Neu5Gc levels (data not shown). The bioreactor temperature set point in this experiment was lowered as the culture near the end of the exponential growth phase, or approximately day 4 (early-shift, ES), and SB was added to a final concentration of 1 mM in the bioreactors the day after temperature shift. The data from two separate experiments using duplicate bioreactors, or $n = 4$ overall, is summarized in Table I and Figure 1. The final titer with SB was 11% lower than control (0.88 vs. 0.99); however, SB improved the sialic acid profiles. Even though the total sialic acid (sum of both ratios) did not change significantly ($P = 0.3$ in t -test) with the addition of SB, Neu5Gc levels decreased from 0.13 to 0.05 ($P = 0.0001$ in t -test) and Neu5Ac levels increased from 0.87 to 0.94 ($P = 0.01$ in t -test). An increase of 8% in Neu5Ac level and a decrease of 62% in Neu5Gc level indicated an improvement in protein quality with the addition of SB.

Since SB inhibited cell growth, the addition of SB was also tested in duplicate bioreactors where the temperature shift and SB addition were both delayed to allow the culture to grow to a higher VCD before being inhibited by SB (Fig. 1A). Instead of a 11% decrease in final titer under the early-temperature shift condition discussed above, delay of the temperature shift and SB addition resulted in an 8% increase in final titer (1.13 vs. 1.05) compared to the control (Table I). This result indicates that timing of SB addition was critical to avoid decreasing harvest titer. The effect of SB on sialic acid content was also confirmed for this condition as shown in Figure 1B–D. The harvest Neu5Gc levels decreased by 50% with SB compared to the control (0.05 vs. 0.10). Thus, addition of SB was more applicable under the mid-temperature shift condition, which not only slightly

increased protein titer but also significantly decreased Neu5Gc level.

Sialic acid levels were determined on days 10, 12, and 14 of the culture, and the trends for each time point were similar with respect to Neu5Ac, Neu5Gc, and total sialic acid (Fig. 1B–D). Overall, 9–13% of the total sialic acid present on Protein B1 was Neu5Gc for the control conditions and addition of SB reduced this percentage to 5%. In addition, while SB addition decreased Neu5Gc content, the total sialic acid content of the recombinant glycoprotein was similar with and without SB.

Addition of SB resulted in an approximate 30% increase in specific productivity (Table I) for both temperature shift conditions, which was lower than other published reports using SB (Hendrick et al., 2001; Jeon and Lee, 2007; Lamotte et al., 1999; Sung et al., 2004). The relationship between specific productivity and Neu5Ac or total sialic acid with SB addition reported here was not consistent with that of Etcheverry and Ryll (1998), who reported decreased sialylation with increasing specific productivity for a recombinant protein. This may be due to the relatively small increase in specific productivity (Table I) by SB in this case, or other potential effects of SB on the glycosylation pathway.

Effect of Temperature Shift Timing

The bioreactor temperature in the fusion glycoprotein B1 process is decreased during the process in order to extend the culture and increase harvest titers. A comparison of the early- and mid-temperature shift control conditions (ES and MS (mid-shift)) from the above SB experiments (Table I and Fig. 1C) showed that the Neu5Gc levels were lower when the

Table I. Summary of experimental condition, peak VCD, and protein productivity for bioreactor experiments.^a

				Harvest viability and productivity		
Treatments	Description of conditions	<i>n</i>	Peak VCD ($\times 10^6$ cells/mL)	Viability	Titer ^b	Specific productivity (<i>q_p</i>) ^b
Sodium butyrate addition in 1-L bioreactors						
Early-shift	Temperature shift at end of exponential growth	4	10.6 ± 0.4	89 ± 1	0.99 ± 0.10	12 ± 0
Early-shift/butyrate	SB added 1 day after early-temperature shift	4	7.2 ± 0.5	77 ± 14	0.88 ± 0.11	15 ± 1
Mid-shift	Temperature shift one day after early-shift	2	11.7 ± 0.1	86 ± 1	1.05 ± 0.01	12 ± 0
Mid-shift/butyrate	SB added 1 day after mid-temperature shift	2	9.5 ± 0.2	80 ± 2	1.13 ± 0.01	16 ± 0
Timing of temperature shift in 1-L bioreactors						
Early-shift	Temperature shift at end of exponential growth	2	8.6 ± 0.8	90 ± 1	1.06 ± 0.04	15 ± 0
Mid-shift	Mid-shift	2	9.6 ± 0.7	90 ± 0	1.06 ± 0.03	13 ± 1
Late-shift	Temperature shift at beginning of stationary phase	2	11.4 ± 0.1	87 ± 1	1.12 ± 0.02	13 ± 0
Bioreactor pCO ₂ concentration in 1-L bioreactors						
Low pCO ₂	pCO ₂ at 20–80 mmHg	3	7.7 ± 0.3	92 ± 0	0.96 ± 0.04	14 ± 1
High pCO ₂	pCO ₂ at 140 mmHg	4	7.2 ± 0.6	92 ± 1	0.95 ± 0.04	14 ± 1
Comparison of base used for pH control in 7-L bioreactors						
NaOH	pH control with 1 M NaOH	6	10.8 ± 0.4	85 ± 3	1.08 ± 0.06	15 ± 1
Na ₂ CO ₃	pH control with 1 M Na ₂ CO ₃	6	12.1 ± 0.9	80 ± 5	1.01 ± 0.05	12 ± 1

^aValues of each parameter are reported as average $\pm$ difference/2 ($n = 2$) or average $\pm$ standard deviation ($n > 2$).

^bAll titer values were normalized using the same divisor.

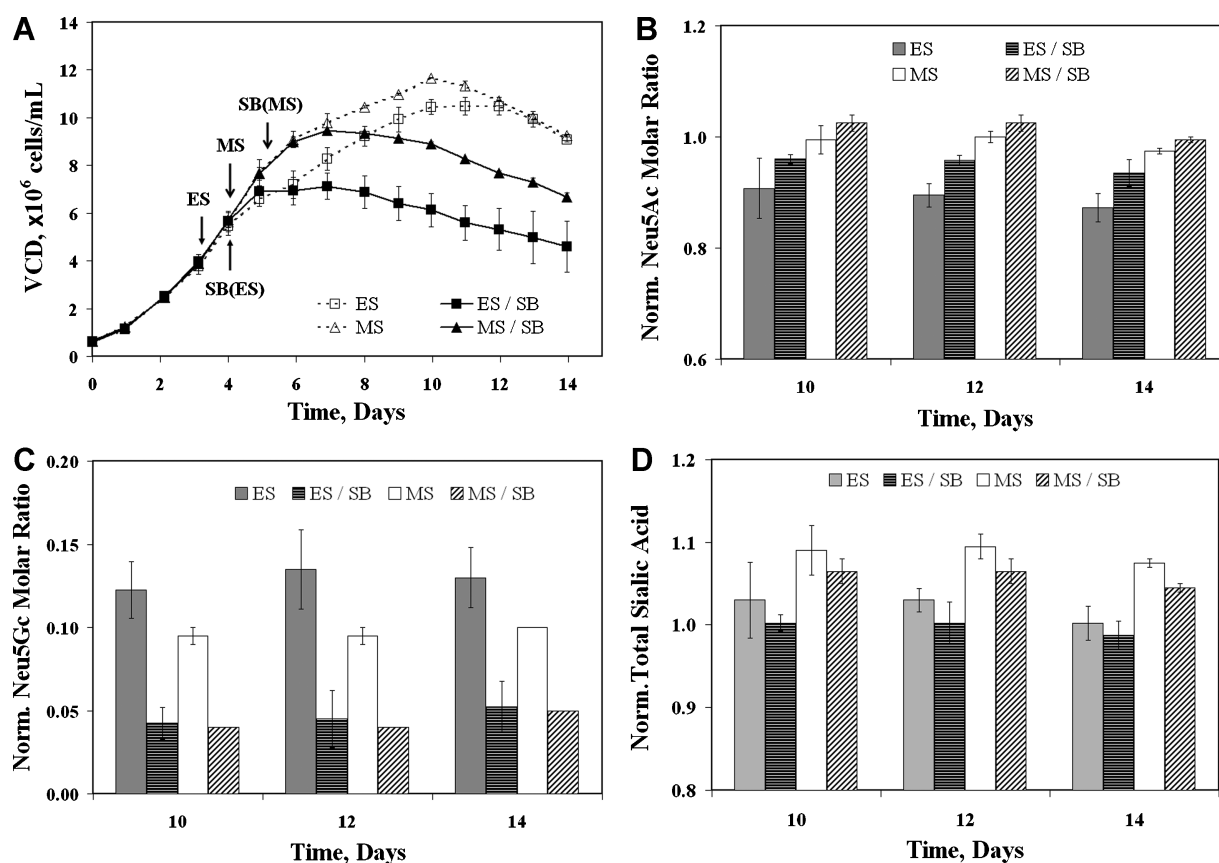


Figure 1. Effect of SB addition on viable cell density (A) and sialic acid content (B–D) in 1-L bioreactors. Definitions of early-temperature shift (ES, $n=4$) and mid-temperature shift (MS, $n=2$) are described in Table I. The arrows in panel A indicate timing of temperature shifts and SB additions. All mole sialic acid form per mole of glycoprotein were normalized using the same divisor. Values of each parameter are reported as average $\pm$ difference/2 ($n=2$) or average $\pm$ standard deviation ($n=4$).

bioreactor temperature was shifted to the lower set point later in the culture. The purpose of this next experiment was to compare the effect of temperature shift timing on Neu5Gc. Duplicate 1-L bioreactors were shifted to the lower temperature set point as the cultures reached a low VCD near the end of the exponential growth period (ES), a mid-level VCD (MS), and a higher VCD as the culture approached the stationary phase (late-shift). All cultures used sodium hydroxide as the base for pH control.

The peak VCD increased with the corresponding higher VCD values of the later temperature shifts (Fig. 2A), although harvest titers were minimally affected (Table I). The main difference among these conditions was the resulting Neu5Gc levels. Neu5Gc levels at harvest were 0.17, 0.12, and 0.07 for the early-, mid-, and late-temperature shift conditions, respectively (Fig. 2C). The corresponding Neu5Ac levels for the early-, mid-, and late-temperature shifts were 0.81, 0.86, and 0.86, respectively (Fig. 2B). Total sialic acid content was similar among the temperature shift conditions with values of 0.98, 0.98, and 0.92 for the early-, mid-, and late-temperature shift conditions, respectively (Fig. 2D). Sialic acid levels were also measured on days 10

and 12, and the trends were similar to those discussed for the harvest data (Fig. 2B–D).

While delaying the temperature shift did not affect harvest titers or total sialic acid levels, Neu5Gc levels were significantly reduced by 29–59% for the mid- and late-temperature shift conditions. As a fraction of total sialic acid, Neu5Gc represented 17% of the total sialic acid present for the early-temperature shift condition, but was reduced to 8% by delaying shift timing.

Effect of High pCO₂

The effects of low-, mid-, and high-pCO₂ concentrations of 20–40, 40–80, and above 140 mmHg were studied in duplicate 1-L bioreactors. The high pCO₂ level of 140 mmHg was chosen to be consistent with that previously reported for this process in 5,000-L bioreactors (Zhu et al., 2005). The pCO₂ was controlled by adjusting the headspace (or overlay) air flow rates for CO₂ stripping and by addition of CO₂ to the bioreactor sparge gas. The pCO₂ profiles of all seven bioreactors are shown in Figure 3A. To limit the effect

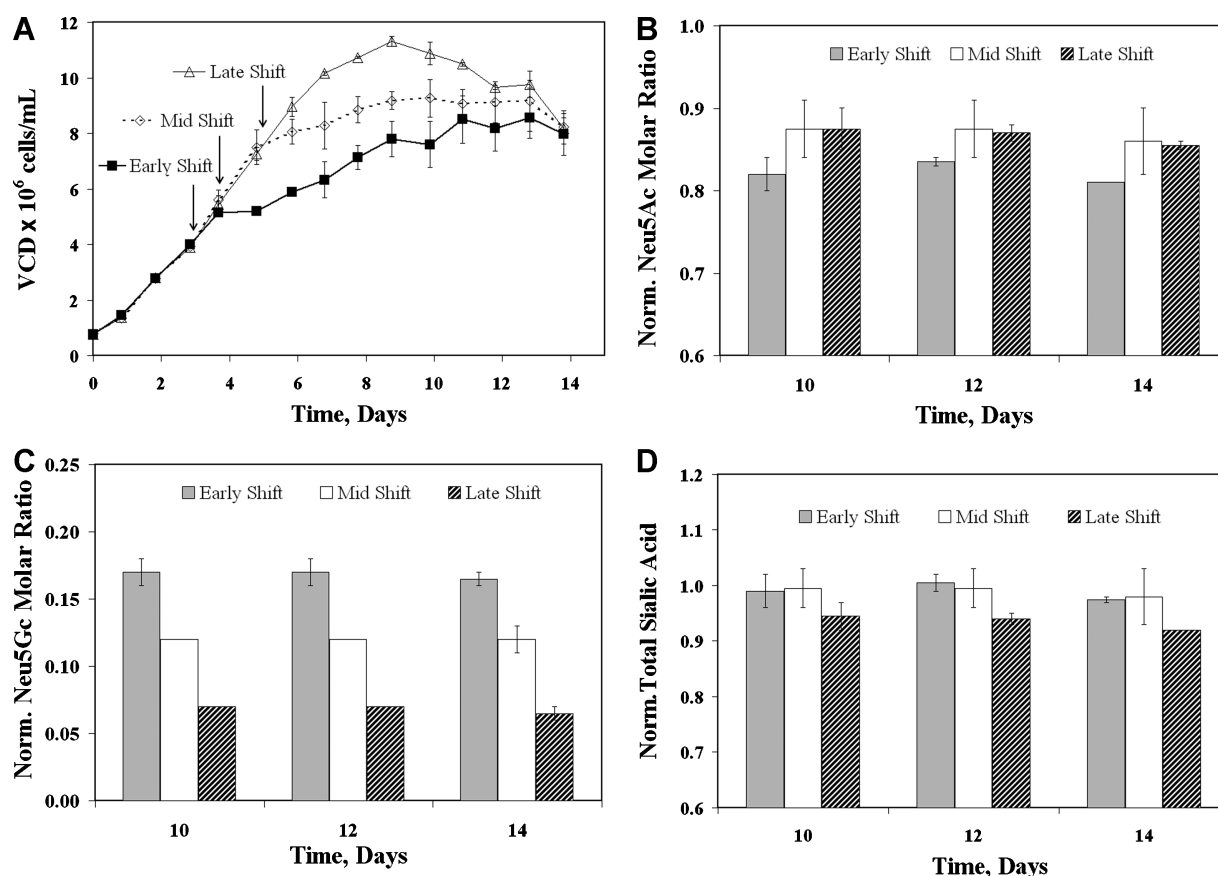


Figure 2. Effect of temperature shift timing on viable cell density (A) and sialic acid content (B–D) in 1-L bioreactors. The arrows in panel A indicate timing of temperature shifts. Definitions of temperature shift schemes are described in Table I. All mole sialic acid form per mole of glycoprotein were normalized using the same divisor. Values of each parameter are reported as average $\pm$ difference/2 ($n = 2$).

of high $p\text{CO}_2$ inhibiting cell growth, the bioreactors were shifted to high $p\text{CO}_2$ later in the culture period on days 3 and 6. In this experiment, the temperature was shifted lower at a VCD near the end of exponential growth (ES criteria).

The $p\text{CO}_2$ toxicity threshold level of 111 mmHg as reported for these cells (Xing et al., 2008) was used to define the low and high $p\text{CO}_2$ conditions as shown by the dashed line in Figure 3A. Thus, the seven cultures were segregated into low $p\text{CO}_2$ (20–80 mmHg, $n = 3$) and high $p\text{CO}_2$ (shifted to 140 mmHg on days 3 and 6, $n = 4$) conditions (Table I). The pH values were controlled at 6.90 ± 0.05 in all seven bioreactors as shown in Figure 3B. Osmolality remained within normal ranges and well below the toxicity threshold limit of 382 mOsm/kg (Xing et al., 2008). The average osmolality values at harvest were 303, 313, 347, and 336 mOsm/kg for the low $p\text{CO}_2$, moderate, ES high, and late-shift high $p\text{CO}_2$ conditions, respectively.

Final values for culture viability, titer, and cell-specific productivity were not significantly different among the $p\text{CO}_2$ conditions (Table I). However, Neu5Gc levels at high $p\text{CO}_2$ were consistently lower (Fig. 3E). Neu5Gc at harvest averaged 0.13 for moderate $p\text{CO}_2$ levels of 20–80 mmHg,

while the conditions shifted to high $p\text{CO}_2$ on days 3 and 6 averaged 0.07 ($P = 0.0004$ in t -test). Conversely, Neu5Ac did not change significantly with increasing $p\text{CO}_2$ as the Neu5Ac at harvest averaged 0.78 at moderate $p\text{CO}_2$ levels and 0.75 at high $p\text{CO}_2$ ($P = 0.1$ in t -test). The net result for total sialic acid was a decrease from 0.91 at moderate $p\text{CO}_2$, to 0.82 at high $p\text{CO}_2$ ($P = 0.008$ in t -test). The trends for sialic acid levels measured on days 10 and 12 were also similar to those reported at harvest (Fig. 3E and F). Overall the harvest data showed equivalent Neu5Ac levels but a decrease of 54% in Neu5Gc level, which indicated an improvement in protein quality under the high $p\text{CO}_2$ levels.

Effect of Base Used for pH Control

Sodium carbonate and sodium hydroxide are commonly used for pH control in bioreactors. Sodium carbonate is potentially more desirable as it is less damaging to the cells than sodium hydroxide. A comparison of these two bases for controlling pH in 7-L bioreactors shows peak VCD (Table I) and overall VCD curves (Fig. 4A) trended slightly higher

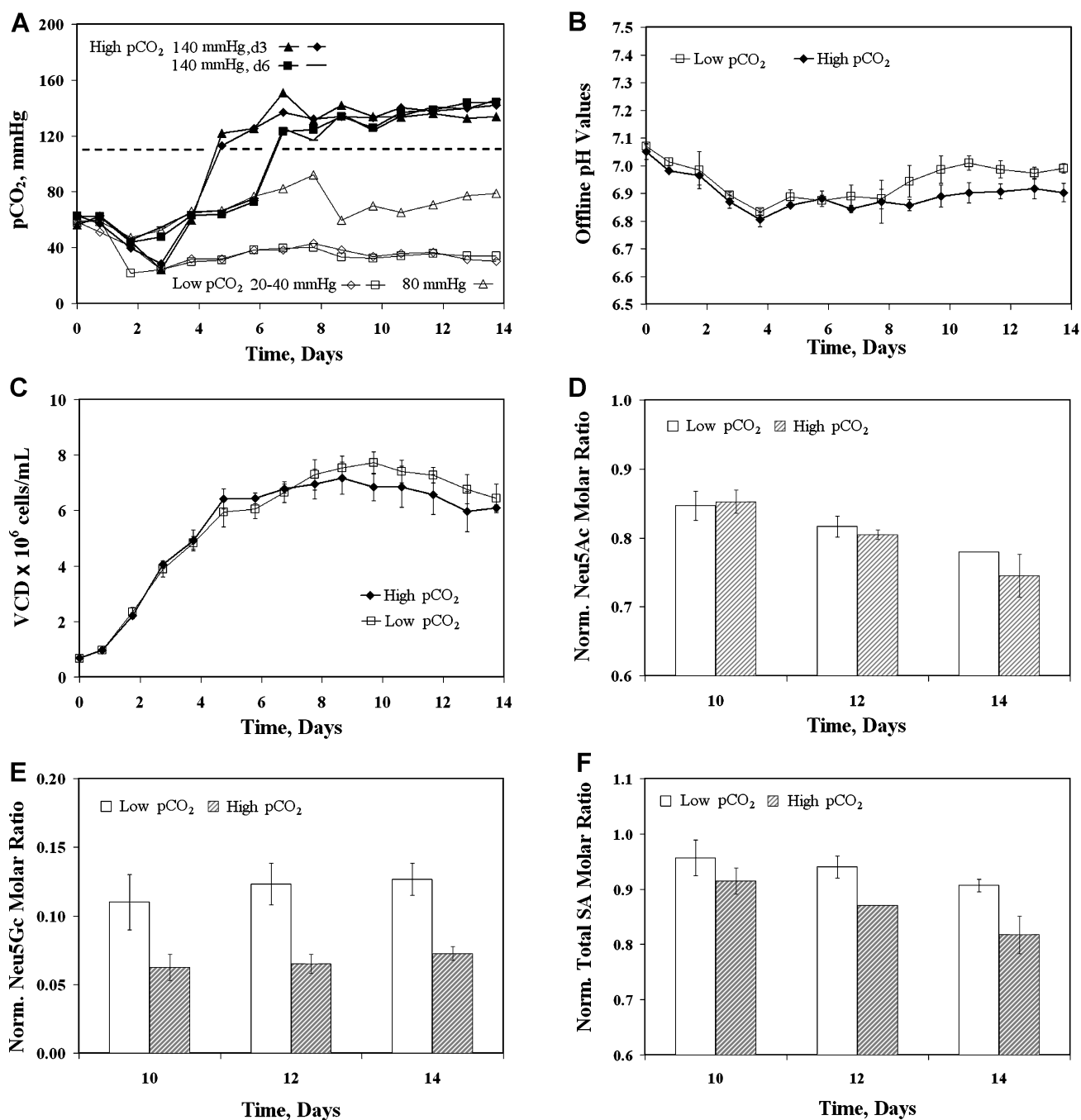


Figure 3. Effects of bioreactors controlled at different pCO₂ levels (A) on pH (B), viable cell density (C), and sialic acid content (D–F) in 1-L bioreactors. The pCO₂ were controlled in the range from 20 to 80 mmHg (low pCO₂, $n = 3$) and 140 mmHg (high pCO₂, $n = 4$). The pCO₂ threshold that inhibits cell growth (110 mmHg) (Xing et al., 2008) is presented as a dashed line in panel A. All mole sialic acid form per mole of glycoprotein were normalized using the same divisor. Values of each parameter are reported as average $\pm$ standard deviation.

when using sodium carbonate. With respect to final culture viability or titer, there were no advantages to use either sodium carbonate or sodium hydroxide. The main differences between the two different bases were that both Neu5Ac and Neu5Gc were consistently higher in the bioreactors using sodium carbonate for pH control (Fig. 4B–D). Neu5Ac at harvest averaged 0.80 with sodium hydroxide

compared to 0.90 with sodium carbonate ($P = 0.0004$ in t -test), while the harvest Neu5Gc averaged 0.10 with sodium hydroxide compared to 0.15 with sodium carbonate ($P = 0.0001$ in t -test). Figure 4C and D shows all of the sialic acid data from days 10 to 14 were consistent. Additional comparisons of sodium hydroxide and sodium carbonate as the bases used for bioreactor pH control have

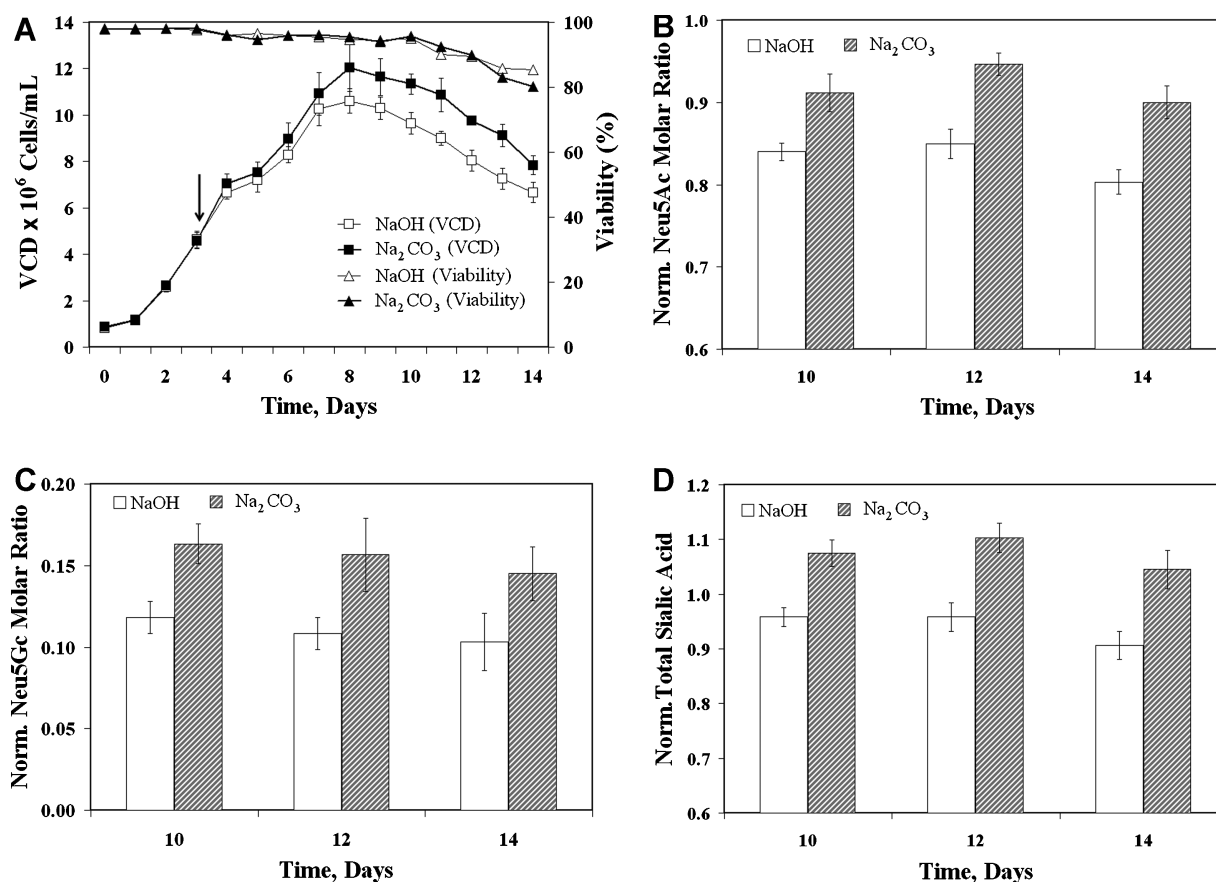


Figure 4. Average viable cell density, viability (A) and sialic acid content (B–D) in 7 L using either sodium carbonate or sodium hydroxide. The arrow indicates timing of the temperature shift. All mole sialic acid form per mole of glycoprotein were normalized using the same divisor. Values of each parameter are reported as average $\pm$ standard deviation ($n=6$).

also been tested at 20-L scale as well as pilot plant (500 L) and manufacturing scales (5,000 L) with similar outcomes (data not shown). The increase in N-linked sialylation was also confirmed with data showing decreased percentages of the non-sialylated form and increased levels of the higher sialylated forms (bi-, tri-, and tetra-sialylated) of the N-linked oligosaccharides under conditions using the sodium carbonate for pH control (data not shown). The type of base did not affect final osmolality as the difference between the carbonate and hydroxide conditions in each case was less than 6% and pCO₂ levels generally remained below 60 mmHg (data not shown).

Discussion

Anti-Neu5Gc antibodies have been reported in 85% of healthy humans (Zhu and Hurst, 2002), and the immunogenicity (Noguchi et al., 1995) and clearance issues relating to Neu5Gc content (Flesher et al., 1995) for new glycoprotein therapeutics are still unknowns. Thus, it is sensible to minimize expression of this epitope known to be immunogenic in humans. In addition, minimizing changes

in Neu5Gc, or any other quality attributes characterizing glycoproteins, are critical for establishing comparability of protein therapeutics when making process changes. This report focused on culture parameters that were found to affect Neu5Gc levels of a recombinant fusion glycoprotein expressed in CHO cells. We confirmed that high pCO₂ does reduce Neu5Gc levels similar to the findings of Kimura and Miller (1997), but report on additional environmental conditions that affected Neu5Gc levels. SB has been reported to affect glycosylation and sialylation (Hendrick et al., 2001; Jeon and Lee, 2007; Lamotte et al., 1999; Sung et al., 2004), but this is the first known report of SB affecting specifically Neu5Gc levels. The timing of the temperature shift in the CHO cell process and the type of base used for bioreactor pH control were initially thought to be innocuous parameters with respect to glycosylation; however, both were found to affect Neu5Gc levels of the final product. These results are of practical importance for increasing understanding and the ability to control Neu5Gc levels in cell culture manufacturing processes.

To understand why these environmental parameters could modulate Neu5Gc and/or Neu5Ac, the source of both

sialic acid forms must be considered. Malykh et al. (1998) found expression of the CMAH enzyme varied between species and tissue, and that incorporation of Neu5Gc into glycoproteins correlated with the amount of the CMAH enzyme present. Once CMP-Neu5Gc is formed from CMP-Neu5Ac, both forms share the same carrier molecule for transport from the cytosol to the Golgi, and once in the Golgi, the sialyltransferases do not exhibit any preference for CMP-Neu5Ac or CMP-Neu5Gc (Lepers et al., 1989). A condition that changes the proportion of Neu5Gc, but with similar overall sialylation would suggest changes in CMP-Neu5Gc content. This could result from changes in CMAH expression levels, CMAH activity in the cytosol, changes in cellular NADH levels (a cofactor for CMAH), all of which could affect CMP-Neu5Ac levels. Changes that involve sialyltransferase activity would result in changes to total sialic acid content and would not necessarily affect either Neu5Ac or Neu5Gc independently of the other.

This general explanation may give indications as to where the environmental changes reported here may affect the intracellular process relating to Neu5Gc. The three factors reported here that involve changes to Neu5Gc with minimal effects on either Neu5Ac or total sialic acid content were SB addition, timing of the temperature shift, and high levels of $p\text{CO}_2$ in the bioreactor. Such trends would be expected where the environmental parameter affected intracellular formation or transport of CMP-Neu5Gc. In contrast, the type of base used for bioreactor pH control affected both Neu5Gc and Neu5Ac levels.

SB has been reported to have a variety of effects on glycosylation in other cell systems (Hendrick et al., 2001; Jeon and Lee, 2007; Lamotte et al., 1999; Sung et al., 2004); however, there have been no reports of SB affecting Neu5Gc. Santell et al. (1999) reported lower sialic acid content with a 4-fold increase in specific productivity. Chotigeat et al. (1994) reported a 6.9-fold increase in specific productivity with SB as well as increases in both sialic acid and sialyltransferase activity. In the present study, the effect of SB on productivity was small compared to the other studies but the effects on sialylation were unique. Neu5Gc levels decreased by 48–62% with SB, while Neu5Ac levels increased marginally, resulting in similar levels for total sialic acid with and without SB. The changes here were more consistent if SB altered the levels or activity of the CMAH enzyme producing CMP-Neu5Gc or increased production of CMP-Neu5Ac. Crowell et al. (2008) reported increased levels of CMP-Neu5Ac with SB treatment of HT-1080 cells expressing EPO. A change in the ratio of CMP-Neu5Ac and CMP-Neu5Gc could potentially change Neu5Gc expression.

The timing of the temperature shift affecting Neu5Gc levels was not anticipated. The data reported here showed that Neu5Gc levels were 59% lower when temperature was shifted later near the stationary phase of the culture compared to an early-temperature shift, near the end of the exponential growth phase. Nam et al. (2008) showed the presence of Neu5Gc in placental alkaline phosphatase from CHO cells when the culture temperature was shifted to 33°C

after exponential growth; whereas Neu5Gc was not evident in cultures maintained at 37°C. Together these data suggest that low temperature may promote Neu5Gc, but the data reported here also suggest that the amount of Neu5Gc was affected and can be modulated by the timing of the temperature shift. This is an important finding as shifts to lower incubation temperatures are commonly used in industry to increase culture duration and titer. One may still retain the positive effects of incorporation of a temperature shift to increase culture duration and titer, yet modulate potential negative effects of increased Neu5Gc due to low temperatures by selection of shift timing closer to the stationary phase. The decreased Neu5Gc content and increasing Neu5Ac levels reported here were again consistent with changes in the reaction forming the CMP-Neu5Gc. The timing of the temperature shift closer to the stationary phase of the culture could affect how cells accumulate at different phases of the cell cycle creating different metabolic states, a situation that could affect CMAH and/or NADH levels.

High $p\text{CO}_2$ (140 mmHg) was found here to decrease Neu5Gc by 46% with minimal effects on Neu5Ac. These results were consistent with those of Kimura and Miller (1997) who reported that the proportion of Neu5Gc in tissue plasminogen activator (tPA) from CHO cells decreased from 2.3–4.0% under 36 mmHg to 1.5–2.2% under 250 mmHg with no changes in total sialic acid content. The effect of high $p\text{CO}_2$ was consistent with a mechanism affecting mainly CMAH or NADH levels. The effect may be more complex than $p\text{CO}_2$ affecting CMAH activity by lowering intracellular pH (pH_i). High $p\text{CO}_2$ has been reported to decrease pH_i by 0.1–0.2 pH units (deZengotita et al., 2002); however, the activity of mouse liver CMAH was measured to be at the optimal activity in the physiological range between pH 6.8 and 7.4 (Shaw et al., 1992). Thus, the effect may be related to cellular NADH levels, as originally proposed by Kimura and Miller (1997) or possibly due to other enzymes such as cytochrome b_5 , and NADH-cytochrome b_5 reductase that are required for the hydroxylation reaction of CMP-Neu5Ac by CMAH (Kawano et al., 1994).

In contrast to the above cases, both Neu5Gc and Neu5Ac levels increased when sodium carbonate was used as the base for pH control compared to bioreactors using sodium hydroxide. The Neu5Gc levels were 50% higher and Neu5Ac levels were 13% higher in the bioreactors using sodium carbonate in the case reported here, which has been repeated at various scales with similar outcomes. A change resulting in increased Neu5Ac as well as Neu5Gc suggests this effect may be due to increases in sialyltransferase activity. Both osmolality and $p\text{CO}_2$ levels were eliminated as the causes for changes to Neu5Gc levels as they were similar between the conditions. The use of sodium hydroxide resulted in a slightly lower culture VCD and higher specific productivity by 20%. These differences may indicate that changes in Neu5Gc induced by the bases could be more general in nature similar to reports associating increasing specific

productivity rates with decreased sialic acid content (Etcheverry and Ryll, 1998). Overall, additional data is required to assess any relationship between specific productivity and either Neu5Ac or Neu5Gc content. Additional testing is also required to address whether coupling the parameters affecting Neu5Gc levels could be additive.

The effects seen with evaluation of temperature shift timing and the base used to control bioreactor pH does emphasize that seemingly simple changes to culture conditions may lead to changes in product quality. Thus, it is essential to identify potential sources for variation as well as methods to control Neu5Gc levels, or glycosylation in general, for glycoprotein therapeutics. Implications for cell culture development and manufacturing, is that such information can then be incorporated into evolving development strategies to understand and modify current processes, and to incorporate this information for the control and identification of design spaces for future products.

Conclusion

The results reported here showed that both significant changes to culture conditions such as SB addition and high carbon dioxide conditions, as well as seemingly subtle changes such as the timing of the shift to a lower culture temperature, and the type of base used for pH control can affect Neu5Gc levels of a recombinant glycoprotein expressed in CHO cells. These results provide additional understanding for controlling Neu5Gc content in glycoproteins through environmental parameters. Understanding the implications of different culture parameters on controlling Neu5Gc is now an additional consideration in formulating process development plans when using a QbD approach.

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