

The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody

by

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## ABSTRACT

The glycosylation pattern of a chimeric heavy chain antibody (EG2) produced from CHO cells was affected by the glucose concentration (0-25 mM) of cultures established at high density ( $>10^6$  cells/ml) over 24 h. The resulting proportion of non-glycosylated Mab was directly correlated to the exposure time of cells to media depleted of glucose. Deprivation of glucose for the full 24 h resulted in a 52% non-glycosylated Mab fraction. Analysis of steady state levels of intracellular lipid-linked oligosaccharides (LLOs) showed that under glucose limitation there was a reduction in the amount of full length LLO ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ ), with a concomitant increase in the smaller mannosylglycans ( $\text{Man}_{2-5}\text{GlcNAc}_2$ ). Glycan microheterogeneity was quantified by galactosylation and sialylation indices (GI and SI), which showed a direct correlation to the cell specific glucose uptake. The high GI of the chimeric antibody (EG2) may be due to its low molecular weight and unusual structure. These findings are important in relation to the low substrate that may occur in fed-batch cultures for Mab production.

## ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
ALG	Acetylglucosaminyl phosphate transferase
BHK	Baby hamster ovary
CDR	Complementarity determining region
CH1, CH1, CH3	Constant regions of the heavy chain of immunoglobulin
cHCAb	Chimeric heavy chain antibody
CHO	Chinese hamster ovary
CL	Constant region of the light chain of immunoglobulin
Dol-PP	Dolichol-pyro-phosphate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno-sorbent assay
ER	Endoplasmic reticulum
Fab	Antibody binding fragment of immunoglobulin
FDA	Food and Drug Administration
Gal T	GalNAc transferase
GDP	Guanosine diphosphate
GI	Galactosylation index
Gluc I	$\alpha$ – 1,2 glucosidase I
Gluc II	$\alpha$ – 1,3 glucosidase II
HCAb	Heavy chain antibody
HILIC	Hydrophilic interaction liquid chromatography
IgG	Immunoglobulin G
LLO	Lipid-linked oligosaccharide
Mab	Monoclonal antibody
Man I	Mannosidase I
MALDI-TOF	Matrix-assisted laser desorption/ionization with time of flight
MW	Molecular weight
NeuAc	Sialic acid
OST	Oligosaccharyltransferase
Q <sub>glc</sub>	Specific glucose consumption
Q <sub>p</sub>	Protein specific productivity
SI	Sialylation index
UDP	Uridine diphosphate
VH	Variable region of the heavy chain of immunoglobulin
V <sub>H</sub> H	Variable region of the heavy chain of a single-domain antibodies
VL	Variable region of the light chain of immunoglobulin
<b>Amino Acids</b>	
Asn	Asparagine
Ser	Serine
Thr	Threonine

**Monosaccharides**

Fuc, F

Gal, G

Glc

GlcNAc

Man

NeuAc, S

Gal

Galactose

Glucose

N-acetylglucosamine

Mannose

Sialic acid (N-acetylneuraminic acid)

## CHAPTER 1 INTRODUCTION

### 1.1 Overview

The “magic bullet” postulation was first proposed by Paul Ehrlich (Fig. 1.1.1) at the beginning of 19th century. In his postulation, he described a compound which could be synthesized specifically to recognize a disease-causing organism. A toxin to that organism could then be delivered with the compound based on its selective properties. Due to the lack of biological and molecular information at that time, his postulation remained as a dream until the discovery of monoclonal antibodies (Mabs).



**Figure 1.1.1. Paul Ehrlich (1854-1915)<sup>1</sup>**

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<sup>1</sup> [http://en.wikipedia.org/wiki/File:Paul\\_Ehrlich\\_1915.jpg](http://en.wikipedia.org/wiki/File:Paul_Ehrlich_1915.jpg)



The first monoclonal antibody, Orthoclone, was approved by the Food and Drug Administration (FDA) for treatment of solid organ transplant rejections, 1986. There are currently more than 34 FDA-approved Mabs in the market for clinical use (Aggarwal, 2011). The use of Mabs in therapeutic applications has grown tremendously. Approaches for Mabs include oncology, asthma, anti-inflammatory disorders, poisoning, viral infections and other diseases (Berger et al., 2002). The two best-selling approaches for Mabs are used in the treatments of cancer and anti-inflammatory disorders. Anti-inflammatory Mabs now constitute almost half (46%) of all sales, and Mabs for oncological use are taken 41% of the market (Aggarwal, 2011). The global market for Mabs in 2011 was estimated at US\$44.6 billion, and the market sales are predicted to increase to US\$58 billion in 2016. As high doses of drugs and large number of patients are usually involved in each anticipated treatment, the total production demand is often multiple tons per year (Gasser and Mattanovich, 2006). The pressure for high yield recombinant Mabs production has focused on finding the perfect antibody expression system.

## **1.2 Antibody expression systems**

Mammalian cells are the most widely used expression system for industrial production of recombinant proteins, and among all mammalian cell lines, the Chinese hamster ovary (CHO) cell line is predominant. There are over 100 biopharmaceuticals produced by CHO cell lines approved by FDA. CHO cell line is well characterized, which has no clear adverse effects during continued usage over several decades. A published “draft” genomic sequence showed 2.45 Gbases and 24,383 predicted genes in the CHO-K1 ancestral cell line (Xu et al., 2011). In Xu’s study, a genome-scale analysis

identified homologs to share 99% of the human glycosylation-associated transcripts, with 53% of them expressed. The high coverage of homologs provides the CHO-K1 cell line the ability to perform human-like post-translational modification on a recombinant protein, and is also a unique opportunity for glycoform manipulation. The importance of glycoform on a therapeutic protein is significant and will be discussed in detail later. A final noteworthy advantage of CHO cells is the lack of viral entry genes, which results in strong viral resistance.

There are several other antibody expression systems. Transgenic animals or plants, insect cells, bacteria and yeast are currently under study as potential antibody expression system alternatives, due to one or more of several advantages, including a lower cost of goods, shorter process periods, higher productivity and lower contamination risk, as well as a lack of unidentified or unforeseen infectious agents. However, one vital drawback of these expression systems is the lack of proper post-translational modification, mainly glycosylation on the final product (Fig.1.2.1).



translational modification, mainly protein glycosylation, is known to be significant, therefore, studying and understanding the N-glycosylation mechanism is very important for many purposes, which are discussed later in this chapter.

### ***1.3.1 Early stage of N-glycosylation***

In eukaryotes, the early steps in the N-glycosylation process are highly conserved. The early steps involve the synthesis of the completed N-glycosylation precursor  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  on the Dolichol-pyro-phosphate (Dol-PP) carrier. Lipid-linked oligosaccharide (LLO) biosynthesis starts on the cytoplasmic side of the endoplasmic reticulum (ER) membrane, with the transfer of sugar residues from nucleotide-activated sugar donors, such as uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man) onto a dolichol phosphate carrier. In the initial step, GlcNAc-P donated by UDP-GlcNAc is transferred onto Dol-P, resulting in GlcNAc-PP-Dol by N-acetylglucosaminyl phosphate transferase (ALG) (Kelleher and Gilmore, 2006). This is followed by the addition of another GlcNAc and five Mannose residues using UDP-GlcNAc and GDP-Man, respectively. These enzymatic reactions result in generation of the heptasaccharide  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  (Fig. 1.3.1).

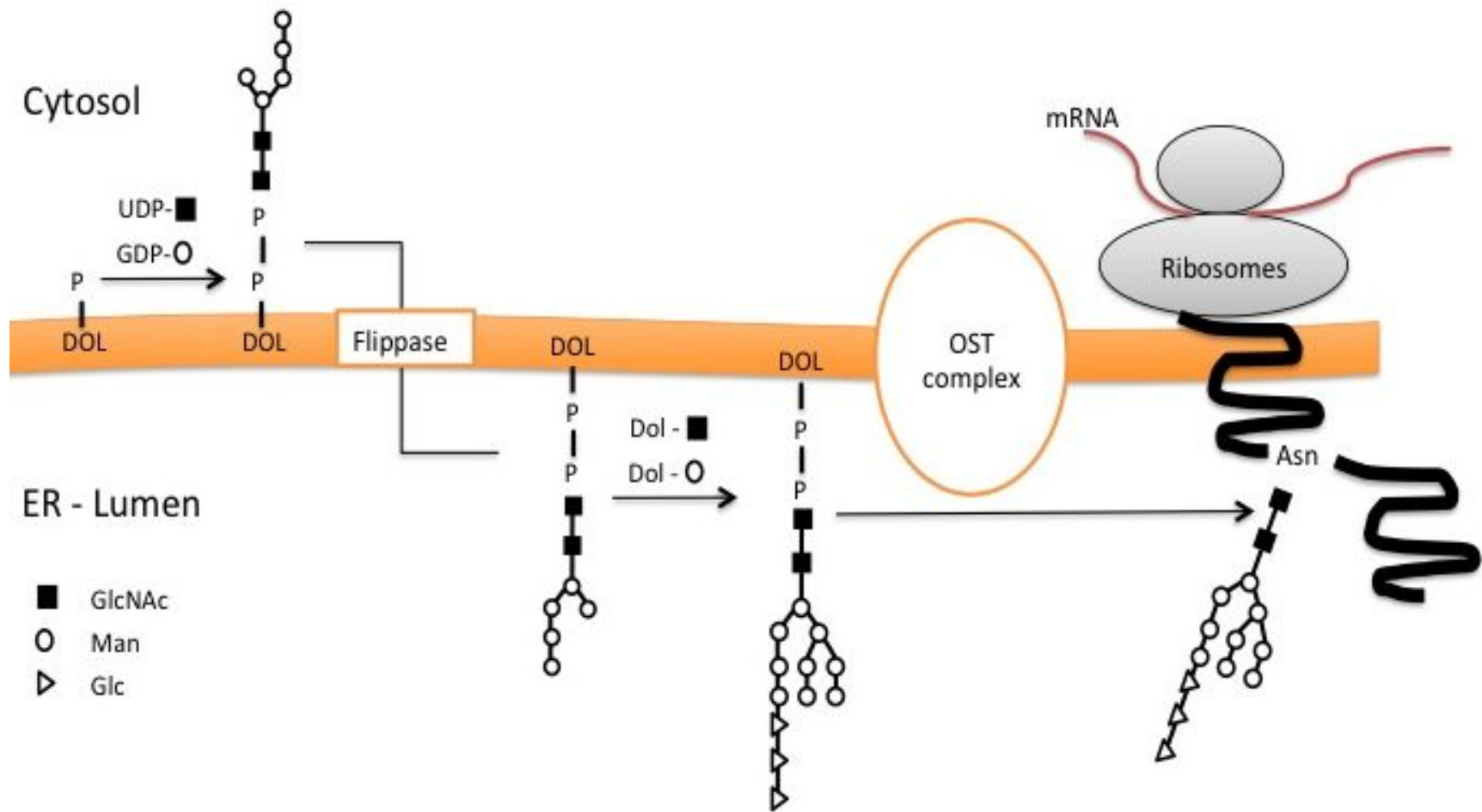


Figure 1.3.1. Early stage of N-glycosylation in the ER.

This heptasaccharide  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  is subsequently flipped to face the lumen of the ER. This translocation is facilitated by an enzyme called “flippase”, which was recently identified as Rft1 in *Saccharomyces cerevisiae* by Helenius et al (2002). The presence of dolichol has been shown to induce destabilization of phospholipid bilayers of the ER membrane (Abeijon and Hirschberg, 1992).

Once  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  is translocated to the luminal side of the ER. Four more Man residues and three glucose (Glc) residues are added onto the LLO chain. The synthesis of the completed N-glycosylation precursor,  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  from  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  occurs through sequential enzymatic reactions, utilizing Dol-P-Man and Dol-P-Glc, which are synthesized via reactions between dolichol phosphate and GDP-Man or UDP-Glc, respectively. These monosaccharide donors are also translocated across the ER membrane to the lumen with help from flippases (Weerapana and Imperiali, 2006).

### **1.3.2 Transfer of the dolichol-linked oligosaccharide to the nascent polypeptide**

The completed N-glycosylation precursor,  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  is transferred *en bloc* from the Dol-PP carrier onto a nascent polypeptide with the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Kornfeld and Kornfeld, 1985). This transfer is facilitated by oligosaccharyltransferase (OST), which binds to LLO and cleaves the phosphoglycosidic bond between the oligosaccharide and Dol-PP carrier. This is followed by the formation of an N-glycosidic linkage between  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  and the amino group of the asparagine (Asn) residue. The Dol-PP carrier is released and de-phosphorylated becomes Dol-P (Jenkins et al., 1996; Jones et

al., 2005; Varki et al., 2009), and then diffuses or is flipped back to the cytoplasmic side of the ER. The dolichol carrier is recycled and can be reused for additional rounds of early stage of N-glycosylation (Rush et al., 2008; Schenk et al., 2001).

### ***1.3.3 Early processing steps: from Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>Asn to Man<sub>8</sub>GlcNAc<sub>2</sub>Asn***

Ensuing the covalent attachment of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> onto Asn-X-Ser/Thr in a nascent polypeptide, a serial of enzymatic trimming takes place inside the ER (Fig.1.3.2). The process of trimming of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>Asn begins with sequential removal of three glucose residues by  $\alpha$ -1,2 glucosidase I (Gluc I) and  $\alpha$ -1,3 glucosidase II (Gluc II) in the ER, and one mannose residue by ER mannosidase I (Man I). These reactions intimately associate with glycoprotein folding. Then, the newly synthesized glycoprotein is transported to the Golgi cisternae by means of vesicles.

These sequential trimming steps that occur in the ER has been thought of as a quality control mechanism required for the formation of the tertiary structure of a protein (Ellgaard and Helenius, 2003). Glycoprotein folding is facilitated by the lectins, calnexin and calreticulin, and the efficiently glycan trimming only occurs if the protein is assumed an appropriate folding pattern and that determines whether the glycoprotein continues to the Golgi for further process or degradation. Oligosaccharide is processed in the Golgi apparatus and becomes a high mannose, hybrid or complex glycan structure.

### ***1.3.4 Late processing steps: from Man<sub>8</sub>GlcNAc<sub>2</sub>Asn to complex N-glycans in CHO cell line.***

Figure 1.3.2 illustrates the correctly folded protein being transferred through the compartments of the Golgi, where Man<sub>8</sub>GlcNAc<sub>2</sub>Asn moiety undergoes various enzymatic trimming and transferring. These modifications create a high mannose, hybrid

or complex glycan structure, depending on organism specificity. These reactions start with the removal of three more mannose residues to form  $\text{Man}_5\text{GlcNAc}_2\text{Asn}$  ( Kornfeld and Kornfeld, 1985).  $\text{Man}_5\text{GlcNAc}_2\text{Asn}$  is the substrate for enzyme N-acetylglucosaminyl transferase I (GnTI) to add the first GlcNAc (Schachter et al., 1983). This hybrid structure is critical during the last stage in N-glycosylation since it is the substrate for several glycosyltransferases and one mannosidase. If this hybrid structure is processed by glycosyltransferase, it will remain in its hybrid form; however, if the hybrid structure is subjected to mannosidase, it will lead to the formation of a complex-type glycan, instead. All N-linked oligosaccharides contain a core of  $\text{Man}_3\text{GlcNAc}_2\text{Asn}$ , and the composition of the outer branches determines whether the glycan is classified as high mannose, hybrid or complex (Fig. 1.3.3). If only mannose residues are found on the outer branches, glycan is referred to as a high mannose glycan; while complex glycan has GlcNAc and Gal residues. Hybrids have at least one complex branch and one high mannose branch. In mammalian cells, the most common type of glycan found is the complex-type glycans (Schachter et al., 1983). In the complex-type glycans, monosaccharides such as GlcNAc, galactose, fucose and sialic acid are sequentially added onto the core structure  $\text{Man}_3\text{GlcNAc}_2\text{Asn}$  through a series of glycotransferases present in the Golgi during the late glycan processing steps, as shown in figure 1.3.2.



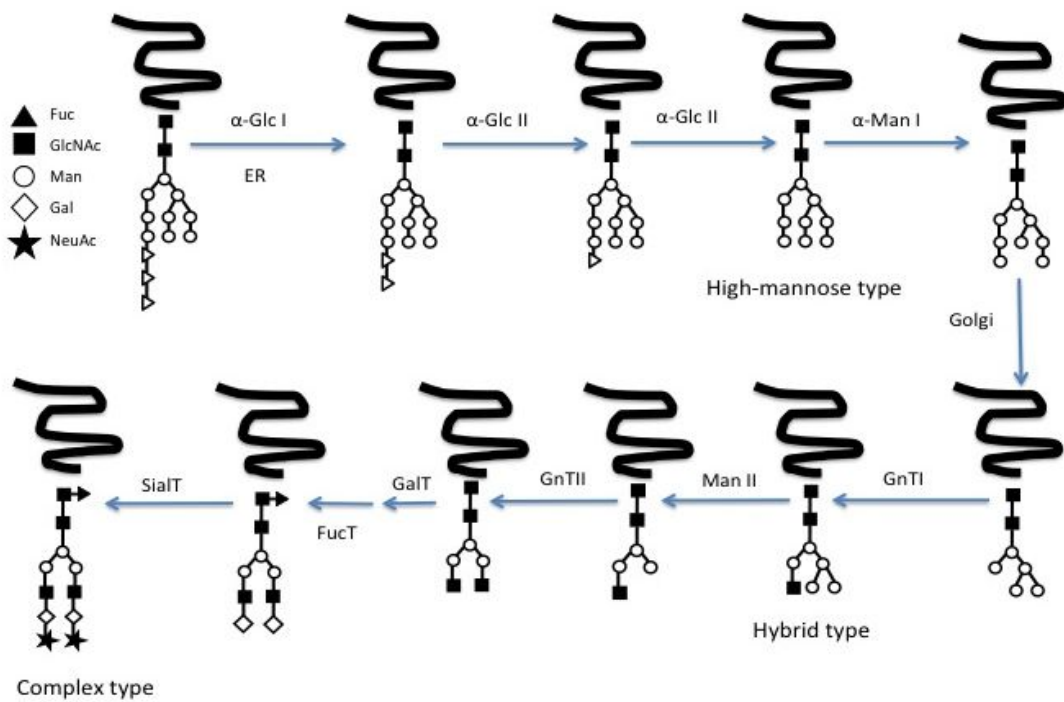


Figure 1.3.2. N-glycosylation processing stages pathway.

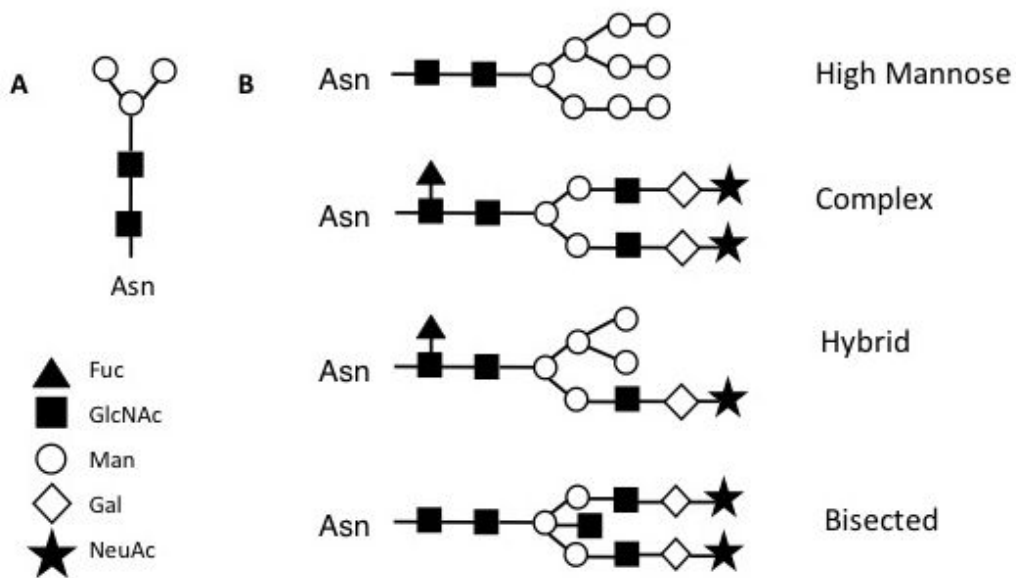


Figure 1.3.3. Glycan structures. (1) Core N-glycan structure and (B) Types of N-glycan structure commonly found on glycoproteins.

### *1.3.5 Heterogeneity in N-glycosylation pathway*

The enzymatic reactions during N-glycosylation do not always proceed to completion, and so give rise to heterogeneity in the N-glycosylation pathway. The first potential source of heterogeneity is during translocation of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> from the dolichol carrier to the nascent polypeptide. This type of heterogeneity is referred to as site occupancy heterogeneity or macroheterogeneity. Another type of heterogeneity arises in the late N-glycosylation processing steps in the Golgi (microheterogeneity), which results in variable antennarity, terminal sialylation, galactosylation, fucosylation of the innermost core, GlcNAc or the outer arm GlcNAc to the central core Man residues.

Heterogeneity in N-glycosylation is considered as a characteristic of a certain cell type or cell line. These effects depend on several cellular factors: (1) enzyme repertoire and localization (Ferrara et al., 2006; Meynial-Salles and Combes, 1996); (2) competition between different enzymes for one substrate (Umaña and Bailey, 1997); (3) transit time of the glycoproteins through the Golgi apparatus (Hooker et al., 1999; Nabi and Dennis, 1998); (4) levels of nucleotide sugar donors (Nyberg et al., 1999; Valley et al., 1999); and last but not the least (5) competition between different glycosylation sites on the protein for the same pool of enzymes (Schachter et al., 1983).

In humans, the glycan structure on a glycoprotein is typically complex sialylated bi-antennary type oligosaccharides (Rich and Withers, 2009). On the other hand, in lower eukaryotes such as fungi and yeast, high mannose structures are the most commonly seen on the end products. In some cases, hybrid structures have been found (Rich and Withers, 2009). Plants synthesize oligosaccharides that are similar to the human type, but which have a  $\alpha$ -1, 3 linked (as opposed to an  $\alpha$ -1, 6 linked) fucose attached to the proximal

GlcNAc and a xylose residue linked to the core mannose which can cause immunogenic effects in human, as have the exposed terminal mannose residues found on yeast glycoproteins (Rich and Withers, 2009). N-glycans of recombinant glycoproteins expressed in insect cells mainly contain high mannose or tri-mannose structure, which are truncated forms of the glycans found in humans, and impact pharmacokinetics as therapeutics (Chang et al., 2003). In mammalian cells, these high mannose intermediates are usually found in the early steps of the N-glycosylation pathway in the ER, and processed prior to translocation to the Golgi.

#### **1.4 Overview of O-linked Glycosylation**

O-glycosylation is another post-translational modification of mammalian glycoproteins. The most common form of O-glycan is the mucin-type, which involves the addition of GlcNAc to a Ser/Thr of the fully folded protein by a GalNAc transferase (Gal T) from UDP-GalNAc (Van den Steen et al., 1998). Further elongation and termination relies on a series of glycosyltransferases that leads to a large number of structures. No consensus sequence has been identified, and it has been found that O-glycosylation depends on the primary, secondary, and tertiary structure of the glycoprotein, so that this event mostly happens due to the accessibility of a protein to GalNAc transferase, which is responsible for the process initiation (Van den Steen et al., 1998).

#### **1.5 The importance of glycosylation to therapeutic antibodies**

Glycan exerts a profound influence on both physical and chemical properties of an antibody, although the carbohydrate moiety of an antibody accounts for only 2 to 3 percent of its total mass. The most obvious function of glycans to a protein is to facilitate

protein solubility and stability. De-N-glycosylated fibrinogen and de-O-glycosylated human granulocyte colony-stimulating factor form aggregates resulting in a loss of biological activities (Weisel et al., 1988; Oh-eda et al., 1990). In addition, during therapeutic treatment, glycosylation protects proteins from proteolytic degradation by forming hydrogen bonds between the hydrophilic amino acids and the glycans, resulting in a steric protection of the peptide moieties (Fiten et al., 1994).

In therapeutic glycoproteins, not only the presence of the N-glycan, but the precise structure of the attached N-glycans, is critical for highly efficient biological activities, also. Enhanced sialylation is found to prolong the circulation of recombinant erythropoietin in serum, which is a considerable clinical advantage (Kiss et al., 2010). Additionally, the presence of sialic acid on glycan moiety can also attenuate the cytotoxic capability of an antibody, but does not interfere with its anti-inflammatory response (Kaneko et al., 2006). Increasing in terminal galactosylation and decreasing in fucosylation in the Fc region of IgG resulted in an elevation in antibody-dependent cell-mediated cytotoxicity, which is the most common mode of action of Mabs *in vitro* and *in vivo* (Niwa et al., 2005; Werner et al., 2007). The expression of N-acetylglucosaminyltransferase III, an enzyme, which transfers 1,4-GlcNAc to a core, mannose (Man) residue, results in increasing potency of the ADCC, and suggests the need for lower therapeutic doses *in vivo* (Shinkawa et al., 2002). Possible reason for specific glycoforms of monoclonal antibodies affect ADCC activity is because that it can interact solely with the Fc—RIIIa receptor of natural killer cells, so that a lack of or deformed glycan structure in immunoglobulin G (IgG) can result in decreased Fc-mediated ADCC activities, or even induce an immunogenic response, etc. (Kanda et al.,

2006; Nimmerjahn et al., 2007; Shields et al., 2002). Altogether, the production of a bio-therapeutic monoclonal antibody needs to be focused not only on the Mab itself, but an appropriate N-glycan structure attachment is also required to maximize therapeutic potential.

### **1.6 Culture parameters that may affect glycosylation**

Structural heterogeneity of N-glycans attached to glycoproteins may be host cell specific and protein specific; however, during mammalian cell culture production, numerous parameters may have impacts as well. It is very important to control culture parameters in order to achieve a consistency of glycosylation of a recombinant protein in a culture bioprocess, especially for those used as therapeutic drugs. Culture conditions such as accumulation of ammonia, dissolved oxygen level, nutrient contents, pH level and temperature, may have great effects on the distribution of glycan structures (microheterogeneity and macroheterogeneity) found on the final recombinant protein products. Those factors may cause one or more mechanisms in the following: (1) the depletion of the cellular energy state (Rearick et al., 1981; Valley et al., 1999); (2) disruption of the local ER and Golgi environment, such as pH (Andersen and Goochee, 1995) or redox potential (Restelli et al., 2006); (3) alteration of glycosyltransferase activities (Chen and Harcum, 2006; Chotigeat et al., 1994); and (4) changes in the N-glycosylation precursor pool, such as nucleotides, nucleotide sugars (Kochanowski et al., 2008; Nyberg et al., 1999) and lipid precursors (Stoll and Krag, 1988).

This of course raises our attention for producing consistent biopharmaceuticals. Knowing the importance of each parameter during cell culture production is extremely helpful to ensure the consistency of glycosylation between batch runs.

### **1.6.1. Glucose**

Glucose is the primary carbon source added into mammalian cell culture, and is mainly metabolized through the glycolytic pathway, producing lactate as a by-product (Tsao et al., 2005). Lactate has a detrimental effect on cell growth and productivity. During industrial production, substrate (glucose, glucosamine) limitation is commonly used to prevent accumulation of a large quantity by-product (lactate, ammonium ion, respectively). Glucose starvation is known to cause an intracellular depleted state or a shortage of glucose-derived precursors for glycosylation (Rearick et al., 1981). A study found that in a glucose-limited chemostat cultures, although glucose concentration could be restored to normal level by pulsed addition rapidly, periodic intervals of glucose depletion increased heterogeneity in N-glycosylation site occupancy on gamma-interferon (Hayter et al., 1992). Reduced N-glycan occupancy was also shown in immunoglobulin synthesis from mouse myeloma cells at low glucose concentration (<0.5 mM) (Stark and Heath, 1979). In addition, abnormal glycosylation was found on viral proteins derived from CHO cells grown in a glucose-limited culture (Davidson and Hunt, 1985).

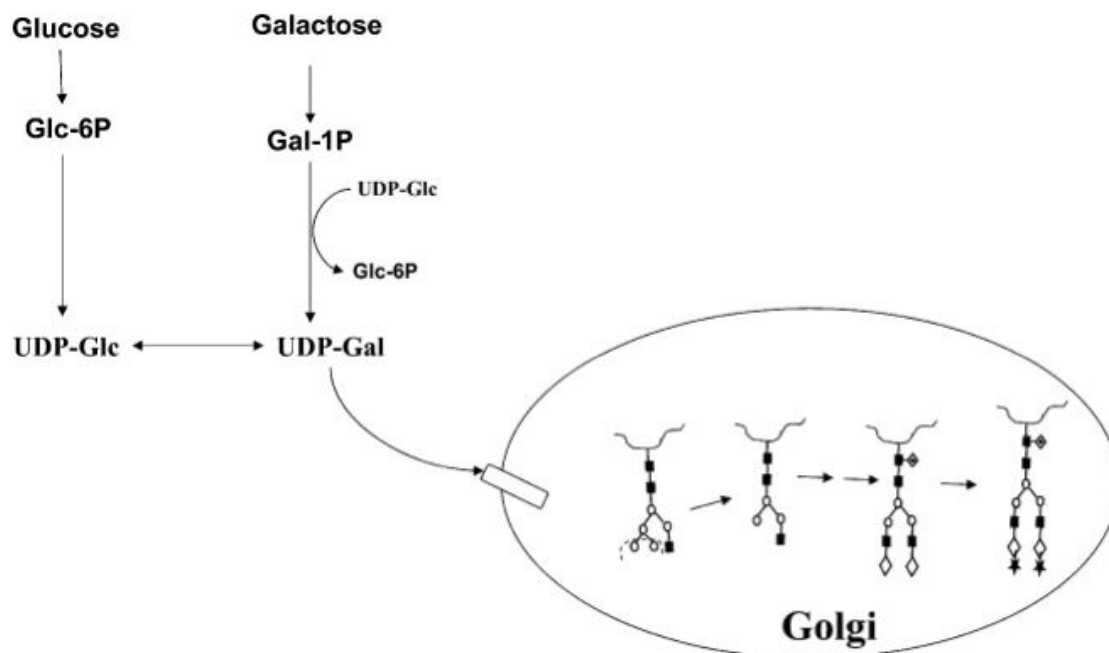
### **1.6.2. Glucosamine**

Glucosamine is a precursor for UDP-GlcNAc. UDP-GlcNAc is an important intracellular nucleotide sugar in late process of N-glycosylation pathway. Study found that adding glucosamine into the culture medium could elevate the intracellular UDP-GlcNAc pool, especially added in conjunction with uridine (Baker et al., 2001). Sufficient UDP-GlcNAc pool resulted in enhancement of antennarity of glycan structures produced in baby hamster kidney (BHK) cells, probably through a stimulation of the

specific GlcNAc TIV and TV (Gawlitze et al., 1998; Grammatikos et al., 1998; Valley et al., 1999). However, this phenomenon is not universal for all cell lines, such as NS0 cells, which have been reported to have no positive feedback for glucosamine addition (Baker et al., 2001). It is noteworthy that since UDP-GlcNAc competes with CMP-NeuAc for transport into the Golgi, elevated UDP-GlcNAc appears to cause a decline in sialylation (Rijcken et al., 1995).

### **1.6.3. Galactose**

Improvement of galactosylation is one of the most important goals during mammalian cell culture production. A galactose feeding strategy has been proposed and practiced to ensure a high galactosylation on recombinant proteins. Galactose feeding was shown to increase the UDP-galactose pool up to 20 x compared to the control levels, and corresponded to a concentration of 7 fmol/10<sup>5</sup> viable cells with a concentration of 36mM galactose addition (Butler, 2005). However, a different study suggested that galactose-feeding may potentially decrease protein sialylation (Clark et al., 2005). In 2011, Gramer's group proposed a feeding regime of uridine, manganese and galactose, which significantly promoted galactosylation and sialylation in CHO cells (Gramer et al., 2011). This finding was later verified in CHO-EG2 cells by Butler's group (Liu et al., 2014). The metabolic pathway in which UDP-Gal as a N-glycosylation precursor is transported into the Golgi by a specific transporter prior to addition to the N-glycan on the protein is shown in figure 1.6.1 (Butler, 2005).



**Figure 1.6.1.** Pathway of galactosylation from galactose feeding (Butler, 2005).

#### 1.6.4. Ammonia

Glutamine, as an energy source for cells as well as an essential precursor for nucleotide synthesis, is often added to a culture medium at a concentration of 2-10 mM. Ammonia ( $\text{NH}_3$ ) or ammonium ion accumulates in a culture as a by-product of glutamine. The accumulation of  $\text{NH}_3$  or ammonium is raised from either thermal decomposition of the glutamine or from deamination or metabolic deamination. Accumulated  $\text{NH}_3$  provides two major changes to the intracellular environment, resulting in heterogeneity in N-glycosylation during protein production: (1) pH elevation in Golgi, which can decrease the activity of selected enzymes, such as sialyltransferase (Valley et al., 1999); (2) an increase in the UDP-GlcNAc pool, which competes with CMP-NeuAc for the transporter, resulting in a sialylation decrease (Andersen and Goochee, 1994).



#### **1.6.5. pH**

The optimal pH for maximum glycan occupancy has been determined to be between 6.9 and 8.2 (Borys et al., 1993). Adverse external pH condition can result in changes of internal pH of Golgi. Acidic pH of Golgi lumen is known to be critical for the correct glycosylation, transport and sorting of proteins during their transit, through its highly organized structure and subcompartmentation (Paroutis et al., 2004). A study showed that the pH of the medium had an impact on the distribution of the glycoforms of a murine hybridoma secreted IgG (Rothman et al., 1989). Mislocalization of certain Golgi glycosyltransferases has been suggested to be the primary reason for the pH-induced glycosylation defects (Rivinoja et al., 2009).

#### **1.6.6. Dissolved oxygen concentration**

Oxygen is a limited nutrient during the cell culture due to its low solubility in the medium. The control of the dissolved oxygen (DO) level is one of the key parameters to ensure optimal cell metabolism and growth, as well as N-glycosylation patterns on a protein. DO concentration during production is set between ranges of 1-100% air saturation. Studies found that low DO concentration resulted in a decrease in galactosylation (Kunkel et al., 1998). The mechanism causing this effect remains unclear. One possible explanation is that reduced DO leads to reduced synthesis or reduced efficiency of UDP-Gal transport, resulting in a decline in availability of UDP-Gal (Butler, 2005). A second explanation is based on the redox environment of the ER or the Golgi. Low DO may impede disulfide bond formation on the inter-heavy chain, which is known to be critical for the extension of galactosylation (Rademacher et al., 1996).

#### **1.6.7. Specific cell growth rate and protein productivity**

Glycoprotein synthesis is based on cooperation between two mechanisms, protein translation and N-glycosylation. The addition of N-glycan onto the sequon by OST is time sensitive. The heterogeneity in glycan site occupancy is affected by the rate of the polypeptide elongation step. It has been shown that glycosylation site occupancy in C127 cells increased 60% due to the addition of cycloheximide, which is highly specific in its inhibition of protein elongation and provision of extra time for glycosylation synthesis (Shelikoff et al., 1994). This effect was also found in the production of human tissue-type plasminogen activator. A decline in growth rate induced by the supplementation of butyrate resulted in an enhanced glycosylation site occupancy (Andersen et al., 2000). This suggests a mechanism by which glycosylation efficiency improves by an escalated exposure time of glycan site to the OST in the ER.

Meanwhile, an extended residence time of proteins in the Golgi can also promote the elongation of N-glycan. By incubating a cell at 21 °C, which previously shown to cause the accumulation of glycoproteins at the trans-Golgi, Wang's group successfully increased N-acetyllactosamine by 100%, compared to the controls by slowing down the flow of glycoprotein through Golgi (Wang et al., 1991). This suggests that increasing the retention time of glycoprotein inside the Golgi helps improve N-glycosylation due to the extended exposure time to glycosyltransferase enzymes.

#### **1.7 Choice of Monoclonal Antibody**

IgG, as the most common class developed for monoclonal antibody production, is widely used in the treatment of diseases such as autoimmune diseases, transplant rejections, cancer and many other illnesses in various stages of clinical trials. IgG is

comprised of four polypeptides: two heavy chains and two light chains. For each heavy and light chain, there are constant and variable regions. The amino-terminal domains of light and heavy chains are constant in sequence and termed CL and CH; whereas, the domains with the variable sequence of each chain are termed VL and VH. There are three domains in each antibody, including two identical antigen-binding (Fab) moieties that recognize and bind a foreign antigen specifically on target cells. Two identical CH regions are important for recruitment of effector cells to activate clearance mechanisms, such as antibody dependent cell-mediated cytotoxicity (ADCC) and the complement cascade (Fig. 1.7.1) (Strome et al., 2007).

IgG has one N-linked glycan structure at Asn297 on the Fc region within the CH2 domain. The structure of oligosaccharide normally consists of a “core” heptasacchride with variable additions of outer arm sugar residues. The complex sugar of galactose may be added sequentially to the GlcNAc resulting in a structure with no galactose (G0), one galactose (G1) or two galactose (G2) (Fig. 1.7.2). Additional microheterogeneity occurs with the addition of bisecting GlcNAc ( $\beta$ 1-4 to the core Man), fucose ( $\alpha$ 1-6 to the primary GlcNAc) or terminal sialic acids (NeuAc or Neu5Gc to Gal). High mannose glycans are often observed (Arnold et al., 2007). As well, glycosylation in the Fab region has been seen at a lower frequency (Roy Jefferis, 2007; Royston Jefferis, 2005).

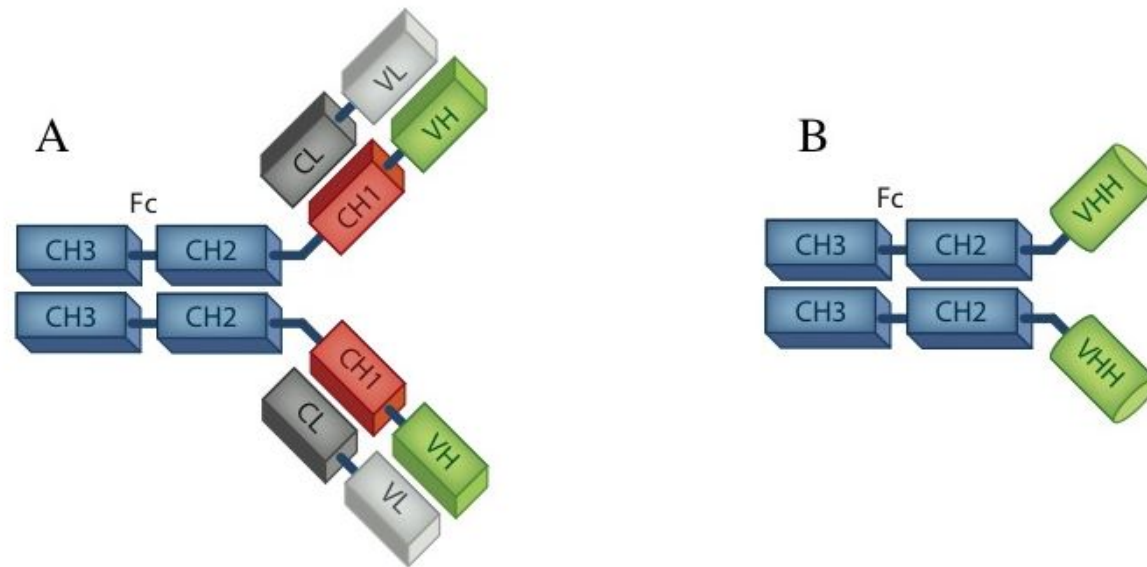
IgGs have become successful in biopharmaceutical sales due to their high specificity and low toxicity. However, due to its large size, an IgG often appears to have undesirable pharmacokinetics and poor tissue penetration which prevent it from more effective therapeutics (Jain et al., 2007). Efforts have been made to optimize the

molecular size of the antibodies, using biological modifiers to modulate these impediments.

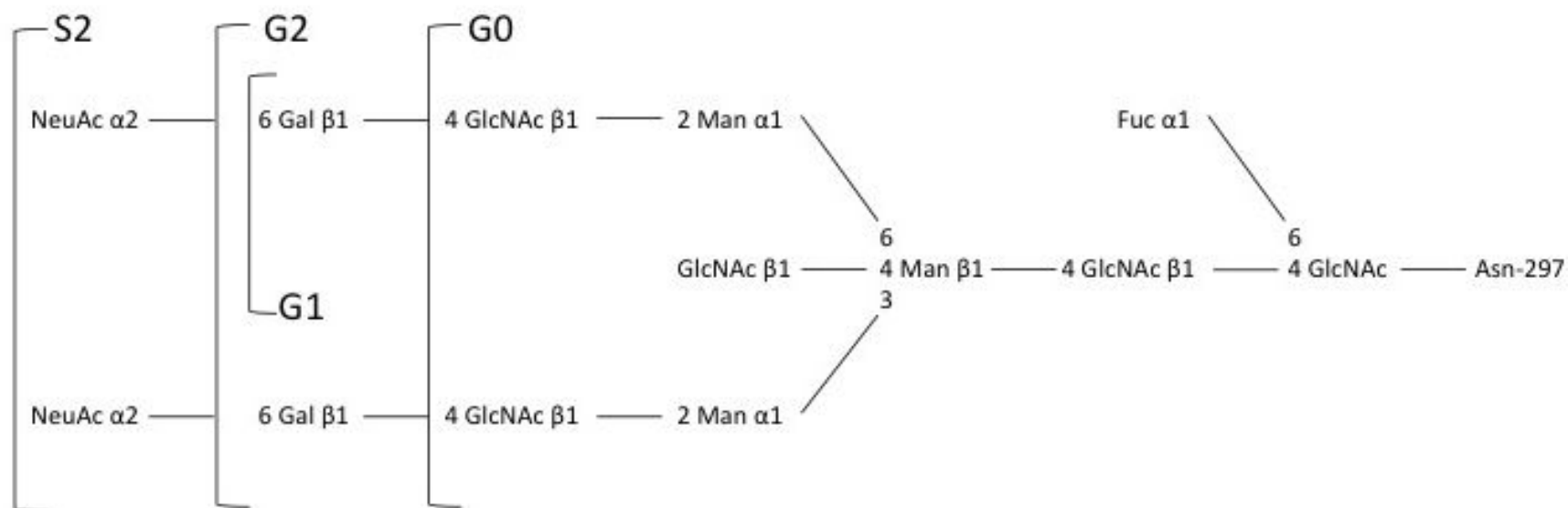
The antibody used in this thesis, the EG2 antibody, is a modified IgG, which is a fusion of the complementarity determining region (CDR) of a single domain antibody (sdAb) against the human epidermal growth factor receptor (EGFR) ectodomain with an intact human Fc region, resulting in a ~80kDa chimeric heavy chain antibody (cHCAb) that was shown to penetrate a solid tumor in a xenograft mouse model.

The reason to refer EG2-hFc as chimeric is that it consists of a human Fc and llama sdAb. The absences of IgG light chains and CH1 in the heavy chain are the main characteristics of camelid HCABs (Fig. 1.7.1). Their smaller size with an approximate molecular weight of 80,000, instead of conventional IgGs at 150,000, results in improved therapeutic activity, such as (1) better tissue penetration; (2) greater ability to access a hidden target not readily accessible to IgGs because of its size (Muyldermans, 2013); (3) a higher level of expression due to its simpler structure; and (4) lower dose required due to its lower MW (Zhang et al., 2009). Meanwhile, the antigen-specific, single variable domain derived from camelid HCABs, comprises other features. Full antigen-binding capacity is maintained for months when they are stored at 4 °C or at -20 °C. Good thermal stability allows several weeks incubation at 37 °C without compromising its activity (Ghahroudi et al., 1997). Moreover, they can tolerate stringent conditions and resist chemical and thermal degradation (Dumoulin et al., 2002). In addition, the presence of the intact human Fc domain, cHCAb is expected to inherit major advantages of conventional IgGs, which enables strong neutralization, effector function for ADCC and CDC activities, as well as a long serum half-life (Zhang et al., 2009). The stability of

cHCAb is essential for tumor targeting *in vivo*, and the EG2 presents better therapeutic potential and a valuable model for this project.



**Figure 1.7.1.** Schematic representation of conventional antibody IgG (A); and chimeric heavy chain antibody (cHCAb) EG2 (B). IgG contains two light (L) chains (VL and CL) and two heavy chains (includes VH, CH1, hinge, and CH2 and CH3 domains). CH3 and CH2 domains form the Fc portion, which is in charge of effector function. EG2 is generated from conventional antibodies, comprised of an intact human Fc region with a single-domain; antigen-specific V<sub>H</sub>H derived from a llama HCAb and contains only heavy chains (includes antigen-binding, single domain the V<sub>H</sub>H, hinge, and CH2 and CH3 domains).



**Figure 1.7.2.** Schematic representation of the full N-glycan structure attached to Fc region of an antibody. S2 indicates full sialylation of N-glycan. G2 represents full galactosylation of N-glycan. G1 represents N-glycan with one galactose attached. G0 represents the “core” heptoligosaccharide of glycoform.

## 1.8 Objectives of the present research

The demand for a high yield recombinant protein production has focused on feed strategies that minimize the concentrations of media components, such as glucose. Although this may prolong cell culture and maximize protein productivity, it is important to ensure that the production quality, such as glycosylation, is maintained.

The first step in the glycosylation pathway involves the synthesis of lipid-linked oligosaccharides (LLOs) via the addition of sugars using nucleotide sugar donors. Glycan macroheterogeneity is introduced when a completed N-glycosylation precursor is cleaved off the dolichol carrier and transferred to a nascent polypeptide. Further modification of the oligosaccharide can occur through processing reactions in the Golgi, where some sugars are removed and additional sugars added, utilizing nucleotide sugar donors. This series of reactions produces microheterogeneity of the final glycan pool. Both macroheterogeneity and microheterogeneity may be affected by the availability of precursors.

Glucose provides energy for cellular metabolism as well as precursors for the synthesis of intracellular nucleotide-sugars, which are essential substrates in N-glycosylation pathway. Another importance of glucose concentration to the recombinant protein production has been well recognized for its effect on glycosylation patterns. It is known that limited glucose levels during protein production can result in the accumulation of truncated N-glycosylation precursors, with only 15 min turn-over time (Rearick et al., 1981). Low glucose concentration has been proven to cause low N-glycosylation site-occupancy in IFN- $\gamma$  (Hayter et al., 1992; Nyberg et al., 1999). However, there is a lack of understanding of the effect of glucose-limited conditions in



relation to the entire N-glycosylation pathway, as well as its potential impairment of the N-glycosylation machinery. This connection has significant implications on the strategic design of a commercial bioprocess for the production of therapeutic antibodies in which the substrate levels are maintained at a low concentration to maximize cell productivity. In particular, during the fed-batch cultures, periodic intervals of glucose depletion may occur, it is very important to find the critical concentration of glucose to balance the protein productivity as well as the quality of N-glycosylation.

For this reason, in the present study, variable concentrations of glucose 0-25 mM were set up to investigate the effects of varying periods of glucose starvation on the profile of intracellular LLO and the final glycan pool of a chimeric Mab produced by CHO-EG2 cells.

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 Cell line

A stably transfected CHO (DUXB II) cell line expressing a chimeric human-llama heavy chain monoclonal antibody (cHCAb) against epidermal growth factor receptor (EGFR) was gratefully received from Yves Durocher, NRC, Montreal (Zhang et al., 2009). The antibody (designated EG2) is a fusion of a camelid single domain antibody to a humanized Fc fragment and has a molecular weight ~80 kDa. Three amino acid mutations are present in the Fc fragment of this antibody namely, D270G, Y278H and A432G as compared to Cetuximab as a standard.

### 2.2 Cell culture

#### 2.2.1 Materials

- Biogro-CHO cell media (25 mM glucose)
- Biogro-CHO cell basal media (0 mM glucose)
- Individual wrapped sterile serological pipettes
- Falcon tubes (Fisher Scientific: Catalog #09-761-34, Supplier #430320)
- 250 mL sterile shaker flasks

#### 2.2.2 Reagents

- Trypan blue stain 0.4% (Gibco by Life Technologies, REF:1520-061)
- Dulbecco's phosphate-buffered saline (PBS)

#### 2.2.3 Procedure

Batch cultures were established in 250 mL shake flasks with a working volume of 80 mL Biogro-CHO media (Biogro Technologies Inc, Winnipeg, Canada) with 0.5 g/L yeast extract (BD, Sparks, MD) containing 25 mM glucose and with an inoculation of  $5 \times 10^5$  cells/mL. The shake flasks were held in a humidified incubator at 120 rpm, 10% CO<sub>2</sub> and 37 °C for 3 days.

On day 3, cell density and viability were tested using trypan blue exclusion assay with Cedex.  $2.1 \times 10^8$  cells were removed from each cell culture and subjected to 24 h glucose test. Firstly, calculated volume of culture contained  $2.1 \times 10^8$  cells was spun at 1500 rpm for 5 min to be able to remove the supernatant. The cell pellet was re-suspend in 10 mL fresh Biogro-CHO media with respective glucose concentration (0-25 mM) and transferred into new 250 mL shake flask and media with respective glucose concentration was added until 80 mL reached. Each culture condition was run in pairs.

### 2.3 Cell culture sampling

Total cell counts and cell viability were measured at regular intervals by Trypan dye exclusion (0.5%) using Cedex Image Analyzer (Innovates AG, Bielefeld, Germany). The percentage of non-stained cells represents the viable cell population.

$$\text{Cell viability} = (\text{number of non stained cells} / \text{total cell count}) \times 100\%$$

Glucose and lactate concentrations were determined enzymatically using a YSI 2700 MBS Bioanalytical System (YSI Life Sciences, USA).

## 2.4 Antibody determination by ELISA

ELISA followed SOP (MNSOP0009) of quantitatively measure the production of CHO-EG2 hFc antibody from cell culture, generated by Natalie Okun.

Briefly, the EG2 antibody concentration was quantified by enzyme linked immunosorbent assay (ELISA). Goat produced anti human IgG (Fc specific) antibodies (Sigma-Aldrich, St. Louis, USA, I21136) were used for coating ELISA plates (Invitrogen, Carlsbad, USA) in phosphate buffered saline (PBS) (Invitrogen, Carlsbad, USA) and blocked with PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, USA) and 0.04% (w/v) Tween 20 (Sigma-Aldrich, St. Louis, USA, P1379). Supernatants of the culture samples were diluted with dilution buffer containing PBS, 0.5% (w/v) BSA and 0.04% Tween 20 and loaded onto the plates. An anti-human IgG (Fc specific) peroxidase-conjugated antibody produced in goat (Sigma-Aldrich, St. Louis, USA, A0170) was used for detection of EG2 antibody. The detection reagent consisted of 3,3',5,5'-tetramethylbenzidine dihydrochloride tablet (Sigma-Aldrich, St. Louis, USA, T3405) and 4 µl of 30% hydrogen peroxide in 20 mL phosphate-citrate buffer (Sigma-Aldrich, St. Louis, USA, P4809). Sulphuric acid (2 M) was used as a stop solution. The titer was measured using an ELISA reader (Biotek, Winooski, USA) EL808 at 450 nm and Magellan software according to the instruction manual. The colorimetric measurements were converted to concentrations using a standard antibody that was quantified by a Bradford assay and confirmed by a Lowry protein assay and ultraviolet absorption (A280).

## 2.5 Lipid-linked oligosaccharide (LLO) analysis

### 2.5.1 Required Reagent

- Dulbecco's phosphate-buffered saline (PBS)
- Methanol (EMD: Catalog #MX0475-1)
- Chloroform (Sigma-Aldrich: Catalog #67-66-3)
- MilliQ water
- CM (2:1 chloroform: methanol)
- CMW (10:10:3 chloroform: methanol: water)
- CMW + 3 mM Ammonium Acetate (NH<sub>4</sub>OAc) (Sigma-Aldrich: Catalog #631-61-8)
- CMW + 300 mM Ammonium Acetate (NH<sub>4</sub>OAc)
- DEAE cellulose (Sigma) converted to acetate form
- 0.1 N HCl in 50% isopropanol (Fisher: Catalog #67-63-0)
- 1:1 mixture of butanol (Sigma-Aldrich: Catalog #308250-6X1L) and water.
- Acetonitrile (Fisher Scientific: Catalog #A998-4)
- Glacial acetic acid (Fisher Scientific: Catalog #35127-212)
- 2-Aminobenzamide (2-AB or Anthranilamide) (Sigma: Catalog #A89804)
- Dowex 50WX8-200 cation exchanger resin (Sigma-Aldrich: Catalog #69011-20-7) hydrogen form, store at 4°C
- AG1-X8 anion exchanger resin (Bio-rad: Catalog #140-1454), formate form, store at 4 °C.
- 30% Acetic acid solution (30% acetic acid, 70%water (v/v).

- 96% Acetonitrile solution (96% Acetonitrile, 4% water (v/v)).

## **2.5.2 Procedure**

### **2.5.2.1 Quenching**

The first step of LLO extraction was cell quenching to stop any metabolic activities inside the cells. Well-mixed 50mL cell culture was divided and quickly poured into 2 x 50 mL falcon tubes (Fisher Scientific: Catalog #09-761-34, Supplier #430320) containing 25 mL, -20 °C quenching solution. Each tube was inverted a few time and centrifuged @ 2000 x g for 1 min (Eppendorf, Hamburg, Germany) (Series: 5810R). Quenching/supernatant solution was quickly and completely removed and the cell pellet was subjected to flash freeze with liquid N<sub>2</sub>. Frozen cell pellet was re-suspended in 2 mL ice-cold methanol and sonicated 2 x 30 seconds bursts on ice at level 8 with narrow probe sonicator (Qsonica, LLC, Newtown, USA) (Series: XL-2000) at the top of the liquid. Combined two 2 mL cell pellet/methanol from the same culture condition and store at -70 °C if not process immediately.

### **2.5.2.2 Extraction of the LLO**

Cell with methanol was dried under N<sub>2</sub> gas until samples were slightly damp with no liquid visible. Added 3 mL of CM to the tube and briefly sonicated in a water bath sonicator (Branson, Danbury, USA) (Series: 8200) and then cell pellet was dried under N<sub>2</sub> gas. This step was repeated twice, followed by addition of 10 mL of CM into each tube and water sonicated for at least 10 min. The cell mixture was centrifuged at 2000 x g

for 10 min and the supernatant was removed. This fraction was containing LLOs with less than 3 sugars (MPD, GPD, GlcNAc<sub>1-2</sub>P-P-Dol). This step was repeated once with the pellet.

The pellet was re-suspended in 2 mL methanol followed by brief sonication in a water bath sonicator and then dried under N<sub>2</sub> gas.

The next washing step was using water. Firstly, 10 mL water was added into each tube and sonicated for 10 min using water bath sonicator. Then the tubes were centrifuged at 2000 x g for 10 min and supernatant was discard. This fraction contained free sugars, phosphosugars, nucleotide sugars and free oligosaccharides. This washing step was repeated once.

The pellet was re-suspended in 2 mL methanol followed by brief sonication in a water bath sonicator and dry under N<sub>2</sub> gas.

10 mL CMW was added into each tube and sonicated for 10 min using water bath sonicator. Mixture was mixed well during 5 min incubation. Then samples were centrifuged at 2000 x g for 10 min and the supernatants were saved. This CMW extract contained the LLOs with more than 3 sugars (Glc<sub>0-3</sub>Man<sub>1-9</sub>GlcNAc<sub>2</sub>-PP-Dol). This extraction step was repeated four times. And saved supernatants from respective sample were combined in a 50 mL tube.

#### 2.5.2.3 Purification of the LLO

Supernatants were purified using DEAE-cellulose column. Columns were prepared by adding 1.0 ~ 1.2 mL (bed volume) of resin to empty Poly-prep columns and equilibrating with 20 mL of CMW. Supernatants were loaded onto the column (5 x 2

mL). Discarded flowthrough. Then washed the column using 5 x 2 mL, followed by 4 x 5 mL CMW (30 mL total). The flowthrough was discarded. The next column wash was using 5 x 2 mL then 4 x 5 mL with CMW + 3 mM ammonium acetate (30 mL total). Discarded flowthrough.

The columns were transferred onto desired the containers (50 mL tube), and LLOs eluted with 5 x 2 mL CMW + 300 mM ammonium acetate. Then LLO extract was dried under N<sub>2</sub> gas flow.

Desalting was using butanol /water mixture. Vortexed samples were centrifuged at 1000 x g for 5 min. Lower phase was discarded and the upper phase was dried under N<sub>2</sub> gas flow. Glycans were cleaved off from dolichol carrier by 2 mL of 0.1 N HCl in 50% isopropanol and incubated in 55 °C water bath for 1 hour. Samples later were dried under N<sub>2</sub> gas flow. And samples were desalted again using butanol/water mixture. Then samples were centrifuged at 1000 x g for 5 min and removed the lower phase into 1.5 mL Eppendorf tube (Fisher Scientific: Catalog #05-408-129, Supplier #05408129). This fraction contained glycans Glc<sub>0-3</sub>Man<sub>1-9</sub>GlcNAc<sub>2</sub>.

Last desalting step was using 200 ul of AG50W-X8 cation exchange resin. Sample mixed with resin for 5 min, then centrifuged (Baxter, Deerfield, USA) (Series: Biofuge B), and solution was filtered by pushing through a 0.45 µm syringe-driven filter (Fisher Scientific: Catalog #SLCR013NL) with a 1mL syringe (Fisher Scientific: Catalog #B309659), into a new 1.5 mL eppendorf tube. This step was repeated with 200 µL of AG1-X8 (formate form) anion exchange resin. Elution were dried completely in SpeedVac (Thermo Fisher Scientific Inc., Waltham, USA).



#### 2.5.2.4. 2-AB labeling and Cleaning-up

2-AB labeling and cleaning-up was followed pre-established protocol. Briefly, 5  $\mu$ L of 2-AB reagent was used to label each glycan samples. Samples with 2-AB were incubated at 65 °C (Fisher Scientific: Isotemp 125D) for 2 h and cooled to room temperature.

GlycoClean S cartridges (PROzyme: Catalog #GKI-4726) were prepared by washing through with 1mL Milli Q water, 5 mL acetic acid solution, 3 mL acetonitrile and an additional 1 mL acetonitrile, respectively. Samples were reconstituted with 100  $\mu$ L acetonitrile and spotted onto a freshly washed cartridge membranes for 15 min. Then each cartridge was washed with 1ml of acetonitrile, followed by 5 x 1 ml of 96% acetonitrile solution, allowing each aliquot to drain before the next was applied. Flowthrough was discarded into a suitable waste container. Next, each cartridge was placed onto a new 1.5 mL eppendorf tube, and glycans were eluted with three washes of 0.5 mL water. Then samples were dried in Speedvac or stored at -20 °C if not needed immediately.

#### 2.5.2.5. HPLC analysis

Hydrophilic interaction liquid chromatography (HILIC) analysis section in the SOP (MNSOP0005) generated by Natalie Okun was followed.

Briefly, HPLC instrument consisted of Waters 2475 binary pumps and a column heater, a Waters 717 autosampler, and a Waters 1425 fluorescent detector (set at excitation wavelength of 330 nm and an emission wavelength of 420 nm). The pumps and detector were controlled by Waters Empower software. The glycans were separated

on a Waters X-Bridge 3.5 um amide column (4.6 X 250 mm) using 50 mM ammonium formate buffer (Buffer A) and acetonitrile (Buffer B). A linear gradient was run from 20% to 50% buffer A over 48 min (flow rate 0.86 mL/min) followed by a linear gradient to 100% A over 3 min (0.86 mL/min). The column temperature was controlled at 30 °C. The elution was calibrated to glucose units (GU) values using a 2-AB labeled glucose ladder (glucose homopolymer) and then assigned glucose unit values by fitting a fifth order polynomial distribution curve (developed by a PHD student in our lab) to allocate GU values from retention times (Figure. 2.5.3.5.1) 2-AB-labeled glycan were separated on HILIC-HPLC. The structures were assigned using standard GU values from the GlycoBase database (NIBRT.ie) (Campbell et al., 2008).

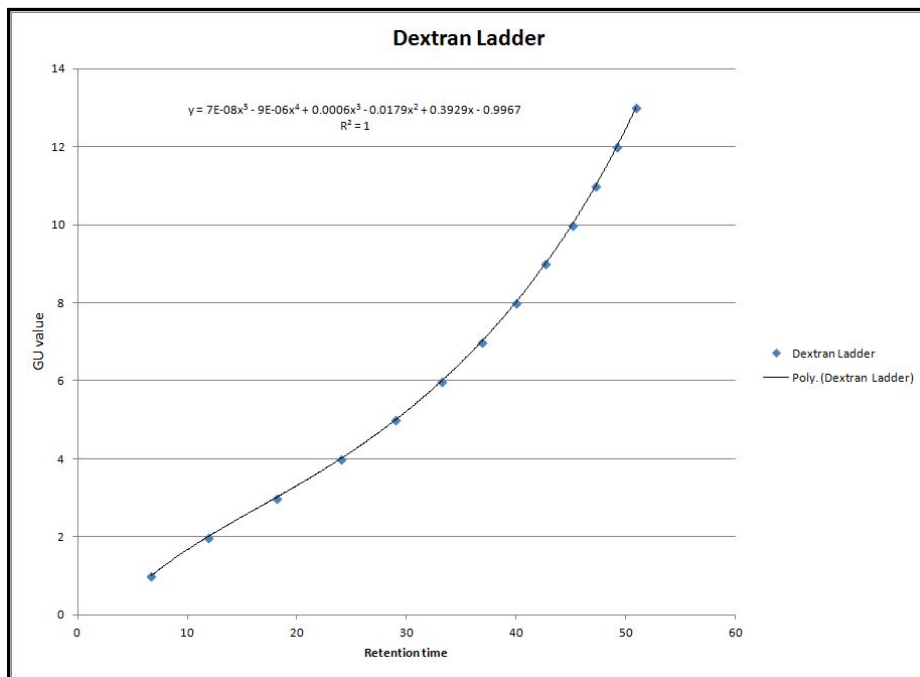


Figure 2.5.2.2.1. Example of the standard curve generated by the data of a dextran ladder. The trendline is a best-fit curve from a fifth order polynomial which is allocated GU values from retention times (x).

## 2.6 Macroheterogeneity determination

### 2.6.1 Required Reagent

- 5 x Running Buffer (2 L)

Tris base (Fisher Science: Catalog #BP152-1) 30 g

Glycine (Sigma G7403) 144 g

SDS (Bio-Rad Catalog # 161-0301) 10 g

Add MilliQ water up to 2 L

pH should be 8.3 without pH adjustment

- 10% (w/v) SDS (100 mL)

SDS (Bio-Rad Catalog #161-0301) 10 g

Add MilliQ water up to 100 mL

- 0.5M Tris-HCl, pH 6.8 (100 mL)

Tris base (Fisher Scientific: Catalog #BP152-1) 6.06 g

Milli Q water 80 mL

Adjust pH to 6.8 with concentrated HCl

Add Milli Q water up to 100 mL

- Laemli Sample Buffer (8 mL)

Bromophenol blue (Fisher Scientific: Catalog #B392) 0.01 g

0.5 M Tris-HCl, pH 6.8 1 mL

Glycerol (Fisher Scientific: Catalog #G33-4)	1.5 mL
--	--------

10% (w/v) SDS	1.6 mL
---------------	--------

Milli Q water	4 mL
---------------	------

For reduced sample buffer beta-mercaptoethanol must be added (details in procedure).

- 2- Mercaptoethanol ( $\beta$ Me) (Sigma M3148-100 mL)
- **N-Glycosidase F**, 250 units (Roche Ref #11 365 193 001), dissolved in 250  $\mu$ L of milliQ water

- Coomassie Brilliant Blue Stain (500 mL)

Brilliant Blue Coomassie R-250 (Sigma B0149-25G)	1.25 g
--	--------

Methanol (EMD: Catalog # MX0475-1)	250 mL
------------------------------------	--------

Concentrated acetic acid (Fisher Scientific: Catalog #35127-212)	37.5 mL
--	---------

Milli Q water	212.5 mL
---------------	----------

- Destain #1 (2 L)

Methanol (EMD: Catalog # MX0475-1)	1 L
------------------------------------	-----

Concentrated acetic acid (Fisher Scientific: Catalog #35127-212)	140 mL
--	--------

Milli Q water	860 mL
---------------	--------

- Destain #2 (2 L)

- |  |         |
|--|---------|
| Methanol (EMD: Catalog # MX0475-1)                               | 100 mL  |
| Concentrated acetic acid (Fisher Scientific: Catalog #35127-212) | 140 mL  |
| Milli Q water  | 1760 mL |
- Wash buffer (20 mM NaHCO<sub>3</sub> pH 7.0) (1 L)
 

Sodium Bicarbonate (NaHCO <sub>3</sub> pH 7.0) (Sigma S4019-1 KG)	1.68 g
Milli Q water	800 mL

Adjust to pH 7.0

Add Milli Q water up to 1 L

### 2.6.2 Required Materials

- Criterion™ TGX™ Precast Gels (8-16%) (Bio-Rad: Catalog #567-1104) or smaller prep gels
- Bio-Rad Criterion gel box (or mini gel box)
- Precision Plus Protein™ Dual Color Standards, 500 µL (Bio-Rad: Catalog #161-0374)
- Power supply for running gels
- Containers for staining/destaining gels
- Shaker for staining/destaining gels
- Sponge to soak up dye
- Gel imager
- White background working area (paper towel, white plastic, etc)

- 1.5 mL microcentrifuge tubes (Fisher Scientific: Catalog # 05-408-129, Supplier # 05408129)
- 1000  $\mu$ L pipette tips (Fisher Scientific: Catalog # 14222694, Supplier # T1000CL)
- 200  $\mu$ L pipette tips (Fisher Scientific: Catalog # 14222812, Supplier # TR222CL)
- 10  $\mu$ L pipette tips (Fisher Scientific: Catalog # 21-277-2A, Supplier # 104FIS)
- Pipetmans –1000  $\mu$ L; 200  $\mu$ L; 20  $\mu$ L; 10  $\mu$ L
- Vortex
- Rotary speedvac vacuum centrifuge
- Millipore™ syringe-driven filter unit (Fisher Scientific: Catalog #SLCR013NL)
- 37 °C incubator
- 65 °C – 95 °C Heating block

### 2.6.3 Procedure

Antibodies purification was followed SOP (MNSOP0004) of protein A antibody purification generated by Natalie Okun. 5  $\mu$ L of N-Glycosidase F was added to 40  $\mu$ L of protein A purified sample from 25 mM glucose culture, vortexed it and quickly moved samples to 37 °C incubator for 10, 20 or 30 min incubation. 4  $\mu$ L of  $\beta$ Me (in fume hood) was added to 80  $\mu$ L sample buffer to make a reduced sample buffer. 45  $\mu$ L of reduced sample buffer was used to stop enzymatic activity of N-Glycosidase F and sample was heated at 95 °C for 5 min. 5  $\mu$ L sample buffer was added to 5  $\mu$ L of N-Glycosidase F and heated at 95 °C for 5 min as control. Then, corresponding amount (display in the table below, volumes needed for 14-well gels were adjusted based on results from Nanodrop) of protein A purified sample was added to equal amount of reduced sample buffer and

heated at 95°C for 5 min. And all samples were loaded onto 8-16% SDS-PAGE gels, then gels were stained with Coomassie Brilliant Blue (CBB) and subjected to densitometry.

Initial glucose concentration (mM)	Approximate sample volume* (μL)	Loading volume (With reduced sample buffer) (μL)
0	20	40
5	10	20
10	7	14
12.5	5	10
15	5	10
17.5	5	10
25	5	10
Sample prior to glucose test	5	10
PNGase digested	5	10
N-Glycosidase F	5	10

## 2.7 Microheterogeneity determination

Microheterogeneity analysis is followed SOP (MNSOP0004) of protein A antibody purification and SOP (MNSOP0005) of N-glycan analysis generated by Natalie Okun.

Briefly, Mabs were purified from culture supernatants with a Protein A affinity column (HP SpinTrap from GE Healthcare) and run under denaturing conditions on a 8-16% SDS-PAGE gels (Biorad, Hercules, CA) and stained with Coomassie Brilliant Blue (CBB). The protein bands were removed from the gel by scalpel, washed, and treated with Peptide-N-Glycosidase F (PNGase F; Roche Diagnostics) for 18 h to remove the attached glycans and the isolated N-linked glycans were labeled with 2-AB (Royle et al., 2006). The structures of the N-linked glycans attached to the purified antibodies were determined as described above using standard GU values from HILIC analysis in GlycoBase. Glycan structures were confirmed using by exoglycosidase enzymatic

digestion arrays using sialidase from recombinant *A. ureafaciens*; bovine kidney fucosidase; bovine testis  $\beta$  (1-3,4)-galactosidase and jack bean  $\beta$ -N-acetylhexosaminidase (Prozyme, Hayward, CA) according to method of Royle et al. (2006).

## 2.8 MALDI-MS

Protein bands were recovered from SDS-PAGE gel and sent to Chemistry department for MALDI-MS analysis.

## 2.9 Calculations

- Cell metabolic coefficient = (Lactate)/(original [glucose] – [lactate])
- Cell specific glucose uptake ( $Q_s$ ) was calculated from the growth data using the following equation:

$$Q_s = \frac{\Delta S}{\int_0^t Nv \cdot dt}$$

Where  $\Delta S$  = change in substrate concentration between time 0 and t

$\int_0^t Nv \cdot dt$  = integral of viable cell number between time 0 and t

- The degree of galactosylation and sialylation of glycans was quantified by calculation of an index:

$$GI = \frac{G2 + 0.5 * G1}{G0 + G1 + G2}$$

Where the galactosylation index (GI) is a value from 0 to 1 calculated from the proportional areas of peaks of non-galactosylated (G0), mono-galactosylated (G1) and di-galactosylated (G2) structures.



- Similarly, the sialylation index (SI) was calculated from the proportional areas of all non-sialylated (S0), mono-sialylated (S1) and di-sialylated (S2) structures.

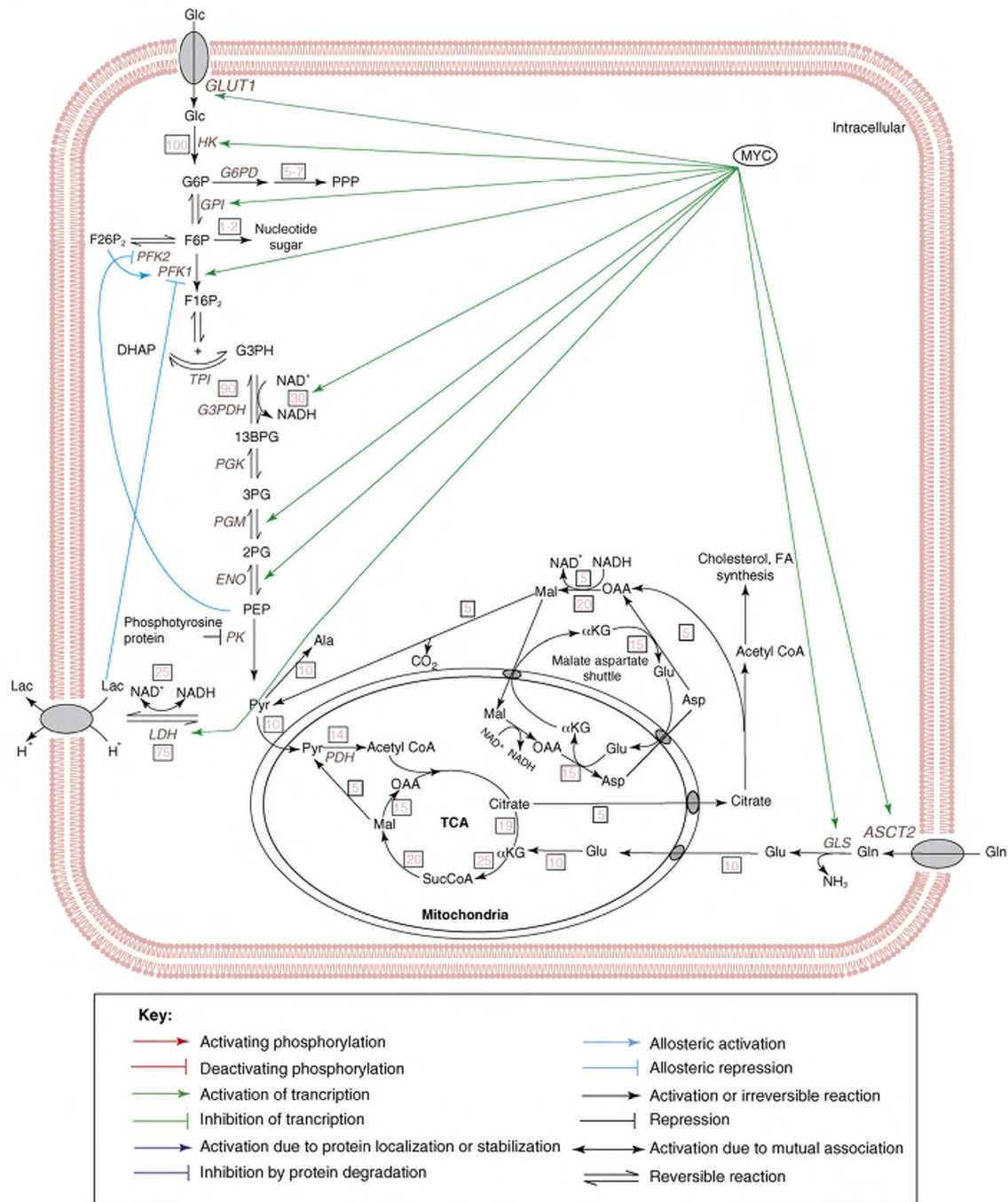
$$SI = \frac{S2 + 0.5 * S1}{S0 + S1 + S2}$$

## CHAPTER 3 CELL GROWTH, METABOLITES AND PROTEIN PRODUCTIVITY

### 3.1 Introduction:

Glucose is the most commonly used carbon source in mammalian cell culture and is mainly metabolized through the glycolytic pathway (Fig. 3.1.1), producing a lactate by-product. Under normal conditions, mammalian cells display an inefficient metabolic phenotype, characterized by high rates of glucose-to-lactate conversion, in which nearly 90% of glucose is converted to lactate during aerobic glycolysis (Mulukutla et al., 2010). In industrial cell culture, lactate can accumulate in the medium to a level of  $> 50$  mM, which causes a reduction of culture cell density and production titer. Reducing lactate production has shown to increase peak cell density, extend process duration, and promote protein productivity (Ma et al., 2009).

Many attempts have been made to regulate glucose metabolism in mammalian cell culture, which direct their glucose consumption towards oxidative respiration. Cellular engineering attempts to manipulate glucose metabolism through (1) constraining glycolytic fluxes through reaction networking; (2) regulation of metabolic enzymes; and (3) regulation of metabolism by signaling proteins and growth control elements (Mulukutla et al., 2010). However, high oxidative metabolism associates with slow cell growth or no growth, which is commonly observed at the late stage of cell culture (Mulukutla et al., 2010).



TRENDS in Biotechnology

**Figure 3.1.1.** Glucose flux map and regulation of metabolic enzymes. Numbers in red indicates the moles of carbon flowing through each reaction for every 100 mol C consumed by the cells as glucose (Mulukutla et al., 2010).

Another approach involves employment of a nutrient-limited fed-batch culture to reach a more efficient metabolic phenotype. Feeding strategies have been reported in the literature to reduce lactate production. Limited glucose supply is deliberately induced to moderately high cell density in order to prolong the productive stationary phase (Cruz et al., 1999; Zhou et al., 1995). However, this is complicated by the fact that nutrient deprivation has an adverse effect on cell growth, productivity and may even sabotage product quality.

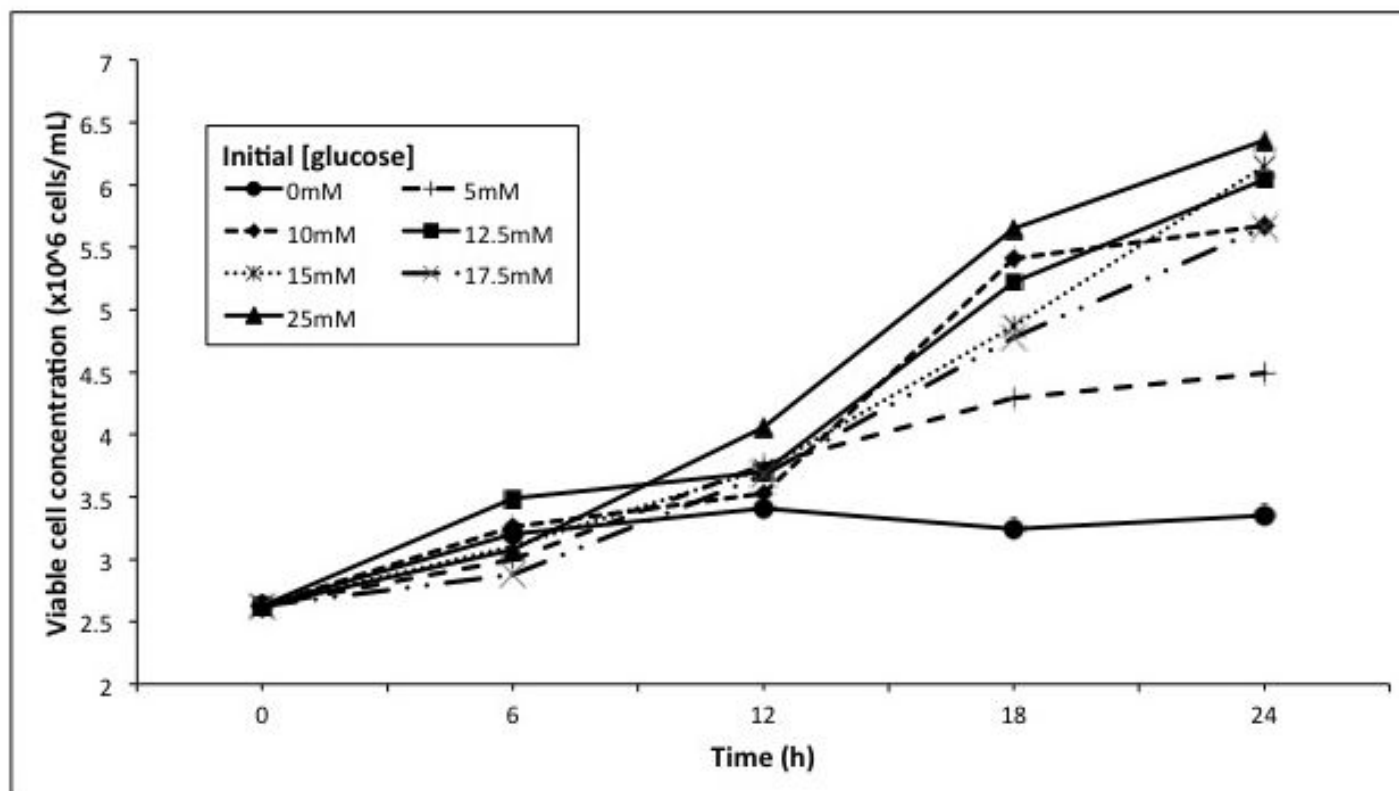
This thesis is mainly investigating glucose deprivation and its effects on the N-glycosylation pathway in CHO-EG2 cells. This chapter will introduce the experimental set-up, initial glucose concentration determination, and illustrate how a designated glucose deprivation state can affect CHO-EG2 cell growth and productivity.

### **3.2 Experimental set-up and initial glucose concentration determination**

Due to the fact that a minimum of  $1 \times 10^8$  cells are required for LLO extraction and a relatively large quantity of cell culture is needed for N-glycosylation analysis,  $2.1 \times 10^8$  cells were originally transferred into the media supplement with (0-25 mM) glucose concentrations. The initial glucose concentrations selected were 0 mM (negative control), 5 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM and 25 mM (positive control).  $2.1 \times 10^8$  cells were incubated under standard culture condition as described in Chapter 2 for 24 h. During incubation time, cell density, viability, and glucose and lactate concentrations were closely monitored. As seen in figure 3.2.1, cell concentrations increased under all glucose conditions. Viable cell density was at least double the amount of the inoculation density, and maintained a high viability (~98%). At 5 mM glucose, the viable cell

concentration increased by 54% with a final viability of ~98%. Cell density in 0 mM glucose culture increased by 21%, but the cell viability dropped to ~90%. Figure 3.2.2 shows the trend of glucose concentration during 24 h assay. The glucose concentration decreased over the incubation period. Complete glucose consumption occurred at a different time point in each culture with an initial glucose concentration equal to or below 15 mM. In figure 3.2.2, cell culture with  $\leq 15$  mM glucose were exposed to glucose deprivation for time periods between 2 and 24 h. For cultures supplemented with 17.5 mM glucose, the substrate was not depleted during 24 h incubation. The initial cell specific glucose consumption ( $Q_{glc}$ ) was related to the glucose concentration, and ranged from a maximum of 8.1 pmol/cell/day for the culture at 25 mM glucose to 4.5 pmol/cell/day for the culture at 5 mM glucose. These initial  $Q_{glc}$  values decreased as glucose was consumed over the 24 h incubation. The average glucose consumption for each culture condition over this 24 h incubation is summarized in table 3.2.1.

Lactate accumulation increased during the 24 h incubation in all cultures with glucose supplementation (Fig. 3.2.3). At 15 mM glucose, the metabolic coefficient (Lactate/glucose) reached the highest value (1.62). Cells with  $\leq 12.5$  mM glucose, utilized lactate when glucose depleted. In 0 mM glucose culture, the overall lactate production was negligible and might be by-product of glucose residual from pre-culture.



**Figure 3.2.1.** Viable cell concentrations of CHO-EG2 cell grown in Biogro CHO media supplemented with various initial glucose concentrations for 24 h. For each of 3 experiments cells were taken from late stage exponential phase and inoculated at  $2.6 \times 10^6$  cells/mL into duplicate shake flasks (250 mL) containing 80 mL of media. The initial glucose concentrations varied from 0-25 mM as indicated.

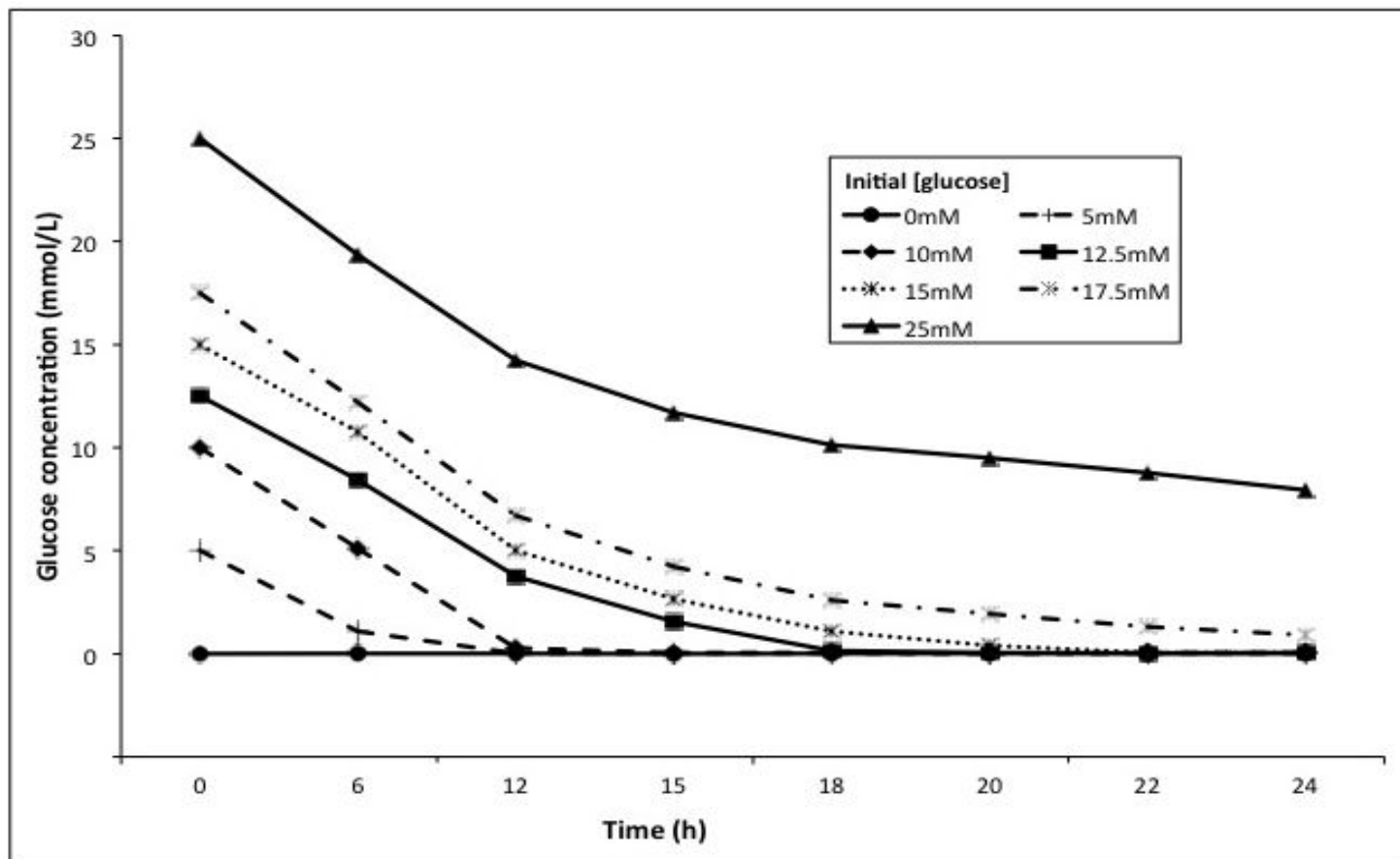
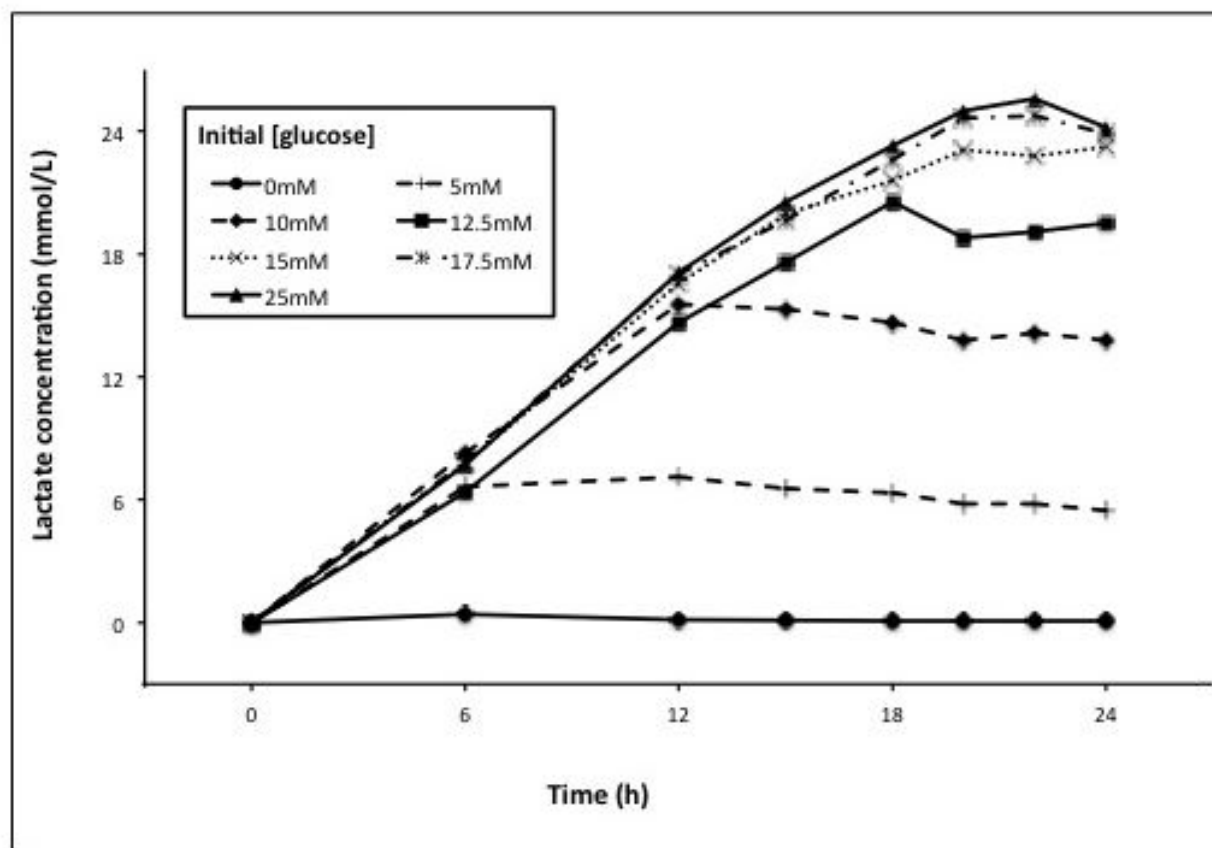


Figure 3.2.2. Analysis of glucose. The concentration of glucose was measured enzymatically at regular intervals from samples (1 mL) taken from the culture described in Fig. 3.2.1. Glucose concentration was analyzed by YSI. Standard deviation is too small to show on the graph.



**Figure 3.2.3.** Analysis of lactate. The concentration of lactate was measured enzymatically at regular intervals from samples (1 mL) taken from the culture described in Fig. 3.2.1. Lactate concentration was analyzed by YSI. Standard deviation is too small to show on this graph.



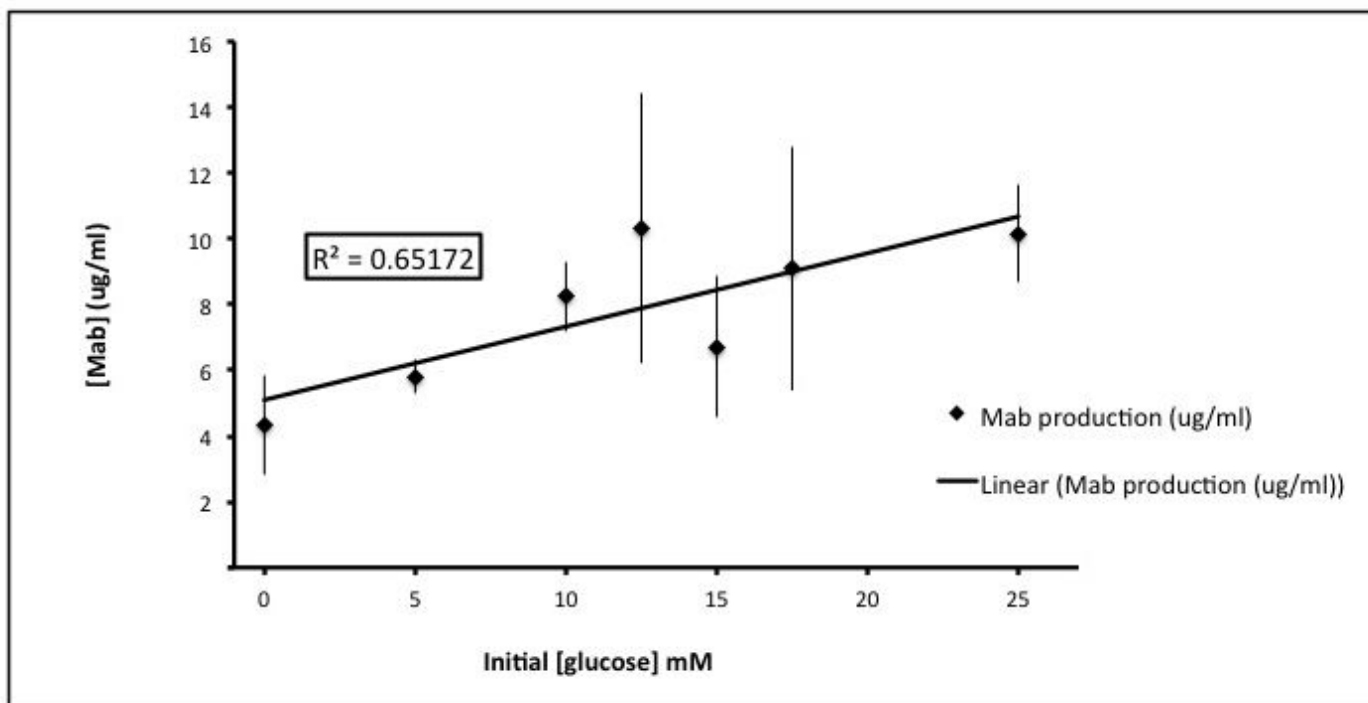
**Table 3.2.1.** Culture measurements following 24 h incubation at various glucose concentrations<sup>2</sup>. The initial cell density was  $2.6 \times 10^6$  cells/mL in 80 mL of media.

<b>Initial glucose mM</b>	<b>Time period of depleted glucose (h)</b>	<b>Viable cell density at 24 h ( <math>\times 10^6</math> cells)</b>	<b>Glucose consumption (pmol/cell)</b>	<b>Mab yield after 24 h (<math>\mu\text{g/ml}</math>)</b>	<b>Specific productivity (pg/cell/day)</b>
<b>0</b>	24	3.4	0	4.3	1.20
<b>5</b>	14	4.5	1.34	5.8	1.81
<b>10</b>	12	5.7	2.4	8.2	1.78
<b>12.5</b>	6	6.0	2.93	10.3	2.51
<b>15</b>	2	6.2	3.67	6.7	2.11
<b>17.5</b>	0	5.7	4.18	9.1	2.34
<b>25</b>	0	6.4	4.07	10.1	2.45

<sup>2</sup> Three trials were performed in total. In each trial, each culture condition was run in pairs. Results shown are the average of the total three trials.

### 3.3 EG2 antibody productivity

Cell culture samples were taken at 6 h intervals during 24 h incubation. Antibody production was investigated by ELISA. The EG2 antibodies accumulated steadily under all conditions to a maximum of 10.1  $\mu\text{g/mL}$  after 24 h in the culture at 25 mM glucose, compared to 4.3  $\mu\text{g/mL}$  in culture with the absence of glucose (Table 3.2.1). Specific productivity ( $Q_P$ ) for cells in 12.5 mM had the highest value of 2.5 pg/cell/day during 24 h incubation, and the cells without glucose experienced the lowest  $Q_P$ , 1.2 pg/cell/day. However, there was significant intrinsic variability in the ELISA assay as reflected in the results. The data showed a positive correlation between initial glucose concentrations in the media and Mab yield ( $R^2 = 0.652$ ) (Fig. 3.3.1).



**Figure 3.3.1.** The effect of initial glucose concentration on Mab production during 24 h incubation. A positive correlation was observed between  $Q_p$  and initial glucose concentrations (0-25 mM) in culture media.

### 3.4 Discussion and conclusion

This chapter has focused on selecting appropriate glucose concentrations for the following experiments which would involve finding the effect of limited glucose concentrations on both macroheterogeneity and microheterogeneity of glycans attached to EG2 antibodies. We selected initial glucose concentrations based on cell growth, viability, glucose starvation time and lactate production. Due to the high seeding density, glucose was depleted at a different time point over the course of the next 24 h in cultures containing  $\leq 15$  mM glucose. The time in which cell were exposed to glucose deprivation varied between 2 to 24 h. Based on the level of lactate accumulation, culture supplemented with 15 mM glucose showed a highest metabolic coefficient, 1.62 among all culture conditions.

Normally, as glucose uptake decreases, lactate metabolism switches from production to consumption. In this chapter, cells in culture with 5 - 12.5 mM glucose, the trend of lactate concentration during 24 h incubation was increasing at beginning and slightly went down when glucose depleted. There was no significant decrease in lactate concentration in this study. However, reports have indicated that once glucose uptake slowed down, and with sufficient lactate supply, cell took lactate over glucose as their main carbon source (Li et al., 2011).

ELISA analysis revealed a positive correlation between initial glucose concentrations and EG2 antibody production. Cells with 12.5 mM glucose concentration exhibited the highest protein productivity. Our result is consistent with previous findings that both high glucose environment and glucose limited condition affect protein productivity. We found that there was significant variability in ELISA assay. Possible

reasons for high variability could be (1) due to the intrinsic variables in ELISA assay; and (2) relatively short glucose deprivation state (0 to 24 h) makes it impossible for cells to adjust their protein production system instantaneously, resulting in a weak regression coefficient ( $R^2 = 0.65$ ) between protein productivity and initial glucose concentrations.

Due to the complexity and difficulty in LLO extraction, it was impossible to work with seven samples and their duplicates all together, based on cell growth, specific glucose consumption rate, four culture conditions were selected for further investigation (0 mM, 5 mM, 15 mM and 25 mM). 25 mM and 0 mM glucose samples were used as our positive and negative controls, respectively. 5 mM glucose sample represented cells underwent glucose depletion for 12 h, half of 24 h experimental time. In 15 mM glucose, cells were under glucose starvation for 4 h, which was shown changed LLO distribution in another CHO cell line in previous study (Rearick et al., 1981).

## CHAPTER 4 EFFECT OF GLUCOSE CONCENTRATION ON LLO DISTRIBUTION

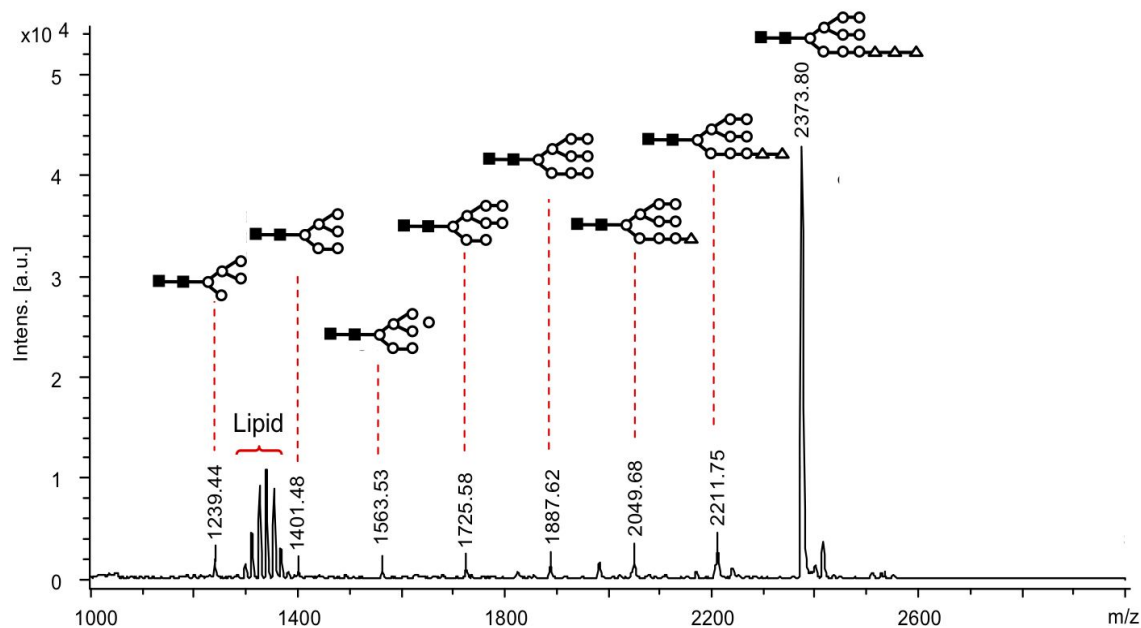
### 4.1 Introduction:

Lipid-linked oligosaccharides (LLOs) are the donors of glycans that modify newly synthesized polypeptide chain within the endoplasmic reticulum (ER) of eukaryotes resulting in formation of N-linked glycoproteins. The most traditional and widely used approach for LLO analysis involves pulse labeling with [2-<sup>3</sup>H] mannose, which is converted successively to [<sup>3</sup>H] mannose-6-P, [<sup>3</sup>H] mannose-1-P, GDP-[<sup>3</sup>H]mannose, and [<sup>3</sup>H]mannose-P-Dolichol. This technique therefore permits efficient labeling of LLOs with multiple mannosyl residues, then LLOs are purified and the oligosaccharides are cleaved from the lipid carriers. The free oligosaccharides are separated via chromatography and detected by liquid scintillation counting or in-line tritium detection (Cacan et al., 1992; O'Rear et al., 1999). Non-radioactive methods have also been used, and these methods involve the release of the oligosaccharide from the lipid carrier with chemical modification using a chromophore, fluorophore, or isotope, followed by chromatographic or electrophoretic separation, and then detect by absorbance, fluorescence, or liquid scintillation spectroscopy (Gao and Lehrman, 2006). In 2006, Lehrman's group developed an alternative method, known as FACE (fluorophore-assisted carbohydrate electrophoresis), using fluorescent labeling of the purified LLOs and followed by a separation via gel electrophoresis and detection using a gel imager with fluorescence detection capabilities.

This chapter details a modified technique for LLO analysis, based on Lehrman's extraction protocol, which has several advantages over the methods previously discussed. This method involves adapted unlabeled LLO purification from cell extracts, followed by acid hydrolysis to remove the oligosaccharide from the dolichol carrier. Oligosaccharides are then labeled with 2-AB and separated on a Waters X-Bridge 3.5  $\mu$ m amide column (4.6 X 250 mm) using 50 mM ammonium formate buffer (Buffer A) and acetonitrile (Buffer B). Peak identity is determined by two methods: (1) glycan structure verification on MALD-MS; and (2) comparing calculated GU values of each oligosaccharide to a GU value database (GlycoBase). These two tools allow us to have accurate detection and quantification of levels of the LLOs in the steady state and how this can be disrupted by glucose-deprivation.

## 4.2 LLO identification

LLOs collected from 25 mM glucose culture using the method described in Chapter 2, were pooled and subjected to mild acid hydrolysis. Lipid-free oligosaccharides were sent to chemistry department for MALD-MS analysis. MS spectrum confirmed the validity of the extraction method (Figure 4.2.1). The LLOs were observed in the mass range 1239 – 2537. All were detected without a free carbonyl reducing-terminus. The most abundant peak was observed at  $m/z$  2373.8, and produced a fragmentation pattern corresponding to a glycan with a Hex<sub>12</sub>GlcNAc<sub>2</sub> composition. The identified structures represent LLO profile at the steady state of cells grown in 25 mM glucose culture after 24 h incubation.



**Figure 4.2.1:** MALDI-MS spectrum of glycans released by acid hydrolysis from a lipid fraction obtained from EG2 cultured in CHO under standard conditions (25 mM glucose). All peaks are detected as  $MNa^+$ . Structural assignments are based on monosaccharide composition, fragmentation patterns and knowledge of the glycan biosynthetic pathway.

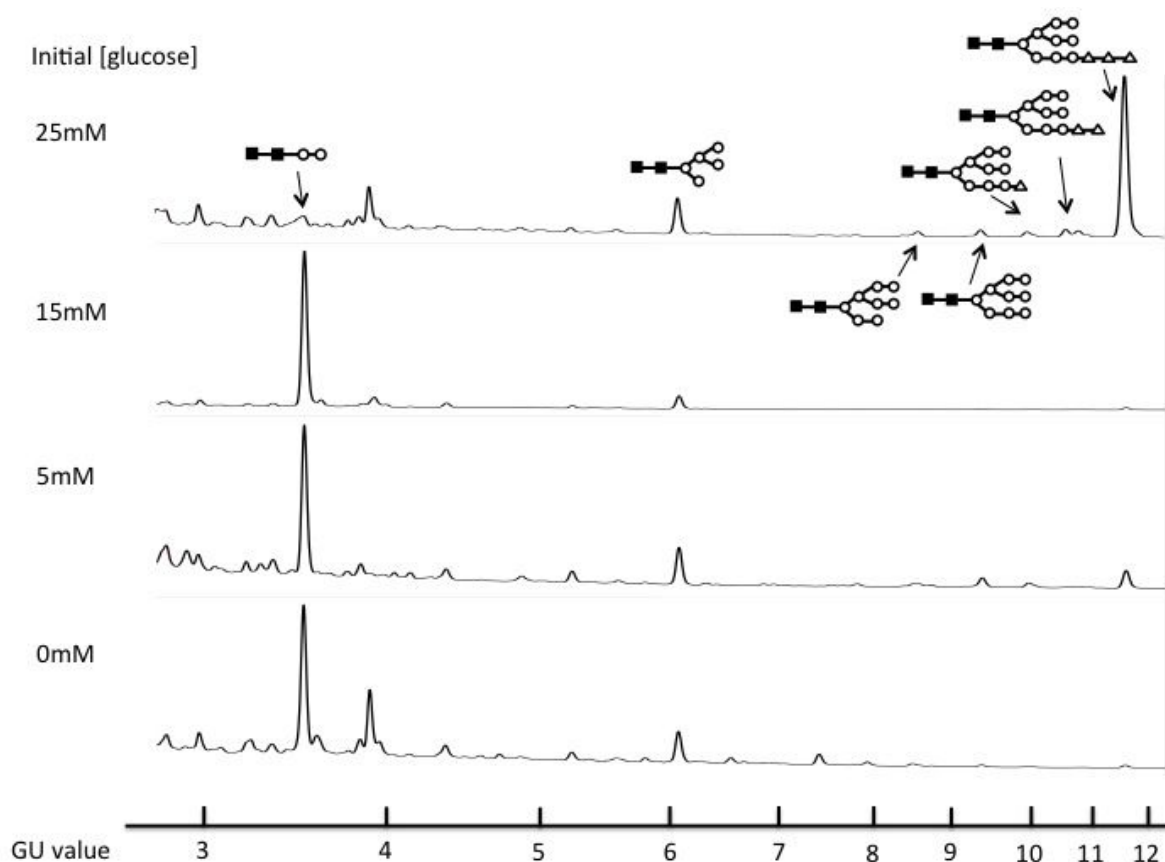
### 4.3 Results from glucose starvation tests

Three biological replicates of LLO extracts from cells grown in each of the 24 h cultures were analyzed by HILIC and structures were assigned from GU values found in the database (GlycoBase). The peaks corresponding to the LLOs from each of the previously described cultures (0-25 mM glucose) were compared (Figure 4.3.1). Samples from cultures containing 25 mM glucose displayed a large prominent peak with a GU value of 11.7, representing 63% of the total LLOs and assigned as the  $Glc_3Man_9GlcNAc_2$  (Man9) structure (Figure 4.3.1). Prominent smaller peaks were identified as  $Glc_2Man_9GlcNAc_2$ ,  $Glc_1Man_9GlcNAc_2$ ,  $Man_9GlcNAc_2$ ,  $Man_5GlcNAc_2$  (Man5) and



Man<sub>2</sub>GlcNAc<sub>2</sub> (Man<sub>2</sub>) structures as labeled in Fig 4.3.1. The other minor peaks were unidentified. Those below a GU value of 4 were contaminants, also present in the blank.

For cells grown at an initial glucose concentration of equal to or less than 15 mM, the predominant peak was Man<sub>2</sub> with a significant level of Man<sub>5</sub> structure; however, the percentage of the Man<sub>9</sub> structure was reduced significantly to 2.9% of the overall LLOs. It is important to note that these cultures ( $\leq 15$  mM glucose) were under conditions of glucose depletion for at least 4 hours prior to harvest.



**Figure 4.3.1:** Lipid-linked oligosaccharide (LLO) profiles. HPLC profiles show LLOs extracted from cell lysates at the end of each culture described in Figure 1. The glycans from each sample were acid hydrolyzed from the lipid carriers, 2-AB labelled and detected by HILIC. (Glc  $\Delta$ ; Man O and GlcNAc  $\blacksquare$ ).

#### 4.4 Discussion:

The LLO extraction method described in this Chapter has several advantages over some of the more traditional techniques. The quenching method that was modified from previous reports provides reliable results of the instantaneous steady state levels of LLO in cell lines (Jones et al., 2010) or tissues (Gao and Lehrman, 2006) under various conditions.

In traditional [2-<sup>3</sup>H] mannose pulse labeling method, low-sugar labeling media is frequently required to improve the incorporation of the radiolabeled mannose (Gao and Lehrman, 2006). During cell culture, changes in media composition can affect the levels of LLO very rapidly. In 1981, Rearick's group found that cells maintained in a low-glucose medium up to the time of 15 min labeling period exhibited as a great shift from Man<sub>9</sub> to Man<sub>5</sub> as did cells starved of glucose for 15 min and 30 min prior to the [2-<sup>3</sup>H] mannose labeling. Therefore, the radioactive labeling method may not accurately represent the levels of LLO at certain time points, making comparison of LLO profiles in various glucose concentration media even harder. On the other hand, due to the fact that culture conditions can also affect N-glycan site occupancy and the final N-glycan structures on a protein, incubation with low-sugar media may sabotage the following investigations on the correlation between initial glucose concentrations and macro- and microheterogeneity of an EG2.

It is well known that glucose starvation alters the LLO composition. In 25 mM glucose culture, LLO composition is consistent with previous studies (Jones et al., 2010; Quellhorst et al., 1999; Rearick et al., 1981), showing M<sub>9</sub>-P-P-Dol as the primary LLO found in CHO-K1 cells, along with small amounts of Glc<sub>2</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>,

Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>, and Man<sub>9</sub>GlcNAc<sub>2</sub> due to the possible reason that an addition of glucose residues onto the fully mannosylated LLO (Man<sub>9</sub>GlcNAc<sub>2</sub>-P-P-Dol) is the rate determined step (Jones et al., 2010). Glucose starvation can induce an accumulation of truncated LLO intermediates in the early stage of N-glycosylation, such as M5 to M2 (Rearick et al., 1981), which is consistent with LLO pools extracted from cells grown in 0-15 mM glucose.

It has been proposed that under glucose-limited condition, all available carbon is preferentially used for energy production, causing reduced nucleotide biosynthesis (Nyberg et al., 1999; Rearick et al., 1981). Exposure of cells to four or more hours to a glucose-depleted media can have impacted the cell's ability to produce precursors for RNA synthesis, nucleoside triphosphates such as UTP become depleted. The last four mannose and three glucose residues are extremely sensitive to the extracellular glucose level, so that glucose deprivation can limit the production of their sugar donors, Man-P-Dol and Glc-P-Dol, resulting in truncated LLO precursor (Chambers et al., 1977; Forsee et al., 1977; Jensen and Schutzbach, 1986; Kang et al., 1978a; Spencer and Elbein, 1980).

One other very important note is that glucose-starved cells produce truncated LLO intermediates, which are less favorable to OST compared to fully glucosylated donors (Gao et al., 2005; Kelleher et al., 2003; Turco et al., 1977). An accumulation of truncated LLO intermediates can result in the addition of premature N-glycans onto the nascent polypeptides at a low rate or leaving Asn residue completely empty (Jackson et al., 1989; Quellhorst et al., 1999; Sharma et al., 1981). Both premature N-glycans and hypoglycosylation may have an effect on the N-glycosylation profile of the final product, which will be investigated in the next experiments in this thesis.

#### 4.5 Conclusion:

In this chapter we described a novel technique for LLO composition analysis. This method allows for the detection of steady state levels of LLO from suspension culture under any desired growth conditions. Sufficient sensitivity and resolution provide us the detection of all intermediates in the LLO synthesis pathway from M2 to M9, as well as other minor components. However, due to the long and complicated experimental procedures, contaminants were introduced at some points, and the future work can involve investigating the sources of contamination, and attempting to minimize or eliminate them.

Meanwhile, we confirmed that the glucose starvation has a negative effect on the early stage of N-glycosylation pathway. Instead of producing a completed N-glycan structure M9, truncated LLO intermediates were accumulated, and M2 became the predominant structure when cells were in a glucose depleted state for 4 h or longer. M9 structure is essential for OST recognition and glycan transfer, the next experiment will be testing on glucose starvation and its relation to macroheterogeneity and microheterogeneity of EG2.

## CHAPTER 5 MACROHETEROGENEITY OF PURIFIED ANTIBODIES

### 5.1 Introduction:

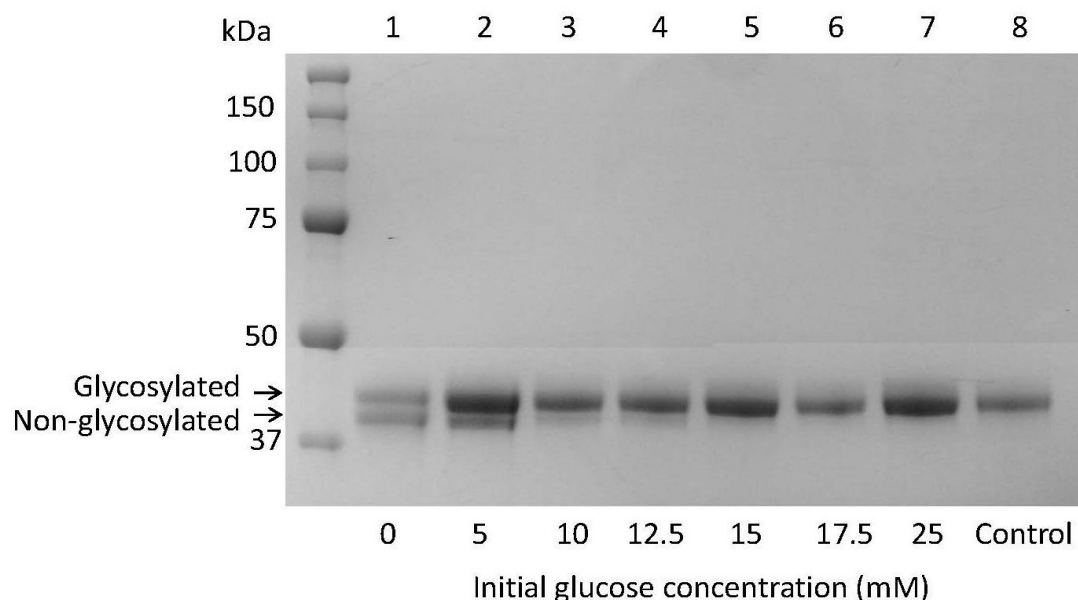
N-glycosylation site occupancy difference has been observed in several glycoproteins (e.g., t-PA, IFN- $\gamma$ , IL-2). Different mechanisms have been found to be potentially responsible for N-glycosylation site occupancy, including the availability of dolichol phosphate (Crick and Waechter, 1994; Rosenwald et al., 1990) and nucleotide sugars (Nyberg et al., 1999), activity of oligosaccharyl transferase (OST) (Hendrickson and Imperiali, 1995; Kaufman et al., 1994), and variable glycosylation site accessibility due to the competition with protein folding (Allen et al. 1995; Holst et al., 1996; Imperiali, 1997; Shelikoff et al., 1996). Limitations in glucose and glutamine have been found to affect IFN-  $\gamma$  site-occupancy in CHO cultures (Hayter et al., 1992; Nyberg et al., 1999). In 2000, Andersen's group found that the site-occupancy of recombinant human t-PA was elevated gradually over the course of batch and fed-batch CHO cultures (Andersen et al., 2000). By controlling cell culture factors, including butyrate and temperature, they also showed the degree of site-occupancy that could be influenced (Andersen et al., 2000). In addition, there was an important correlation between the growth profile of CHO cultures and site-occupancy, in which a decrease in cell growth led to an increase in site-occupancy (Wang et al., 1991). However, all of them failed to reveal the causes for this scenario.

Studies have showed that a broad spectrum of assembly intermediates (GlcNAc<sub>2</sub>-PP-Dol to Glc<sub>2</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol) can be utilized by OST *in vitro* and *in vivo* (Jackson et al., 1989; Sharma et al., 1981). However, the presence of glucose units in the

completed N-glycosylation precursor,  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ , is essential for an efficient transfer reaction, and being preferred by more than 20-fold over nonglycosylated oligosaccharide-PP-Dol in cell free assays (Ballou et al., 1986; Parodi, 2000). The previous chapter illustrated that glucose deprivation state could result truncated LLOs accumulation in the early stage of N-glycosylation pathway. Previous studies showed that truncated LLOs were less favorable to OST and had less chance to be added onto the nascent polypeptides. This chapter is focused on the relationship between truncated LLO intermediates caused by glucose starvation and macroheterogeneity in CHO-EG2 cells.

## 5.2 Results:

Samples from cultures at various glucose concentrations were spotted on reduced (8-16%) SDS-PAGE gel. Protein A purified samples from all culture conditions were run parallel on the gel for comparison. Analyzed results showed visible antibody bands of approximately 40 kDa stained with CBB, representing denatured single heavy chains (HC; Figure 5.2.1). The antibodies produced by cells grown in 17.5 - 25 mM (Lane 6 and 7) displayed one single strong band corresponding to the glycosylated HC. Proteins isolated from cell cultures with less than 15 mM initial glucose concentrations (Lane 1 to 5) showed a faint band underneath the predominant protein band. The proportional density of the lower band increased as the initial glucose concentration decreased. The ratio density was analyzed by densitometry on figure 5.2.1. The lower protein band was taken 26% in 12.5 mM glucose culture and raised up to 52% for 0 mM glucose sample. (Table 5.2.1).



**Figure 5.2.1.** Separation of EG2 antibodies on reduced 8-16% SDS-PAGE gel. Protein A purified antibodies were isolated from 24 h CHO cultures with (lane 1) 0 mM, (lane 2) 5 mM, (lane 3) 10 mM, (lane 4) 12.5 mM, (lane 5) 15 mM, (lane 6) 17.5 mM and (lane 7) 25 mM D - glucose. The purified antibody in lane 8 was isolated from the culture prior to the 24 h incubation. Upper bands in lanes 1 to 4 correspond to glycosylated antibodies, and the lower bands were determined to be non - glycosylated antibodies.

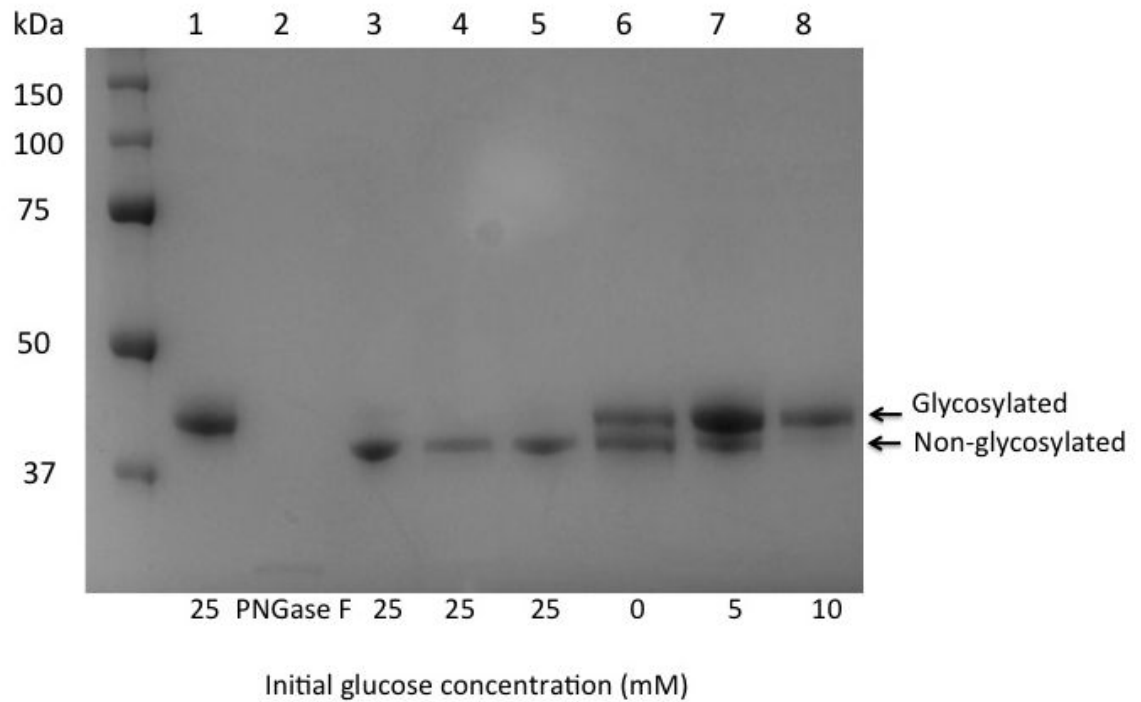
The lower protein bands were suspected to be deglycosylated proteins due to an estimated 2% weight loss, which corresponds to the typical mass of glycan found on IgGs (Deisenhofer, 1981). The identity of the lower band from the reduced gels was confirmed to be non-glycosylated HC by two methods. Firstly, the antibodies purified from control culture (25 mM glucose) were incubated with endoglycosidase PNGase F for either 10, 20, or 30 min respectively, prior to gel electrophoresis. This action caused a protein band migration shown in figure 5.2.2., on which the predominant protein band migrated from 40 kDa position to where the lower protein band was found from samples with low initial

glucose concentration cultures (0-10 mM). The presence of PNGase F (Lane 2) in samples did not interfere with the final result. Secondly, the upper and lower bands from prep-gel with 0 mM glucose sample were well separated and collected, and sent to chemistry department for mass spectrometry analysis by another member of the lab. This showed m/z values of 82,670 and 79,350, which are the expected masses of the glycosylated and non-glycosylated forms, respectively, of the complete antibodies.

**Table 5.2.1.** Quantitative densitometry of Protein A purified EG2 antibodies stained with coomassie blue (n = 5) on 8-16% SDS-PAGE gel shown on figure 5.2.1.

Initial glucose concentration (mM)	% Glycosylated protein	% Non-glycosylation protein
0	48 ± 1	52 ± 1
5	60 ± 4	40 ± 4
10	69 ± 2	31 ± 2
12.5	74 ± 2	26 ± 2
15	100	0
17.5	100	0
25	100	0
Control	100	0





**Figure 5.2.2.** Comparison of PNGase F partial digested glycosylated EG2 antibodies and antibodies from glucose starved cultures on reduced 8-16% SDS-PAGE gel. Protein A purified antibodies from 25 mM culture were subjected to PNGase F partial digestion for 0 (lane 1), 10 (lane 3), 20 (lane 4) and 30 (lane 5). In lane 3 to 5, upper protein bands in those 25 mM glucose samples were completely digested and migrated along with other lower protein bands. PNGase F in lane 2 run further on the gel compared to antibodies indicating no cross contamination between PNGase F and lower protein band. Upper bands in lane 1, 6, 7 and 8 corresponded to glycosylated antibodies, and the lower bands in lane 3 to 8 were determined to be non-glycosylated antibodies.

### 5.3 Discussion:

This chapter has studied the effect of glucose starvation on N-glycosylation site occupancy of a chimeric HC antibody, EG2 in the batch culture of Chinese Hamster ovary cells. We found that glucose limitation could cause low glycosylation occupancy

and we verified this by using N-glycosidase partial digestion and MALDI-MS. In the PNGase F partial digestion experiment, endoglycosidase digested EG2 antibodies originally isolated from 25 mM glucose culture; migrated along with other lower protein bands found in those glucose depleted samples (0-10 mM glucose) on 8~16% SDS-PAGE gel. MALDI-MS analysis verified that the reason for this migration on the SDS-PAGE gel was due to the loss of N-glycan on HC.

We found that the macroheterogeneity of N-glycosylation was correlated with initial glucose concentration, or in another word, the time period of cells exposed to glucose deprivation during 24 h incubation. There is no link found between macroheterogeneity in N-glycosylation and the extracellular hydrolytic degradation (Burteau et al., 2003; Curling et al., 1990); consequently, the cause for the diminution in N-glycosylation site occupancy only relies on the intracellular effects. Our results are consistent with previous findings, as it has been recognized for some time that glucose starvation decreases glycosylation efficiency. In the previous chapter, we found that glucose starved cells synthesized truncated LLOs and this could result a weaken oligosaccharide transfer by OST compared to that happened to a completed N-glycosylation precursor. And this experiment showed that glucose limitation could result in a decrease in N-glycan occupancy according to the appearance of an additional non-glycosylated antibody band found in gel electrophoresis. Table 5.2.1 showed a strong positive correlation between the time periods that the cells were subjected to glucose depletion over the 24 h incubation, and the proportion of non-glycosylated antibodies produced.

This effect of glucose limitation on glycan occupancy has been seen previously in chemostat cultures for IFN- $\gamma$  (Hayter et al., 1992). Glucose limited cells preferentially utilize glucose for energy production rather than replenish the nucleotide pool for glycosylation (Nyberg et al., 1999). Low metabolic energy status of cells also contribute to low glycan occupancy (Kochanowski et al., 2008). However, the possibility of transferring truncated glycan onto proteins by OST will be investigated in the next Chapter.

#### **5.4 Conclusion:**

This chapter has shown that the availability of glucose in the media can enhance macroheterogeneity in N-glycosylation in EG2-producing CHO cells. The proportion of non-glycosylated antibodies produced increases as the time period of cells exposed to glucose starvation was elongated.

## CHAPTER 6 N-GLYCAN MICROHETEROGENEITY OF EG2 ANTIBODIES

### 6.1 Introduction

Recombinant glycoproteins represent the major share of marketed and clinical development phase therapeutic proteins. A well understanding the nature and the functions of N-glycans and their impact on pharmacological properties are essential for discovering and developing safe and efficacious glycoprotein biopharmaceuticals. N-glycan plays an important role in many aspects, including: establishing or maintaining the integrity of glycoprotein, enhancement in protein solubility, optimizing the binding of the Fc domain to its many receptors, affecting pharmacokinetic behavior including serum half-life, maximum and minimum concentrations, and last but not least, immunogenicity to patients.

During industrial production, glycans on most recombinant glycoproteins exist as a pool of a heterogeneous population of glycoforms. For IgG antibody, there is a single glycosylation site at Asn 297 on each heavy chain, and there are as many as 32 different structures can be identified at Asn 297 in human serum (Arnold et al., 2007). Terminal sialylation and galactosylation of the antenna, fucosylation of the innermost core GlcNAc or the outer arm GlcNAc and addition of a “bisecting” GlcNAc to the central core Man residue are signature glycoforms found on N-glycosylation site.

These structures of glycans depend on the levels of specific glycosyltransferases, the availability of corresponding monosacharides and sugar nucleotides donors, as well as the local three-dimensional structure of the protein at glycosylation site. Meanwhile, this microheterogeneity can also be induced by various parameters during the fermentation conditions, such as nutrient content, pH, temperature, oxygen or ammonia as introduced in Chapter 1. Manipulation of those parameters can lead to enhanced glycosylation, reduced batch to batch variation in the production process, and increased therapeutic efficacy. Understanding the parameters that cause the variation in glycosylation is very important, and in this chapter we will be focusing on the effect of glucose starvation on N-glycan microheterogeneity of EG2 antibodies.

## 6.2 Glycan structure identification

The method we employed, involves HILIC-HPLC based analysis of enzymatically released 2-AB labeled N-glycans published in 2012 (Royle et al., 2010) to monitor glycosylation changes on EG2 antibodies corresponding to glucose depletion time. EG2 antibodies produced during 24 h glucose starvation test were isolated from cell cultures with different initial glucose concentrations and purified using Protein A affinity columns. Then, sample was reduced and purified by 8~16% prep-gel. Deglycosylated polypeptide run further on the gel compared to the glycosylated sample due to 2% weight loss. The de-stained glycoprotein band was carefully removed from the gel and subjected to PNGaseF digestion. PNGaseF removes N-glycans with or without  $\alpha$ -1,6 linked core fucose. The released glycans are fluorescently labeled with 2-AB following HILIC HPLC analysis.

Glycans collected from 25 mM glucose culture separated on HILIC HPLC first as control. The elution time was calibrated to GU values using Dextran ladder and then assigned glucose unite values by fitting a fifth order polynomial distribution curve to allocate GU values from retention times. The structures were assigned using standard GU values from the GlycoBase database (NIBRT.ie) (Campbell et al., 2008). Glycan structures were also confirmed by exoglycosidase enzymatic digestion arrays according to the method of Royle et al (2006), and conducted by other members in Dr. Butler's lab. This allowed the identification of biantennary glycan structures with variable sialylation (S), galactosylation (G) and fucosylation (F).

### 6.3 Results from glucose starvation tests

To compare the difference in glycosylation profiles of EG2 antibodies induced by different glucose depletion times, all samples were run parallel and compared by HILIC HPLC. The glycan pool was separated into six major peaks which eluted between 33 and 43 min, with corresponding GU values between 5 and 9 (Figure 6.3.1). The predominant glycan structure on antibodies found in 25 mM glucose culture was a fully galactosylated biantennary and fucosylated structure, G2F, which comprised nearly 60% of the overall glycans. G0F and G1F structures were determined at 6% and 34%, respectively. Meanwhile, sialylated S1G2F and S2G2F were distinguishable in the spectrum.

EG2 antibodies isolated from cell cultures with initial glucose concentrations from 0 to 15 mM, showed that the predominant peak shifted from G2F to G1F and eventually G0F. This loss of galactosylation correlated with increased glucose depletion time. Sialylation depends on prior galactosylation of a glycan and subsequently decreased

as glucose starvation extended. The degree of galactosylation and sialylation of glycans was quantified by the calculation of either the galactosylation index (GI) or sialylation index (SI) based upon the relative peak areas on the HPLC profile. The average GI value of three biological replicates changed from 0.72 (25 mM glucose sample) to 0.35 (0 mM glucose sample) as glucose depletion state proceeds. SI went from 0.058 (25 mM glucose sample) to 0.019 (0 mM glucose sample). There was a strong positive linear correlation between GI and SI value for each sample and the time spent by the corresponding cells in glucose deprived media over the 24 h experimental period ( $R^2 = 0.965$  and  $0.936$  for GI and SI values respectively; Figure 6.3.2).

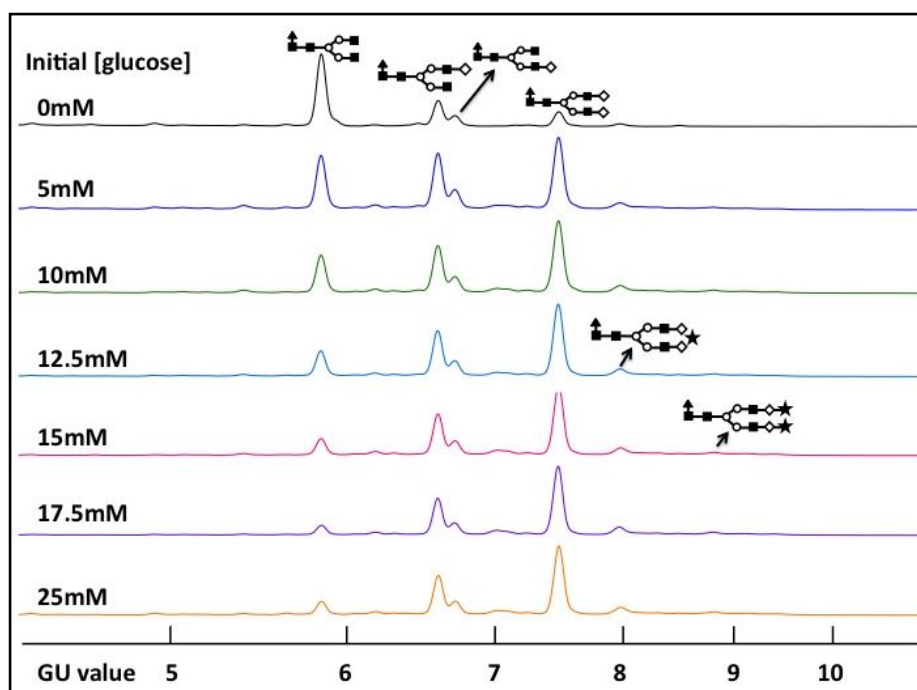
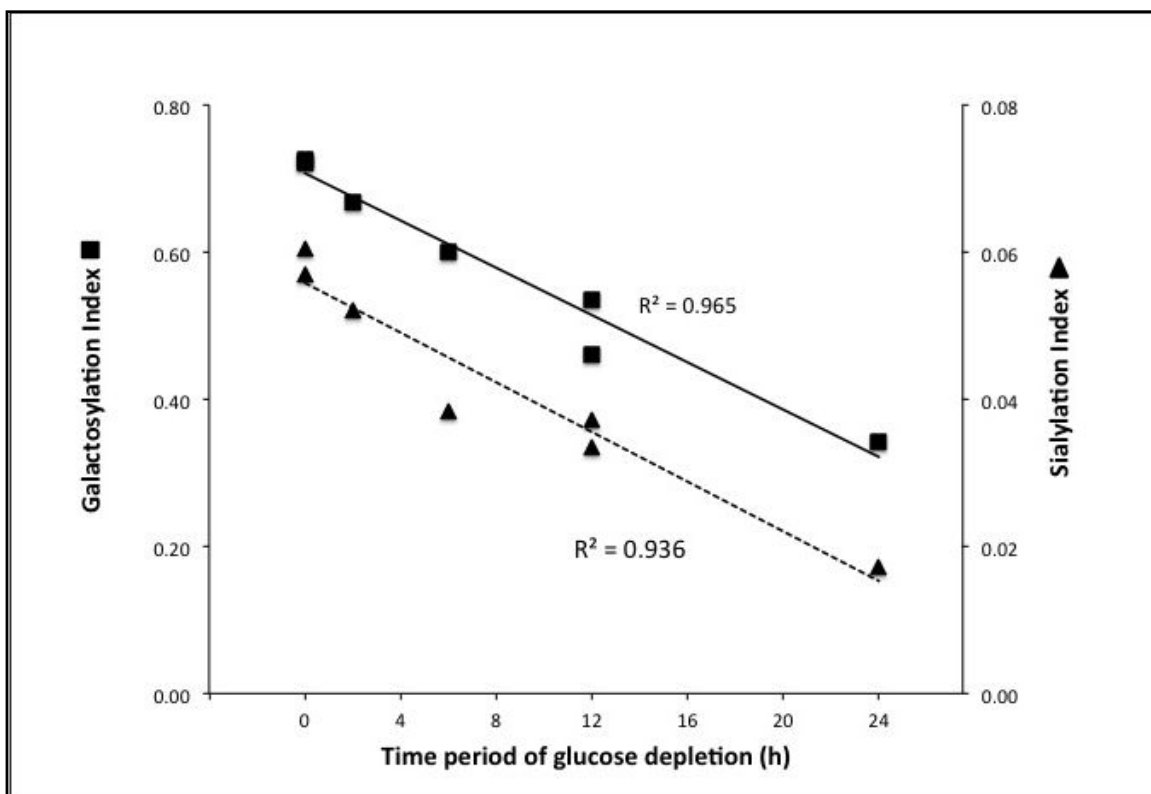


Figure 6.3.1: HPLC profiles of N-glycans isolated from EG2 antibodies produced by CHO cells with various initial glucose concentrations during a 24 h incubation. Antibodies were purified from cultures containing an initial glucose concentration of (A) 0 mM; (B) 5 mM; (C) 10 mM; (D) 12.5 mM; (E) 15 mM; (F) 17.5 mM, and (G) 25 mM glucose. Antibodies were isolated from the supernatants of the cultures after 24 h using a Protein A column. Glycans were released enzymically, 2-AB labeled, and detected via HILIC. Structural assignments of peaks were made as follows: (1) F(6)A2G0, (2) F(6)A2G1(1,6), (3) F(6)A2G1(1,3), (4) F(6)A2G2, (5) F(6)A2G2S1 and (6) F(6)A2G2S2.





**Figure 6.3.2:** The effect of exposure time of cells to media depleted of glucose on the galactosylation (GI; ■) and the sialylation (SI; ▲) indices of EG2 antibodies produced by CHO cells. Calculations used to determine the GI, SI and Qs are described in the Methods section. A statistically significant positive correlation was determined between Qs and both SI ( $R^2=0.884$ ) and GI ( $R^2 = 0.994$ ).

## 6.4 Discussion

The rationale behind the design for this experiment is to correlate the glycan microheterogeneity of CHO-EG2 cells to exposure time in a glucose depletion state in the media. The functional importance of the consensus glycan structure has been well studied. The reduction in galactosylation can result in decrease complement-dependent

cytotoxicity (CDC) in a human antibody (Tsuchiya et al., 1989). Sialylation has been proposed as a natural physiological switch to regulate the immune response upon antigenic challenge (Kaneko et al., 2006). Due to defined relationship between a glycan structure and the biological function of the antibody, it is very important to minimize batch-to-batch variance and promote the preferred glycosylation pattern to meet the desire function.

This chapter describes the effect of glucose starvation on glycan microheterogeneity of EG2 antibodies. Cells were seeded in high density in media containing glucose from 0-25 mM, and incubated for 24 h. In chapter 3 we found that cells in culture with an initial glucose concentration 15 mM had been in a glucose depleted state for nearly 4 h. Cells in lower glucose concentration (<15 mM) were exposed to glucose starvation for up to 24 h. Antibodies were isolated from each culture by protein A purification and a gel-based method. 2-AB labeled antibodies were separated on HILIC HPLC, and compared. Antibodies produced by cells in 25 mM glucose culture displayed predominant glycan structures G2F, G1F and G0F with a galactosylation and sialylation index values, 0.72 and 0.058, respectively. Cells in a glucose-depleted state resulted in a biantennary complex glycan structure with less galactosylation and sialylation. A strong positive correlation was observed between the time periods that cells were exposed to glucose depletion over the 24 h incubation and the GI and SI values with  $R^2 = 0.965$  and  $0.936$ , respectively.

No evidence has been found between extracellular galactosidase and galactose content (Gramer et al., 2011; Jones et al., 2010; Quellhorst et al., 1999; Rearick et al., 1981). The results showed a linear correlation between the time period of glucose

depletion during 24 h and galactosylation and sialylation indexes, suggesting that the decrease in galactosylation and sialylation could be elicited by depletion of activated monosacharides as dolichol phosphate or nucleotide derivatives, mostly glucose-derived substrates. Previous studies revealed that feeding N-glycosylation precursors or substrates could improve sialylation or galactosylation of interferon-  $\gamma$  expressed in CHO (Gu and Wang, 1998; Jones et al., 2010). In addition, a feeding regime of uridine, manganese and galactose as media supplements improves GI of CHO-EG2 cells (Liu et al., 2014). Secondly degalactosylation and desialylation can be caused by inefficient factors associated with intracellular biosynthesis, instigated by Golgi stress induced by energy deficiency (Gramer et al., 2011; Pacis et al., 2011; Rearick et al., 1981). It is possible that ER and Golgi stresses provoked by glucose starvation might stimulate the dysfunction in  $Mn^{2+}/Ca^{2+}$  pumps, in which  $Mn^{2+}$  is essential cofactor for galactosyltransferase (Chambers et al., 1977; Forsee et al., 1977; Gramer et al., 2011; Jensen and Schutzbach, 1986; Kang et al., 1978b; Pacis et al., 2011; Spencer and Elbein, 1980), resulted in low GI of antibodies. However, this conjecture requires further investigation. Results in the present study prompted the previous hypothesis of showing the impact of glucose depletion on N-glycosylation patterns of EG2 antibodies.

## 6.5 Conclusion

This chapter investigated the effect of glucose depletion on N-glycan microheterogeneity of EG2 antibodies. Glucose starvation has a severe impact on galactosylation and sialylation of EG2 antibodies, and the time of glucose depletion during the 24 h period has a negative linear correlation with GI and SI ( $R^2 > 0.9$ ). Results

suggest that the availability of glucose in the media can affect the microheterogeneity of N-glycosylation of a secreted antibody from CHO cells. The results imply an appropriate feeding strategic during commercial bioprocess for the production of therapeutic antibodies might minimize the batch-to-batch variation and manipulate glycan structure to meet the desire function.

## CHAPTER 7 DISCUSSION, CONCLUSION AND PERSPECTIVE

### 7.1 Discussion:

The rationale for this project was to study the variability of antibody glycosylation with respect to nutrient concentrations in culture media, in order to understand the effects of substrate depletion that may occur in mammalian cell cultures, particularly during fed-batch cultures that are designed to maintain low glucose and glutamine levels to prevent the accumulation of metabolic by-products.

The functional importance of the consensus glycan structure on immunoglobulins has been widely recognized. The specific effect of individual sugar residue on the glycan has been introduced in detail in Chapter 1. Briefly, a lack of, or insufficient galactosylation can decrease ADCC and CDC activities of an antibody (Ferrara et al., 2011; Hodoniczky et al., 2005; Tsuchiya et al., 1989). Sialylation has been proposed to have a regulation function to immune response upon antigenic challenges (Kaneko et al., 2006). Due to these defined relationships between the structure and function of the antibody glycans, it is very important at one level to control the consistency of glycosylation in a bioprocess and at a secondary level to promote the preferred glycosylation profile, in order to ensure the targeted function.

In this study, we investigated the effect of glucose depletion on the macroheterogeneity and microheterogeneity of glycans attached to a human-camelid

antibody produced from CHO cells. Cells were inoculated at high density in media containing glucose from 0 to 25 mM for 24 h. Viable cell density, glucose and lactate concentrations, and protein productivity were monitored at 6 h intervals during the 24 h. Not surprisingly, in cultures with initial glucose  $\leq 15$  mM, cells were under glucose depletion for 2 ~ 24 h. Based on the level of lactate accumulation, culture supplemented with 15 mM glucose showed the highest metabolic coefficient, 1.62, among all culture conditions.

Organic solvent extraction of the hydrophobic metabolites of the corresponding cell lysates enabled analysis of the lipid-linked oligosaccharides (LLOs) by HPLC separation and mass spectrometry. This identified a predominant 14-unit oligomer peak  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  under standard conditions of 25 mM glucose and is consistent with a previous study using a similar method (Jones et al., 2010). Quenching method employment ensures that this profile of LLOs represents the metabolic steady state at the time of harvest from these cultures. Metabolic precursor in the early stage of N-glycosylation, such as  $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ ;  $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ ;  $\text{Man}_9\text{GlcNAc}_2$ ;  $\text{Man}_5\text{GlcNAc}_2$  were also found on HPLC spectra as minor peaks. This suggested that assemblage of the 14-unit glycan oligomer  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2 - \text{P-P-Dol}$  occurs rapidly, and the addition of the last three glucose residues onto the high mannose glycan is the time determined step.

LLOs isolated from culture suffered glucose depletion, the content of the completed N-glycosylation precursor decreased significantly. Instead, truncated LLO, such as  $\text{Man}_2\text{GlcNAc}_2$  became the predominant peak on HPLC spectra. Extraction from cell culture with 15 mM initial glucose concentration, cells were under glucose depletion

for at least 2 h prior to harvest. A previous study showed that the turnover time for early stage N-glycosylation synthesis could be as short as 15 min in CHO cells (Rearick et al., 1981). The consistency between our findings and previous studies (Jones et al., 2010; Rearick et al., 1981) indicates the adequacy quenching method we applied, as well as the accuracy of our results to present the metabolic steady state of the time of harvest.

Truncated LLO accumulation suggests that the subsequent steps of the pathway were limited by the lack of availability of a monosaccharide substrate at the time of cell harvest. Dol-P-Man and Dol-P-Glc content were found extremely sensitive to external glucose concentration (Rearick et al., 1981). This is a possible explanation to our findings of the glucose-depleted cultures.

Oligosaccharyltransferase (OST) is a key enzyme in N-glycosylation pathway and responsible for the transfer of glycan from dolichol carrier onto the nascent polypeptide with the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Korner et al., 1998; Kornfeld and Kornfeld, 1985). OST is highly specific, and truncated LLO intermediates found to be less favorable to OST than completed N-glycosylation donor (Kelleher et al., 2003; Turco et al., 1977). Thus, the presence of the LLO intermediates in cells exposed to media with depleted glucose would be expected to lead to reduced glycan occupancy in a synthesized protein (Hülsmeier et al., 2007).

The 14-well SDS-PAGE shows that the depletion of glucose resulted in decreased glycan occupancy by the appearance of the non-glycosylated antibody band followed by MS with no evidence of transfer of the truncated oligomer to the antibody. There was a positive correlation between the time period that the cells were exposed to glucose depletion over the 24 h incubation and the proportion of a non-glycosylated antibody. A

glucose limited culture has been proven to reduce glycosylation site-occupancy, as discussed in Chapter 5. The main reason is that under low glucose concentration culture, cells preferentially use the available substrate for energy metabolism rather than replenishing nucleotide-sugar pools that are the essential precursors of glycosylation (Nyberg et al., 1999). Meanwhile, another study found that under a low metabolic energy status of cells can also contribute to low glycan occupancy (Kochanowski et al., 2008). In this study, we found that under low energy status, insufficient substrate formation for OST could also contribute to macroheterogeneity of N-glycosylation.

Microheterogeneity of N-glycosylation was found in cultures in a glucose-depleted situation. Glycan microheterogeneity was manifested by changes in the galactosylation and sialylation of the biantennary complex glycan structures. The predominant glycans on the antibody were G2F, G1F and G0F. Each sample was assigned an index (0-1) of galactosylation and sialylation based upon the percentage of Gal and NeuAc occupancy on each glycan branches determined by HPLC. As initial glucose concentration decreased, the increased time period of cells that underwent glucose depletion resulted in a shift to lower galactosylation and sialylation on glycan. Our results displayed a strong positive correlation between the time period of glucose depletion and the measured galactosylation ( $R^2 = 0.965$ ) and sialylation ( $R^2 = 0.936$ ) indexes of the purified antibody. This suggests that the observed decrease of galactosylation and sialylation was associated directly with the availability of the precursor.

Table 7.1 listed GI values that have been calculated previous studies of Mabs. The list contains values of antibodies from pooled human serum with GI of 0.36 (Mariño et



al., 2010). A review reported that commercially available Mabs showed that GI from CHO-derived Mabs is lower than that in murine cell derived Mabs (Raju and Jordan, 2012). There are several reports of non-commercialized Mabs from CHO cells with high GI values around 4.5 (Costa et al., 2013), although the intrinsic difference between cell lines remains unclear. For Mab production, no evidence showed a relationship between extracellular galactosidase and external galactose content (Gramer et al., 2011; Quellhorst et al., 1999). However, it is clear that the extent of galactosylation varies with the culture conditions of the producer cell, as well as environmental factors that may change significantly during the course of a batch culture (Gramer et al., 2011).

The control of parameters enables GI values to be altered within a certain range. Dissolved oxygen level from 10 to 100% could shift GI from 0.41 to 0.56 (Kunkel et al., 1998). Human or humanized antibodies showed a maximum GI value of 0.4 whereas two murine antibodies possessed a much higher values of 0.62 shown in table 2. In 2014, Butler's group found that supplements such as uridine, manganese and galactose can directly affect galactosylation, in which culture, GI has significant improvement in both EG2 and DP12 antibodies. Two reported antibodies with mutations in the Fc fragments contain higher values of 0.70 (Rose et al., 2013) and 0.83 (Jassal et al., 2001).

**Table 7.1.** Variable galactosylation of antibodies reported in the previous literature.

Source/cell type	Antibody	Variable parameter	GI range	Publication source
Human serum	Serum IgG	Physiologically normal	0.36	(Mariño et al., 2010)
Murine hybridoma (NS1)	Murine IgG1	Bcl-2 transfection	0.53-0.62	(Majid et al., 2007)
Murine hybridoma (Sp2/0)	Murine IgG1	Dissovled oxygen	0.37-0.56	(Kunkel et al., 1998)
Murine NS0	Humanized IgG1	Redox potential	0.19-0.29	(Dionne & Butler, 2012)
Murine (Sp2/0)	Chimeric and human IgG1	3 commercial Mabs	0.32-0.39	(Raju and Jordan, 2012)
Hamster (CHO)	Human/humanized IgG1	3 commercial Mabs	0.09-0.23	(Raju and Jordan, 2012)
Hamster (CHO K1)	Recombinant human	Mode of growth	0.44-0.45	(Costa et al., 2013)
Hamster (CHO K1)	Recombinant human	Serum/serum-free	0.26-0.32	(Costa et al., 2013)
Hamster (CHO-K1SV)	Human Ig	Uridine/Mn <sup>2+</sup> /Gal feeding	0.03-0.23	(Gramer et al., 2011)
Hamster (CHO K1)	Chimeric human IgG3	F243A mutation in CH2	0.39-0.83	(Jassal et al., 2001)
Human 293 cells	Human IgG1	Y407E mutation in CH3	0.29-0.70	(Rose et al., 2013)
Hamster (CHO)	Human IgG1 (DP12)	Uridine/Mn <sup>2+</sup> /Gal feeding	0.30-0.43	(Liu et al., 2014)
Hamster (CHO DUXB)	Human-camelid EG2	Glucose concentration	0.35-0.72	(Liu et al., 2014)
Hamster (CHO DUXB)	Human-camelid EG2	Uridine/Mn <sup>2+</sup> /Gal feeding	0.73-0.83	(Liu et al., 2014)

High GI value found in the EG2 antibody may be related to structural differences. EG2 is a chimeric antibody with a camelid Fab region fused to a humanized Fc region. The position of conserved glycan in the restricted interstitial space between the Fc domains of the heavy chains of an antibody typically restrains glycosyltransferase access, such as galactosyltransferase and sialyltransferase, which disrupts galactosylation and sialylation of an antibody (Jefferis, 2009). In the EG2 antibody, the lack of light chain pairings, in addition to its smaller size, may contribute to a more relaxed and open structural environment around the glycan site. Furthermore, two amino acid mutation sites around the glycan site, D270G and Y278H could both alter the size and charge of the associated amino acid residue, and contribute to the accessibility of glycosyltransferases, resulting in high GI of the Mab, under standard conditions. The significantly higher levels of GI and SI were also observed on the antibody missing an aromatic amino acid residue at the CH2 (Jassal et al., 2001) or CH3 (Rose et al., 2013) domain of human antibodies.

## 7.2 Conclusion and perspective

The study of the effect of lowered glucose on LLO distribution, both macro- and microheterogeneity revealed many valuable findings:

- Glucose limited culture conditions have an adverse effect on protein productivity.
- Glucose deprivation caused CHO-EG2 cells to produce primary truncated LLO intermediates instead of completed N-glycosylation precursor,  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ .

- Accumulation of truncated LLO intermediates affects N-glycosylation macroheterogeneity due to the possible reason of high specificity of OST and a limited metabolic energy status of CHO-EG2 cells.
- Glucose depletion affects N-glycan microheterogeneity, which was manifested by changes in the galactosylation and sialylation of the biantennary complex glycan structures on EG2 antibodies.

These findings have important implications for the rational design of a commercial bioprocess for the production of the therapeutic antibodies, in which the substrate levels are maintained at a low concentration to maximize productivity. In particular, periodic intervals of glucose depletion during fed-batch cultures could cause the production of non-glycosylation antibodies or low levels of galactosylation and sialylation

The findings of the present study also gave rise to many hypotheses as directions for future research. Investigating of nucleotide sugar content and the changes during glucose starvation states may show how glucose limitation sequentially affects an N-glycosylation pathway. Furthermore, chemostat culture should be recommended to reveal the minimal level of glucose concentration, which can resolve the conflict between prolongations of cell culture and maintain functional and sufficient N-glycosylation synthesis during industrial recombinant protein production.

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