The enhancement of antibody concentration and achievement of high cell density CHO cell cultivation by adding nucleoside

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Abstract Recently, with the dramatic increase in demand for therapeutic antibodies, Chinese hamster ovary (CHO) cell culture systems have made significant progress in recombinant antibody production. Over the past two decades, recombinant antibody productivity has been improved by more than 100-fold. Medium optimization has been identified as an important key approach for increasing product concentrations. In this study, we evaluated the effects of deoxyuridine addition to fed-batch cultures of antibody-expressing CHO cell lines. Furthermore, we investigated the effects of combined addition of deoxyuridine, thymidine, and deoxycytidine. Our results suggest that addition of these pyrimidine nucleosides can increase CHO cell growth, with no significant change in the specific production rate. As a result of the increased cell growth, the antibody concentration was elevated and we were able to achieve more than 9 g/L during 16 days of culture.

Similar effects of nucleoside addition were observed in fed-batch cultures of a Fab fragment-expressing CHO cell line, and the final Fab fragment concentration was more than 4 g/L. This nucleoside addition strategy could be a powerful platform for efficient antibody production.

Keywords Chinese hamster ovary (CHO) cell · Therapeutic antibody · Nucleoside · Cell culture medium

Introduction

At the end of the 1980s, the dominance of monoclonal antibody (mAb) approvals was about 10% of all biologic products approved, while recently they have represented almost 30% of all approvals. Moreover, the market for biopharmaceutical products reached \$140 billion in total cumulative sales in 2013 (Walsh 2014). Among the mammalian cell expression platforms, Chinese hamster ovary (CHO) cell-based systems are commonly used. Recently, about 60–70% of all recombinant therapeutic proteins are produced by CHO cells (Jayapal et al. 2007). There are some advantages to using CHO cells as the host cells for therapeutic protein production. First, CHO cells are able to achieve appropriate post-translational modification, and can thus produce recombinant proteins with the correct glycoforms for compatibility with humans. Second, CHO cells have already been

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demonstrated to be safe host cells for clinical use, potentially making it easier to obtain approval from regulatory agencies. Third, CHO cells can easily be adapted to serum-free suspension culture, which is suitable for large-scale manufacture in bioreactors (Omasa et al. 2010).

Recently, with the dramatic increase in demand for these therapeutic proteins, manufacturing platforms for recombinant protein production using CHO cell culture systems have made significant progress (Omasa et al. 2010). Over the past two decades, recombinant protein productivity has been improved by more than 100-fold (Wurm 2004). Several efforts, including cell engineering, optimization of cell line development, cell culture medium optimization, and process development for scaling up or down, have led to significant improvements in recombinant protein production (Omasa 2002; Omasa et al. 2008; Nakamura and Omasa 2015). Consequently, CHO-based mAb production processes can achieve 1–10 g/L productivity in fed-batch cultures (Kunert and Reinhart 2016).

Medium optimization has been identified as an important key approach for increasing product concentrations. Much effort has been made toward understanding cell nutrient requirements to develop chemically-defined (CD) media that can improve cell culture performance, and toward avoiding the use of animal-derived raw materials and variability associated with hydrolysates (Zhang et al. 2013). Screening of new small molecules as medium additives and balancing of current medium components by studying their interactions have been reported to increase productivity (De Leon et al. 2007; Backliwal et al. 2008; Inoue et al. 2000). Bovine serum was a popular supplement in cell culture medium, because it improves cell growth and productivity. However, regulatory agencies encouraged the removal of animal-derived supplements because of safety concerns (Kishishita et al. 2015). Consequently, non-animal-derived hydrolysates from soy, wheat, and yeast, which can provide similar benefits to serum, were widely used. However, because of their compositional complexity and lot-to-lot variation, hydrolysates led to process and product inconsistencies (Luo and Chen 2007; Zhang et al. 2003). Therefore, many biopharmaceutical companies are now shifting to CD medium formulations. The majority of the biopharmaceutical industry is currently using fed-

batch cultures as a platform technology for biopharmaceutical protein production, because recent advances have resulted in higher final product concentrations (Li et al. 2014).

It is well known that optimization of nutrient concentrations, such as amino acids, vitamins, and trace metals, is very important for improving protein production (Takagi et al. 2001; Kim et al. 2005; Chaderjian et al. 2005). However, few reports have described the application of exogenous nucleoside addition for protein production processes. Purine and pyrimidine nucleotides are essential cellular compounds involved in many biochemical processes. They are indispensable for cell growth because they are required not only for nucleic acid synthesis as building blocks, but also for energy metabolism (Yamaoka et al. 1997). The *de novo* and salvage pathways are the principal pathways for nucleotide synthesis (Lane and Fan 2015). Nucleotide biosynthesis via the salvage pathway induced by exogenous nucleosides is more efficient than *de novo* biosynthesis (Austin et al. 2012). Hence, it is important to investigate the effects of nucleosides on cell growth and protein production. Several studies have reported the influence of nucleosides on CHO cell growth (Chen et al. 2012a; Carvalhal et al. 2003). However, much more attention has been paid to the effects on product quality, especially glycosylation (Gramer et al. 2011; Wong et al. 2010), and little is known about the use of extracellular nucleosides for optimization of recombinant protein production as an effective tool to elevate antibody production. Furthermore, the impact of pyrimidines on CHO cell cultures has been less commonly reported in the literature compared with that of purines (Carvalhal et al. 2011).

In this article, we report an approach to increase the final product concentration through addition of pyrimidine nucleosides, especially deoxyuridine. Moreover, we show that the combined addition of deoxyuridine, thymidine, and deoxycytidine can increase the product concentration compared with single addition of deoxyuridine. As a result of the improvement in cell growth and prevention of decrease in viability in the death phase, we could achieve high product concentrations. The efficacy of this strategy is demonstrated for cell lines expressing a Fab fragment and two different recombinant antibodies, suggesting that it could be a beneficial platform technology for antibody-based therapeutic protein production systems.

Materials and methods

Cell lines and media

The three CHO cell lines used as the model cell lines in this study were generated from CHO-K1SV cells (Lonza Biologics, Slough, UK), which had been adapted to suspension culture and can grow in serum-free medium. The GS Gene Expression System (Lonza Biologics) was used as the expression system for CHO-K1SV cells (GS-CHO system). The expression vectors are constructed by cloning protein coding gene into GS expression vectors (Lonza Biologics) (Barnes et al. 2000). The GS coding sequence is under the control of a weak promoter; meanwhile the protein coding sequence is under the control of a powerful promoter (Bebbington et al. 1992). The three cell lines were transfected with plasmids encoding different protein molecules and established by single-cell cloning. CHO cell line A and CHO cell line B produced human mAb A and mAb B, respectively, while CHO cell line C produced Fab fragment C.

The basal and feed media used in this study were fully in-house CD media. The CD basal and feed media contained trace elements, inorganic salts, energy source, amino acids, vitamins, fatty acids, lipids, and others. Nevertheless, the glucose and other nutrient concentrations should be maintained at appropriate levels during the cell culture process to supply sufficient amounts of energy source and cellular components. Several report described how much nutrient would be needed for GS-CHO culture (Carinhas et al. 2013; Kyriakopoulos et al. 2013; Sellick et al. 2011). Further nucleoside components were added as indicated.

Nucleosides

Deoxyuridine (A16026) and deoxycytidine hydrochloride (L14153) were purchased from Alfa Aesar (Heysham, UK). Thymidine (205-08091) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Deoxycytidine hydrochloride was used as the form for addition of deoxycytidine. All three nucleosides were dissolved in water and sterilized by filtration through a 0.22- μ m filter before addition.

Cell culture methods

Cells were routinely maintained in suspension culture using 125-mL shaking flasks (Corning, Corning, NY,

USA) on a rotary shaker–incubator (Kuhner, Birsfelden, Switzerland) controlled at 36.5 °C, 5% CO₂, and 140 rpm.

Production culture was carried out in the fed-batch culture mode using the 125-mL shaking flasks. Cells were seeded at 1.0×10^6 cells/mL, and subjected to different nucleoside addition strategies. Feeding was operated with glucose and in-house CD media to provide sufficient nutrients to the cells. The feeding of in-house CD media was operated every day from day 2 to the end of the culture. Cell suspension samples were taken at appropriate times, and the cell concentrations, cell viabilities, and protein product concentrations were analyzed.

Analytical methods of cell concentration and mAb concentration

The viable cell concentration (VCC) and cell viability were measured in an automated cell counting device (ViCell-XR; Beckman Coulter, Indianapolis, IN, USA) using the trypan blue exclusion method. The concentrations of mAb A and mAb B in the culture supernatants were measured using an HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector and a protein A affinity column (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of Fab fragment C was measured with a reverse-phase column (Imtakt Corp., Kyoto, Japan) using the same HPLC system.

Calculation of specific growth rate and specific production rate

In fed-batch cultures, the mass balance of total cells, viable cells, and mAb were described by Eqs. (1) and (2) in a previous report (Omasa et al. 1992). Briefly, V , X_t , μ , X_v , F_0 , P , and ρ are the culture volume, total cell concentration, specific growth rate, viable cell concentration, drawing rate via sampling, mAb concentration, and specific production rate, respectively. These parameters are measurable during the culture.

$$\frac{dVX_t}{dt} = \mu VX_v - F_0 X_t \quad (1)$$

$$\frac{dVP}{dt} = \rho VX_v - F_0 P \quad (2)$$

Equations (3) and (4) are led by the integrals of Eqs. (1) and (2). The specific growth rate μ and

specific production rate ρ are calculated from Eqs. (3) and (4).

$$\ln(VX_t) + \int \frac{F_0}{V} dt = \mu \int \left(\frac{X_v}{X_t} \right) dt + \ln(VX_t)_0 \quad (3)$$

$$(VP) + \int F_0 P dt = \rho \int VX_v dt + (VP)_0 \quad (4)$$

Results and discussion

Effects of deoxyuridine on cell growth and mAb production

Fed-batch cultures were initiated at a cell concentration of 1.0×10^6 cells/mL in 125-mL shaking flasks using CHO cell line A. Control cultures were performed without deoxyuridine addition. To determine the effects of deoxyuridine addition on CHO cell growth and mAb production, deoxyuridine was added on day 2 at final concentrations of 10, 25, 50, 100, and 200 mg/L. Fed-batch cultures in shaking flasks with different concentrations of deoxyuridine were carried out for 14 days, and the results are summarized in Fig. 1 and Table 1.

As shown in Table 1, no significant differences in the specific growth rate were observed in the various deoxyuridine concentration cultures. However, addition of deoxyuridine at all concentrations from 10 to 200 mg/L significantly increased the peak VCC. In particular, addition of 25 mg/L deoxyuridine produced a peak VCC of 18.4×10^6 cells/mL,

representing a 75% increase compared with that of the control culture (10.5×10^6 cells/mL). Furthermore, there were differences in cell viability. In the control culture, the cell viability gradually dropped off toward the end of the culture period. As shown in Fig. 1, the viability after day 6 was not decreased following addition of 25 mg/mL to 100 mg/L deoxyuridine. The increases in peak VCC and viability led to higher values of the integrated viable cell concentration (IVCC). The increased final product concentrations could be primarily linked to the increased cell growth. In all cases with addition of deoxyuridine, increases in the mAb concentration were observed compared with no addition of deoxyuridine. The final mAb concentration in the control condition was 3.3 g/L, while the final mAb concentration in the deoxyuridine addition cultures was dramatically improved. For example, in the 25 mg/L deoxyuridine culture, the mAb concentration was 5.5 g/L, representing a 67% higher concentration compared with that in the control culture. Thus, addition of deoxyuridine caused significant increases in the peak VCC of CHO cells and the final product concentration. Furthermore, as shown in Table 1, there were no significant differences in the specific production rate. The elevation of the final product concentration seemed to be mainly caused by the increased IVCC. The final product concentration in cultures was reported to equal the specific production rate multiplied by the IVCC over the culture duration (Birch and Racher 2006). Our findings showed that cells were able to increase their IVCC, without significant loss of the specific production rate.

Fig. 1 Effects of addition of different concentrations of deoxyuridine on cell growth, cell viability, and mAb concentration in CHO cell line A cultures. **a** Viable cell concentration.

b Cell viability. **c** mAb concentration. Control cultures were performed without deoxyuridine addition. Deoxyuridine was added at the initial time of culture

Table 1 Experimental conditions and kinetic parameters for different concentrations of deoxyuridine addition culture

Fed-batch condition	Peak VCC ($\times 10^6$ cells/mL)	Viability at day 14 (%)	IVCC at day 14 ($\times 10^6$ cells h/mL)	MAb concentration at day 14 (g/L)	μ (1/h)	ρ (pg/cell/day) ^a
Control	10.5	59.4	2226.9	3.3	0.024	38.2 ± 3.7
10 mg/L	13.2	48.7	2498.9	3.4	0.027	35.6 ± 2.6
25 mg/L	18.4	73.4	3827.4	5.5	0.027	36.8 ± 3.6
50 mg/L	17.4	73.1	3738.3	5.4	0.026	37.0 ± 3.2
100 mg/L	16.6	66.6	3278.2	4.7	0.026	37.6 ± 3.7
200 mg/L	14.5	48.2	2814.9	3.9	0.025	37.0 ± 2.7

The specific growth rate μ is calculated from the data in Fig. 1 in the exponential phase

The specific production rate ρ is calculated from the data in Fig. 1 in the whole process time

^a With 95% confidence interval

However, the peak cell concentration decreased after addition of more than 100 mg/L deoxyuridine. It was estimated that the optimal range of deoxyuridine concentration to maximize the effects was 25 to 50 mg/L. To simplify further evaluations, 25 mg/L was selected as the added concentration.

Synergistic effects of deoxyuridine, thymidine, and deoxycytidine on cell growth and mAb production

It has been reported that cells have intracellular pools for individual nucleosides (Staub et al. 1988; Russell and Partick 1980; Carvalho et al. 2011). Addition of deoxyuridine at more than 25 mg/L could not further enhance the increase in cell growth. It was supposed that the content of the intracellular deoxyuridine pool might be a limiting factor for this effect. Deoxyuridine, thymidine, and deoxycytidine are categorized as pyrimidine nucleosides. These compounds are metabolized by related pyrimidine salvage pathways (Traut 1994). Thymidine and deoxycytidine were expected to have synergistic effects with deoxyuridine.

To evaluate the combined effects among the three nucleosides, fed-batch cultures were performed at an initial cell concentration of 1.0×10^6 cells/mL with CHO cell line B. The combinations of nucleosides and culture results are summarized in Table 2. To evaluate the effects of combined addition of the nucleosides, fed-batch cultures in shaking flasks were carried out for 14 days. Control culture was performed without addition of any nucleosides. To determine the synergistic effects of deoxyuridine, thymidine, and deoxycytidine, the nucleosides were added at the initial time

at final concentrations of 25 mg/L in various combinations.

As shown in Table 2 and Fig. 2, no significant differences in the specific growth rate were observed between the single nucleoside addition cultures (dU, dT, and dC) and the control culture. However, deoxyuridine or thymidine addition increased the peak VCC and final product concentration. After addition of 25 mg/L deoxyuridine, the peak VCC (21.7×10^6 cells/mL) was 38% higher than that in the control culture (15.7×10^6 cells/mL). Moreover, after addition of 25 mg/L thymidine, the peak VCC (20.1×10^6 cells/mL) was 28% higher than that in the control culture (15.7×10^6 cells/mL). Slight increases in viability were observed from day 6 to day 10 in the deoxyuridine and thymidine addition cultures. As a result of the increases in the peak VCC and viability, the final product concentrations were increased in the deoxyuridine and thymidine addition cultures. The final mAb concentration in the deoxyuridine addition culture (7.0 g/L) was 37% higher than that in the control culture (5.1 g/L). Similarly, the final mAb concentration in the thymidine addition culture (6.8 g/L) was 33% higher than that in the control culture. As increases in the specific production rate were not observed in these conditions (Table 2), the increases in the final product concentration seemed to be mainly caused by the increases in the IVCC.

As shown in Table 2 and Fig. 3, no significant differences in the specific growth rate were observed among the cultures with combined addition of two nucleosides (dU-dT, dU-dC, and dT-dC) and the deoxyuridine addition culture (dU). Moreover, none of the two combined nucleoside cultures showed higher

Table 2 Experimental conditions and kinetic parameters for various combinations of nucleoside addition culture

Fed-batch condition	Nucleoside		Peak VCC ($\times 10^6$ cells/mL)	Viability at day 14 (%)	IVCC at day 14 ($\times 10^6$ cells h/mL)	mAb concentration at day 14 (g/L)	μ (1/h)	ρ (pg/cell/day) ^a
	Deoxyuridine	Thymidine	Deoxycytidine					
Control	–	–	–	72.9	3417.0	5.1	0.026	46.2 $\pm$ 6.8
dU	25 mg/L	–	–	73.1	4900.3	7.0	0.027	43.1 $\pm$ 5.9
dT	–	25 mg/L	–	78.5	4481.7	6.8	0.028	46.7 $\pm$ 6.8
dC	–	–	25 mg/L	74.8	3409.7	5.2	0.026	47.0 $\pm$ 7.4
dU-dT	25 mg/L	25 mg/L	–	69.4	4741.2	6.9	0.027	45.3 $\pm$ 5.7
dU-dC	25 mg/L	–	25 mg/L	77.1	4920.8	7.2	0.028	44.8 $\pm$ 7.2
dT-dC	–	25 mg/L	25 mg/L	75.0	4527.2	6.3	0.029	42.4 $\pm$ 6.2
dU-dT-dC	25 mg/L	25 mg/L	25 mg/L	86.3	5638.3	8.2	0.028	44.0 $\pm$ 7.7

The specific growth rate μ was calculated from the data in Figs. 2, 3, and 4 in the exponential phase

The specific production rate ρ was calculated from the data in Figs. 2, 3, and 4 in the whole process time

^a With 95% confidence interval

peak VCC, viability, specific production rate, and mAb concentration than the deoxyuridine addition culture. Nevertheless, all two combined nucleoside cultures showed a higher peak VCC and mAb concentration than the control culture. Hence, these results showed no synergistic effects among the cultures with two combined nucleosides on the cell growth, viability, and mAb concentration.

As shown in Table 2 and Fig. 4, the three combined nucleoside culture (dU-dT-dC) showed higher viability after day 8 compared with the deoxyuridine addition culture (dU). The viability at day 14 was 86.3% in the three combined nucleoside culture, while that in the deoxyuridine addition culture was 73.1%. The specific growth rates were similar between these two cultures. In addition, the peak VCC was slightly higher in the three combined nucleoside culture. Consequently, the IVCC was higher than that in the deoxyuridine addition culture. There was no significant difference in the specific production rate between these two cultures. As a result of the IVCC increase, the final product concentration (8.2 g/L) was increased by 17% compared with that in the single deoxyuridine addition culture (7.0 g/L). Because of the increased viability at day 14, the three combined nucleoside culture achieved a mAb concentration of 9.2 g/L at day 16. Thus, we were able to attain high mAb production with this nucleoside addition strategy using the GS-CHO system. These observations support the notion that synergistic effects could exist among these three combined nucleosides for the cell growth and mAb concentration. Overall, the mAb concentration at the final culture point (9.2 g/L) was dramatically increased by 80% compared with that in the control culture (5.1 g/L) following the addition of the three nucleosides.

Thymidine was previously reported to have an enhancement effect on cell growth (Chen et al. 2012b). However, this is the first report to identify that deoxyuridine has impacts on the cell growth and final product concentration, and that deoxyuridine, thymidine, and deoxycytidine have synergistic effects on the cell growth and product concentration. The observed effects suggested that the pyrimidine salvage pathway could be one of the key metabolic pathways for improvement of mAb production. The strategy of exogenous nucleoside addition might have advantages for cell growth by supplying precursors of nucleic acids via the salvage pathway. The reason why

Fig. 2 Effects of addition of different single nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. **a** Viable cell concentration. **b** Cell viability. **c** mAb concentration. Control cultures were performed without

nucleoside addition. Each nucleoside was added at the concentration of 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

Fig. 3 Effects of addition of two combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. **a** Viable cell concentration. **b** Cell viability. **c** mAb concentration. Control cultures were performed without

nucleoside addition. Each nucleoside was added at the concentration of 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

synergistic effects were only observed for the combination of deoxyuridine, thymidine, and deoxycytidine remains unclear. Further investigations are necessary to understand the mechanism underlying these effects.

Verification of nucleoside addition effects using a Fab fragment-expressing CHO cell line

It is known that different recombinant CHO cell lines show different responses to external culture environments (Porter et al. 2010; Kim et al. 2005). Furthermore, recombinant CHO cell lines expressing different types of protein show different productivity.

Therefore, it is important to confirm the effects of nucleoside addition using different CHO cell lines that produce different types of protein. To investigate whether the enhancement of cell growth by nucleoside addition is a general phenomenon, CHO cell line C expressing Fab fragment C was evaluated using fed-batch cultures. It was worth evaluating a protein with different characteristics for general application.

The fed-batch cultures were carried out at an initial cell concentration of 1.0×10^6 cells/mL. Control cultures were performed without nucleoside addition. To investigate the effects of the nucleosides, two types of fed-batch culture with 25 mg/L deoxyuridine

Fig. 4 Effects of addition of three combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. **a** Viable cell concentration. **b** Cell viability. **c** mAb concentration. Control cultures were performed without

nucleoside addition. Each nucleoside was added with 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

addition and with deoxyuridine, thymidine, and deoxycytidine addition at 25 mg/L each were performed. Nucleosides were added at the initial time of culture. Fed-batch cultures in shaking flasks were carried out for 14 days. The results are summarized in Fig. 5.

As shown in Fig. 5a, c, 25 mg/L deoxyuridine addition increased the peak VCC and also increased the final product concentration. In the deoxyuridine addition culture, the peak VCC (25.6×10^6 cells/mL) was 84% higher than that in the control culture (13.9×10^6 cells/mL). However, in the deoxyuridine addition culture, a gradual decrease in viability was observed after day 6 (Fig. 5b). As a result of the increase in the peak VCC, the final product concentration was increased in the deoxyuridine addition culture. The Fab fragment concentration in the deoxyuridine addition culture (3.1 g/L) was 40% higher than that in the control culture (2.2 g/L). Moreover, the three combined nucleoside culture showed an 18% higher peak VCC (30.1×10^6 cells/mL) than that in the single deoxyuridine addition culture (25.6×10^6 cells/mL). Interestingly, the viability after day 8 remained higher compared with that in the single deoxyuridine addition culture. Finally, the viability at day 14 in the three combined nucleoside culture was 80.6%, while that in the deoxyuridine addition culture was 57.1%. As a result of the peak VCC and viability increases, the final product concentration (4.2 g/L) was increased by 35% compared with that in the control culture (3.1 g/L). The

synergistic effects of the three nucleosides were also observed in these results. Overall, the enhancement effect of deoxyuridine addition on CHO cell line C growth was confirmed, and the final Fab fragment concentration was increased in CHO cell line C cultures. Furthermore, synergistic effects of deoxyuridine, thymidine, and deoxycytidine were also observed. These results suggest that the effects of deoxyuridine, thymidine, and deoxycytidine addition can form a general strategy for increasing product concentrations among recombinant CHO cell lines. Moreover, the effects of three combined nucleoside addition were also confirmed in CHO cell line A culture. The three combined nucleoside culture showed a 27% higher peak VCC (26.4×10^6 cells/mL) than that in the single deoxyuridine addition culture (20.7×10^6 cells/mL) and 124% higher peak VCC than that in the control culture (11.8×10^6 cells/mL). The viability after day 8 was also higher than that in the single deoxyuridine addition culture. As a result of the IVCC increase, the final product concentration (5.9 g/L) was increased by 28% compared with that in the single deoxyuridine addition culture (4.6 g/L) and increased by 99% compared with that in the control culture (2.9 g/L) (data not shown). The significant increase in cell growth and product concentration were observed in CHO cell line A, CHO cell line B and CHO cell line C from independent experiments. Our nucleoside addition strategy is a simple and reproducible methodology that resulted in an increase in the final product concentration. Desirably, duplicated

Fig. 5 Effects of addition of three combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line C cultures. **a** Viable cell concentration. **b** Cell viability. **c** Fab fragment concentration. Control cultures were performed

without nucleoside addition. Each nucleoside was added with 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

Table 3 Process-relevant data from different CHO production systems for antibody production

Process type	Culture scale	Host cell/gene expression system	Culture media	Peak VCC ($\times 10^6$ cells/mL)	Final product concentration (g/L)	Reference
Fed-batch	100-L pilot	CHO DG44/DHFR	With hydrolysate	25.0–30.0	13.0 (day 18)	Huang et al. (2010)
Fed-batch	10-L bioreactor	CHO-K1SV/GS	CD	10.0–15.0	3.2 (day 15)	Porter et al. (2010)
Fed-batch	2-L bioreactor	DUK-XB11/DHFR	CD	8.0	7.5 (day 20)	Yu et al. (2011)
Perfusion	Not described	CHO DHFR/not described	With hydrolysate	127	3.0 (day 12)	Clincke et al. (2013)
Fed-batch	500-mL shake flask	CHO DG44/not described	CD	21.3	5.8 (day 16)	Reinhart et al. (2015)
Fed-batch	24-deep well plate	CHO-K1SV GS ⁻ /GS ^a	Not described	Not described	7.6 (day 16)	Rajendra et al. (2016)
Fed-batch	125-mL shake flask	CHO-K1SV/GS	CD (nucleoside addition)	23.5	9.2 (day 16)	This study

The final product concentration is shown with the process run time

DHFR *Dhfr*-gene amplification system, *GS* *GS* gene expression system from Lonza Biologics; *CD* chemically-defined medium

^a This study used the original engineered *GS* gene expression system derived from the *GS*-CHO system with the CHO K1SV *GS* knockout cell line

experiments and statistical analysis are preferable for quantitative evaluation of effect of nucleotide addition in future.

Table 3 summarizes the current high productive CHO cell culture processes in the literature. Our study showed a remarkably high product concentration compared with other previous studies. As our new strategy is based on a fed-batch culture system, it is easy to apply common cell culture processes. In particular, among CHO cell culture processes using

GS-CHO systems with *CD* culture media, our data showed a significantly high product concentration. Although the mechanisms of the enhancement are unclear, the nucleoside addition strategy will be a powerful platform for the efficient production of proteins. To elucidate the mechanism underlying the effects of nucleoside addition, further detailed analyses of metabolites will be necessary in future studies. In a previous report, the IVCC and peak VCC were identified as important factors for predicting final

product concentrations (Golabgir et al. 2016). Another report showed enhancement effects of thymidine and hypoxanthine addition on the mAb production process, but their performance was insufficient for current protein production processes (Chen et al. 2012b). Those authors demonstrated elevation of the final product concentration (from 0.18 mg/L to 0.63 mg/L) as a result of increasing the peak VCC from 3.80×10^6 to 6.45×10^6 cells/mL. They optimized additional compounds and concentrations in lower peak VCC cultures than the current process shown in Table 3. A metabolomics analysis showed that metabolism varied in CHO cultures with different growth characteristic (Dietmair et al. 2012). Therefore, it is important to investigate the effects of nucleoside addition in the current high VCC culture process. Recently, a continuous bioprocess based on perfusion technology has been dramatically developed for biopharmaceutical production. To attain this highly-effective continuous bioprocess, maintenance of a high cell concentration during perfusion cell culture is necessary. In the high cell concentration process, the requirements for optimal addition of nutrients and nucleosides might be increased. In the future, our new strategy will be scaled up to bioreactor use, and the quality of the produced proteins will be analyzed.

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