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Effects of nutrient levels and average culture pH on the glycosylation pattern of camelid-humanized monoclonal antibody

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ABSTRACT

The impact of operating conditions on the glycosylation pattern of humanized camelid monoclonal antibody, EG2-hFc produced by Chinese hamster ovary (CHO) cells has been evaluated by a combination of experiments and modeling. Cells were cultivated under different levels of glucose and glutamine concentrations with the goal of investigating the effect of nutrient depletion levels and ammonia build up on the cell growth and the glycoprofiles of the monoclonal antibody (Mab). The effect of average pH reduction on glycosylation level during the entire culture time or during a specific time span was also investigated. The relative abundance of glycan structures was quantified by hydrophilic interaction liquid chromatography (HILIC) and the galactosylation index (GI) and the sialylation index (SI) were determined. Lower initial concentrations of glutamine resulted in lower glucose consumption and lower cell yield but increased GI and SI levels when compared to cultures started with higher initial glutamine levels. Similarly, reducing the average pH of culture resulted in lower growth but higher SI and GI levels. These findings indicate that there is a tradeoff between cell growth, resulting Mab productivity and the achievement of desirable higher glycosylation levels. A dynamic model, based on a metabolic flux analysis (MFA), is proposed to describe the metabolism of nutrients, cell growth and Mab productivity. Finally, existing software (GLYCOVIS) that describes the glycosylation pathways was used to illustrate the impact of extracellular species on the glycoprofiles.

1. Introduction

The manufacturing of monoclonal antibodies is one of the most profitable operations in the pharmaceutical market comprising 7% of it in sales (Elvin et al., 2013).

The key therapeutic properties of Mabs are strongly related to the post-translational process of glycosylation. The glycan conformations are important because they can influence the Mabs' therapeutic properties including secretion, solubility, receptor recognition, antigenicity, bioactivity and pharmacokinetics (Hossler et al., 2009; Butler, 2006).

Mammalian cell culture systems, particularly Chinese Hamster Ovary (CHO) cells, are recognized as efficient hosts for the commercial production of therapeutic glycoproteins because beyond their robustness their protein processing machinery, closely simulates the glycosylation process in humans (Hauser and Wagner, 1997). The complex process of glycosylation that occurs in eukaryotic cells involves several enzymatic reactions whereby oligosaccharide chains are added to the polypeptide through a complex series of reactions in the endoplasmic reticulum (ER) and the Golgi

Abbreviations: [Ala], alanine concentration (mM); [AM], ammonia concentration (mM); [Asn], asparagine concentration (mM); [Asp], aspartic acid concentration (mM); fgr, growing fraction of viable cells; [Glc], glucose concentration (mM); [Gln], glutamine concentration (mM); Kij, model parameters; μ, specific growth rate (day^{-1}); TCA, Citric acid cycle; UDP-GNAc, uridine diphosphate *N*-acetylhexosamine; UDP–GalNAc, uridine diphosphate *N*-acetylgalactosamine; UDP–GlcNAc, uridine diphosphate *N*-acetylglucosamine; Xd, specific death rate (day^{-1}); Xv, total viable cell density; Man I, Mannosyl-oligosaccharide 1,2-a-mannosidase; Man II, Mannosyl-oligosaccharide 1,3-1,6-a-mannosidase; GnT I, a-1,3-mannosyl-glycoprotein, 2-b-Nacetylglucosaminyltransferase; GnT II, a-1,6-mannosyl-glycoprotein 2-b-*N*-acetylglucosaminyltransferase; FucT, Glycoprotein 6-a-Lfucosyltransferase; GalT, b-*N*-acetylglucosaminylglycopeptide b-1,4-galactosyltransferase; SiaT, b-Galactoside a-2,3/6-sialyltransferase.

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apparatus. In N-linked (asparagine-linked) glycosylation, the predominant type in animal cells, a high mannose oligosaccharide is transferred en bloc to asparagine residues from a lipid carrier molecule in the ER and they further processed and modified to add heterogeneity and complexity to the glycoforms. The degree of glycosylation depends on general cell-culture conditions including the mode of culture operation, i.e. batch, fed-batch or continuous, temperature, pH, dissolved oxygen, pCO_2 and shear stress or changes in the specific culture variables including glucose, glutamine, ammonia, lactate and amino acids levels (Butler, 2006; Hossler, 2012).

Among the culture conditions that could affect the final glycan patterns, the nutrient levels and the concentrations of byproducts such as ammonia have been identified as very significant (Restelli and Butler, 2002; Yang and Butler, 2000). To achieve higher cell growth and productivity, mammalian cells need to be cultivated at the higher level of essential nutrients, mainly glucose and glutamine. However, the increased level of initial nutrients results in accumulation of cell metabolism byproducts, in particular lactate and ammonia, which in return can result in lower cell densities and product titres (Nyberg et al., 1999; Taschwer et al., 2012; Yang and Butler, 2000).

Also, nutrient levels influence the final glycan profiles by changing the intracellular nucleotide sugar pools that are the precursors for oligosaccharide synthesis (Nyberg et al., 1999). For example, high levels of UDP–GlcNAc result in significant increase of antennarity and reduction of sialylation in baby hamster kidney (BHK) cells expressing IL-Mu6 glycoprotein (Valley et al., 1999) and for a CHO Epo-Fc fusion producer cell line (Taschwer et al., 2012).

High levels of ammonia also can result in lower glycosylation by perturbing the balance of the nucleotide sugar pool (Chen and Harcum, 2006; Gawlitzek et al., 1999). Different explanations have been proposed for this effect: (i) ammonia induces the synthesis of UDP-GlcNAc that competes with the transport of cytosine monophosphate (CMP)–sialic acid to the cell membrane thus inhibiting the sialylation process (Valley et al., 1999) and (ii) ammonia is correlated with the sialylation level through the resulting changes in internal pH in the Golgi apparatus which causes lower activity of glycosyltransferase enzymes (Andersen and Goochee, 1994; Yang and Butler, 2002).

However, the reports regarding the effects of ammonia and pH on glycosylation are sometimes contradictory and may be highly dependent on the cell line, cultivation process and glycoprotein type (Raju et al., 2000; Butler, 2006; Chen and Harcum, 2006). For example, changes in sialylation of recombinant human granulocyte colony-stimulating factor (Andersen and Goochee, 1994) have been reported for either low ammonia levels of 2 mM or for very high ammonia levels above 9 mM for other glycoproteins (Chen and Harcum, 2006; Yang and Butler, 2000; Borys et al., 1993).

In this study, a transfected CHO (DUXB) cell line expressing a chimeric human-llama heavy chain monoclonal antibody (cHCAb) gratefully received from Yves Durocher, NRC, Montreal (Zhang et al., 2009) a member of MabNet (National Science and Engineering Research Council monoclonal antibody network (MabNet)), was used. The EG2-hFc contains a single N-linked glycan at Asn-297 and its smaller size (~80 kD), longer serum half-life and distinctive tumor accumulation are comparable to that of human IgGs (~150 kD) (Bell et al., 2010).

Our ultimate goal is to produce a model to assess the effects of nutrients, by-products and culture pH on growth, Mab productivity and glycosylation progress and could be used for process optimization (Ohadi et al., 2014). Toward this objective the current study reports a combination of experiments and modeling results that were used to demonstrate and quantify these effects for the particular CHO cell line under study and to understand the particular tradeoffs between Mab productivity and glycosylation levels.

First, the effect of ammonia was studied by conducting batch experiments with different initial levels of glutamine (0, 2, 4 and 8 mM) that result in different ammonia levels rather than directly adding of ammonia chloride extra-cellularly as previously reported in the literatures (Yang and Butler, 2000, 2002). The current study revealed a clear tradeoff between cell growth of CHO-EG2 and the quality of EG2-hFc at appropriate levels of glutamine and resulting levels of ammonia.

Second, to directly evaluate the effects of ammonia on the potential disruption of internal pH of Golgi apparatus and activity of transferases, the average culture pH was lowered along the entire or partial duration of batch culture by lactic acid addition. It was hypothesized that lactic acid will be both effective for lowering the pH as well as serving as nutrient after glucose is depleted.

To quantify the glycosylation of the Mab, which can potentially affect its activity, the galactosylation index (GI) and sialylation index (SI) were defined and monitored during the course of culture under different operating conditions (Majid et al., 2007).

To corroborate and quantify effects of cellular conditions on the cell metabolism and glycosylation process the experimental data was used with two different mathematical models: (i) a dynamic model of extracellular metabolites formulated based on metabolic flux analysis that describe the metabolism of nutrients, growth and productivity and (ii) a static model describing the network of glycosylation reactions (Hossler et al., 2006) was used to identify the reaction pathways relating changes in particular glycans to induced nutrient levels and average pH conditions.

2. Material and methods

2.1. Cell line and culture propagation

The CHO cells were cultivated in BioGro-CHO, a serum-free medium supplemented by varying concentrations of glucose and glutamine (BioGro Technologies Inc, Winnipeg, Canada). 250 ml Polycarbonate shake-flasks were used for cell propagation. Cells were sub-cultured every two to three days with a seeding density of 0.25×10^6 cells ml^{-1} to maintain them within the exponential phase of growth. The incubation conditions were 37 °C and 5% CO_2 with a 120 rpm shaking speed (Thermo Scientific, MaxQ shaker).

2.2. Batch culture

Batch experiments were performed at two levels of glucose (25 and 45 mM) and four different levels of glutamine (0, 2, 4 and 8 mM) in 500 ml polycarbonate shaker flask with an initial cell density of 0.2×10^6 cell ml^{-1}. Samples were taken daily for immediate analysis of pH and viable cell concentration.

The environmental pH changes were implemented only in the culture with 25 mM glucose and 4 mM glutamine concentrations that were found to be optimal for cell growth. Attempts for altering the pH at lower initial glutamine concentration and lower pH than 6.8 ± 0.05 were not successful since the growth and Mab productivity were negligible for these conditions.

Two strategies were applied for manipulating the pH: (i) maintaining the average pH of culture at 6.8 ± 0.05 at the start of the batch operation to be referred hereafter as the reduced-pH strategy and (ii) shifting the average pH to 6.8 ± 0.05 at the time period of peak cell density to be referred hereafter as shifted-pH strategy.

Maintaining the average pH at 6.8 ± 0.05 for reduced-pH and shifted-pH cultures were achieved by daily additions of a predetermined 1 M lactic acid and HCL solutions calculated based on a preliminary calibration curve into each flask to achieve the required pH level of 6.8 ± 0.05. To provide complete CO_2 equilibrium, the medium was incubated in 5% CO_2 and 37 °C while shaking

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