The Effect of Glucose Depletion and Media Supplementation on the Productivity and Quality of Monoclonal Antibodies Production

By

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Dedication

Para ti papá

Mi ejemplo a seguir.
Mi gratitud y admiración
no se pueden expresar con palabras.
Espero algún día llegar a ser
tan grande, leal, humilde, decidido y entregado como usted.
Gracias por siempre impulsarme a ser mejor y
por enseñarme que siempre hay algo
nuevo que alcanzar.
Estos años dedicación son dedicados a usted.
Gracias por confiar en mí.

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Abstract

N-linked glycosylation of the Fc domain is an important posttranslational modification that can have a great impact on the therapeutic efficacy of monoclonal antibodies (Mabs). The oligosaccharide structure can be influenced by a variety of factors. Here, the glycosylation patterns of NS0-IgG1 and CHO EG2-hFc Mabs was evaluated under nutrient limitation and high cell densities, parameters commonly used in the production of recombinant proteins. Glucose depletion occurred in batch cultures containing ≤5.5 mM glucose and seeded at $2.5x10^5$ cells/mL or at ≥$2.5x10^6$ cells/mL at higher glucose concentration of up to 25 mM. Glucose-depleted cultures produced up to 51% of non-glycosylated Mabs along with a GI< 0.43 and decrease in sialylation of up to 85%. Reduced glycosylation was a result of limited synthesis of precursors of glycosylation (0.03-0.23 fmoles/cell) as well as a low adenylate energy charge (AEC < 0.57).

Galactosylation was shown to be a limiting step for sialylation. Thus, culture media was supplemented with manganese chloride (M), uridine (Urd) and galactose (Gal) in an attempt to increase GI of NS0-IgG1 and EG2-hFc Mabs. An improvement in GI of up to 33% was observed in Mabs produced in M+Gal-containing media. In NS0 cells, this increment also elevated by up to 135% alpha-Gal containing glycans. The latter was a response to an increase of up to 3.5-fold in UDP-Gal intracellular concentrations. Correspondingly, sialylated glycans also increased by up to 142.4% and 62% in NS0 and EG2 cultures respectively. Urd improved the GI in both cell lines but not sialylation. In EG2-hFc Mabs, Urd caused a decrease of up to 23.5% was observed in sialylation even after an improvement of 27.7% in GI. Masking terminal Gal residues with sialic acids has shown to increase glycoproteins' serum half-life *in vivo*. Thus, a substrate-based

approach was used to intercept the Sia pathway with different glycosylation precursors. However, supplementation with Ac_4GlcNAc (>50 μM) alone or in combination with Urd induced a decrease in sialylation of up to 33% in sialylation. Similarly, >200 μM Ac_4ManNAc alone or in combination with Cyt decreased both,- the GI by up to 23% and sialylation by up to 25-64% of IgG1 Mabs.

The relative abundance of Neu5Gc was also evaluated in cultures supplemented with glycosylation precursors. In NS0-IgG1, the relative abundance of Neu5Gc with respect to Neu5Ac decreased from 8:2 to 7:3; 6:4; 1:1; and 1:1 in M-; M+Gal-; M+Urd-; and M+Urd+Gal-containing cultures respectively. Similarly, the Neu5Gc:Neu5Ac ratio was reduced to 4:1 and to 1:1 in cultures containing >50 μM Ac_4GlcNAc or >200 μM Ac_4ManNAc respectively. Supplementation with free -Neu5Gc, -Neu5Ac and -Neu5Gc plus Neu5Ac were also evaluated on their effect over Sias proportions in NS0 and EG2 cells. In NS0 cells, Neu5Ac competed with newly synthesized Neu5Gc, changing the Neu5Gc:Neu5Ac ratio of 7:1 to 6:1. Supplementation of Neu5Gc+Neu5Ac also changed Sias ratio but in favor of Neu5Gc (9:1). No differences were found in EG2-hFc Mab. Knowing that the production of CMP-Neu5Gc requires the presence of iron, IgG1 Mab sialylation was evaluated in media supplemented with ferric citrate (FC). A decrease of up to 67.6% and 76.6% in GI and sialylation respectively was observed in IgG1 Mabs produced in chelated iron supplemented media. Furthermore, control's Neu5Gc:Neu5Ac ratio (8:2) changed gradually to 1:1 as the concentration of chelated FC increased from 0.1 to 10 μM.

Serum has been identified as a rich source of salvaged sugars for glycosylation, including the immunogenic Neu5Gc Sia. Thus, the effect of 10% Ultra-Low IgG FBS (UL-FBS) on the relative abundance of Neu5Gc-containing glycans was also evaluated in NS0-IgG and EG2-

hFc Mabs. UL-FBS reduced sialylation by up to 22% and 14% in IgG1 and EG2-hFc Mabs respectively. NS0 cells also showed a decrease of up to 24% in Neu5Gc along with a 5-fold increase in Neu5Ac-resulting in a Neu5Gc:Neu5Ac ratio of 1:1. Thus, fetal bovine serum with <5 μM IgG can be used as an strategy to reduced Neu5Gc in cells in culture.

The results presented in this thesis show that glycosylation patterns were strongly dependent on the cell producers' machinery, protein itself, nutrient availability and culture media supplements. Furthermore, improving IgG1 and EG2-hFc Mabs' *N*-glycosylation required more than just providing the cells with glycosylation precursors.

List of Abbreviations

Ac_4GlcNAc	Peracetylated glucosamine
Ac_4ManNAc	Peracetylated mannosamine
ADCC	Antibody-dependent cellular cytotoxicity
Ade	Adenosine
ADP	Adenosine diphosphate
AEC	Adenylate Energy Charge
AMP	Adenosine monophosphate
Asn	Asparagine
ATP	Adenosine triphosphate
CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary
CMAH	CMP-*N*-acetylneuraminate monooxygenase
CSS	CMP-sialic acid synthetase
CTP	Cytidine triphosphate
Cyt	Cytidine
Dol-	Dolichol
ELISA	Enzyme linked Immuno Sorbent Assay
ER	Endoplasmic reticulum
EUFS	Emission Unit Full Set
Fab	Fragment antigen binding
Fc	Fragment crystallisable
Fuc	Fucose
Gal	Galactose
GalT	Galactosyltransferase
GDP	Guanosine diphosphate
GI	Galactosylation index
GlcNAc	Glucosamine
GNE/MNK	UDP-GlcNAc-2-epimerase/ManNAc-6-kinase
GnT III	*N*-acetylglucosaminyltransferase III

GTP	Guanosine diphosphate
Gua	Guanosine
HAMA	Human anti-mouse antibody response
HEK	Human embryonic kidney cell line
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HT	Fibrosarcoma cell line
Ig	Immunoglobulin
IVCD	Integral viable cell density
Le	Lewis
LLO	Lipid-linked oligosaccharide
Manganese chloride	M
Man	Mannose
ManNAc	*N*-acetylmannosamine
MBL	Mannan binding lectin
MCD	Maximal cell densities
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
OST	Oligosaccharyltransferase
PBS	Phosphate-buffered saline
PEP	Phosphoenolpyruvate
PER.C6	Human embryonic retinal cells
pI	Isoelectric point
q_{Glc}	Specific glucose consumption rate
q_{Lac}	Specific lactate production rate
Ser	Serine
Sia	Sialic acids
ST	Sialyltransferase
Thr	Threonine
UDP-GalNAc	Uridine diphosphate-*N*-acetylgalactosamine
UDP-Glc	Uridine diphosphate-glucose

UDP-GlcNAc	Uridine diphosphate-*N*-acetylglucosamine
Urd	Uridine
UTP	Uridine triphosphate
$Y_{Lac/Glc}$	Yield coefficient, moles of lactate produced per mole of glucose utilized
μ	Specific cell growth rate

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Chapter 1

Introduction

1.1.Protein glycosylation

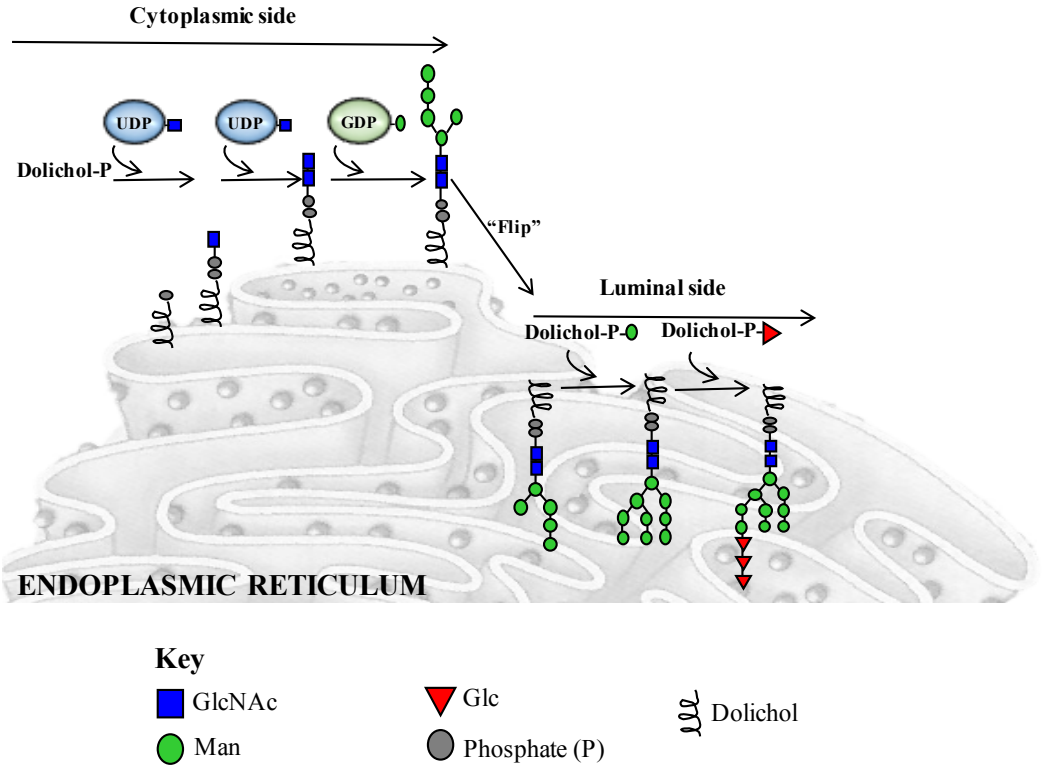
Glycoconjugates such as glycoproteins and glycolipids are made of mono-, oligo-, or polysaccharides attached to proteins or lipids respectively. The sugar portion of glycoconjugates is also referred to as glycans(Berger M. 2011). The biosynthesis of glycans, unlike that of DNA, RNA or proteins, is a non-template driven process that leads to a large diversity and heterogeneity of structures(Raman, Raguram *et al.* 2005). There are two main types of protein glycosylation depending on the amino acid to which the oligosaccharides are attached to: *N*-linked glycosylation corresponds to the oligosaccharides attached to the nitrogen of asparagine (Asn) residues and *O*-linked glycosylation corresponds to the oligosaccharides attached to the hydroxyl oxygen of serine or threonine (Ser/Thr) residues (Berger M. 2011) (Nobuko Hosokawa 2015).

The *N*-glycosylation pathway has been well established and consists of three stages: **1.** Synthesis of a lipid-linked precursor oligosaccharide (**Figure 1.1**); **2.** *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ precursor to the polypeptide (**Figure 1.2)** and **3.** processing of oligosaccharide by action of glycosyltransferases (**Figure 1.3**). First, the dolichol inserted into the lipid bilayer receives two GlcNAc and five mannose residues by action of glycoenzymes along with the nucleotide sugar donors UDP-GlcNAc and GDP-Man respectively (Berger M. 2011). The lipid-linked glycan is then translocated across the membrane where is further processed by the addition of four mannose residues and three glucose residues from dolichol-P- Man and dolichol-P-Glc.

The $Glc_3Man_9GlcNAc_2$ precursor is then transferred to the Asn-Xaa-Ser/Thr (Xaa any amino acid except proline) sequon of a nascent polypeptide by oligosaccharyltransferase complex (OST) (Berger M. 2011, Morihisa Fujita 2015).

Figure 1. 1 Assembly of the lipid-linked oligosaccharide (LLO) precursor $Glc_3Man_9GlcNAc_2$ in the endoplasmic reticulum (ER).

Early stages of LLO synthesis focus on the formation of $Man_5GlcNAc_2$ in the cytoplasmic side of the ER. This seven-sugar precursor is then flipped to the luminal side where it is further modified by the addition of four more mannose residues and three glucose residues. UDP-GlcNAc, GDP-Man, Dolichol-P-Man and Dolichol-P-Glc constitute the sugar donors used by glycosyltransferases.



Following the transfer to the polypeptide, glucosidases I and II are responsible of removing the three glucose residues located at the non-reducing termini of the glycan (Taylor and Drickamer 2006, Nobuko Hosokawa 2015). Trimming of the last glucose residue constitutes a quality control system that monitors proper folding and oligomerization of glycoproteins before exiting from the ER (Helenius 1994, Spiro 2000, Nobuko Hosokawa 2015). Thus, improperly folded proteins are retained in the ER where they are re-glucosylated by an α-glucosyltransferase (Nobuko Hosokawa 2015). The chaperons calnexin and calreticulin prevent premature protein oligomerization along with N-glycan degradation (Varki A 1999, Berger M. 2011).

Figure 1. 2 LLO en-bloc transfer to nascent protein by oligosyltransferase (OST). Once transferred, trimming of sugar residues by glycosidases takes place prior to exit the endoplasmic reticulum (ER). Improperly folded proteins enter the calnexin/calreticulin cycle and are retained in the ER until reaching a proper conformation.

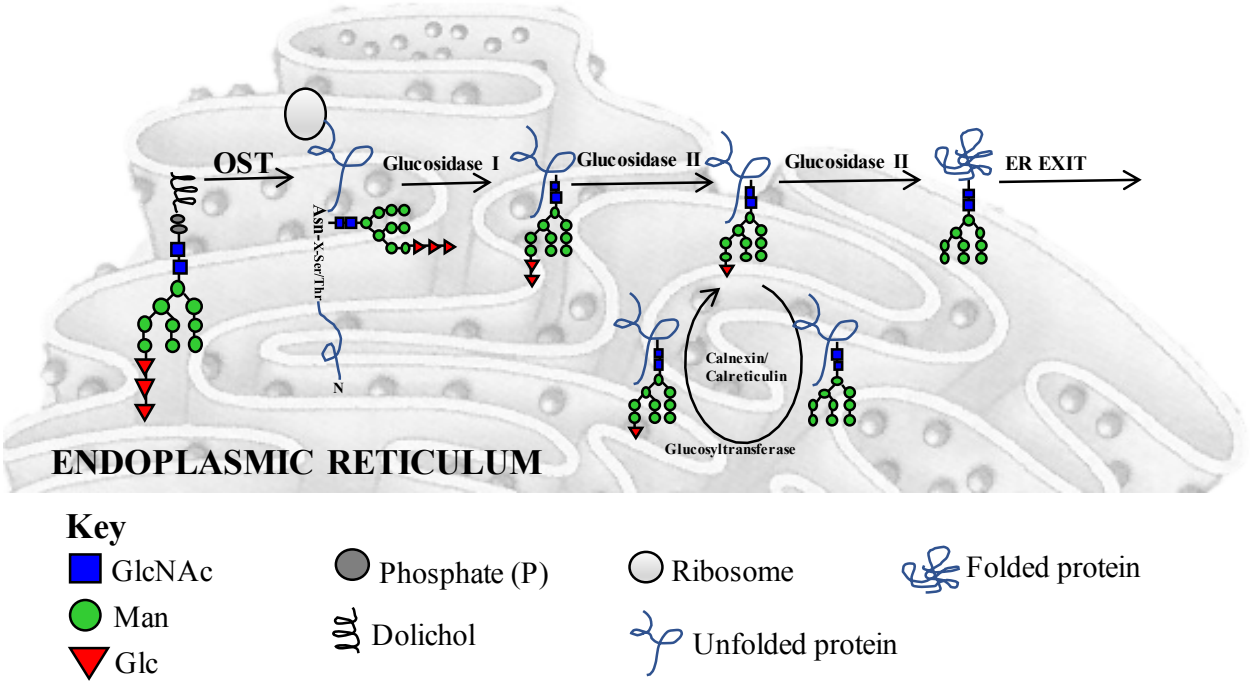
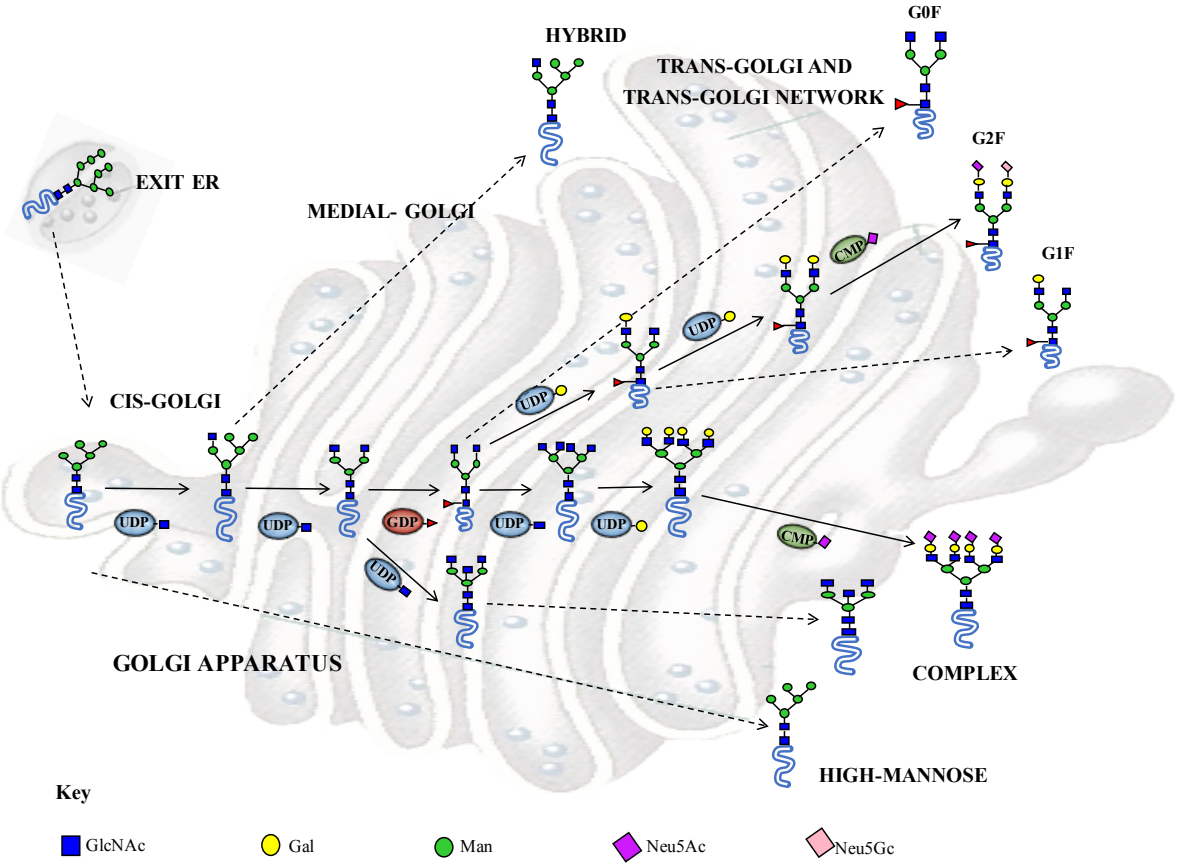


Figure 1. 3 Processing of oligosaccharide by action of glycosyltransferases in the cis-, medial-, and trans- portions of the Golgi Apparatus.
Reactions include the removal of mannose residues and step-wise addition of *N*-acetylmannosamine (GlcNAc), fucose (Fuc), galactose (Gal), and sialic acid (Sia)



Further processing consists in the removal of some or all α1-2 linked mannose residues in the ER and cis portion of the Golgi apparatus(Hristodorov 2013). The resulting structure $Man_5GlcNAc_2$ constitutes the starting point for the synthesis of hybrid- and complex-glycans (Berger M. 2011). Hybrid-glycans can arise when mannosidases fail to remove mannose residues from one of the branches while the other branch is processed by the stepwise addition of GlcNAc, Gal and Sia (Hristodorov 2013). In complex glycans, β1-2-linked GlcNAc is added to the Manα1-

3 arm while the remaining two mannose residues are removed for further addition of one, two, three or four more GlcNAc to produce bi-, tri-, and tetra- antennary structures (Savage 1997). In addition, bisecting GlcNAc can be added to the core oligosaccharide in the *medial* portion of the Golgi, preventing addition of fucose to the GlcNAc residue linked to asparagine (Varki A 1999, Hristodorov 2013). Further elongation consists in the addition of Gal and Sia by galactosyltransferases and sialyltransferases localized in the *trans* portion of the Golgi apparatus. Because nucleotide sugar donors (e.g. UDP-GlcNAc, GDP-Fuc, UDP-Gal and CMP-Neu5Ac) are made in the cytosol or in the nucleus, antiporters are responsible for facilitating the entry of the donors to the luminal side of the Golgi apparatus (Taylor and Drickamer 2006, Hristodorov 2013).

1.2.Sources of monosaccharides

Glycosylation processes require the conversion of monosaccharides into activated sugar nucleotides. For this to occur, monosaccharides are first either imported into the cell, salvaged from degraded glycoconjugates or derived from other sugars within the cell which occur in the cytoplasm (Varki A 2009). Then, nucleotide donors in the form of NTP or NDP react with a sugar or sugar-1-P act by action of a kinase to form high energy donors that can be used by glycosyltransferases in the Golgi. Another scenario is the interconversion of a previously synthesized activated sugar. During the first steps of biosynthesis of the dolichol oligosaccharide precursor $Glc_3Man_9GlcNAc_2$, activated sugars such as UDP-*N*-acetylglucosamine (UDP-GlcNAc) and GDP-mannose (GDP-Man) act as sugar donors to generate $Man_5GlcNAc_2$ at the cytosol. Then, once the $Man_5GlcNAc_2$- Dol precursor flips to the lumen of the ER where enzymes use lipid-linked sugars donors (e.g. Dol-P-Man, Dol-P Glc) to complete the synthesis of $Gal_3Man_9GlcNAc_2$

(Leszek A. 2015). Activated sugars that intervene in glycosylation biosynthesis in the Golgi include: UDP-*N*-acetylglucosamine (UDP-GlcNAc), UDP-galactose (UDP-Gal), GDP-fucose (GDP-Fuc), GDP-mannose (GDP-Man) (Kochanowski, Blanchard *et al.* 2006). In addition, CMP-*N*-acetylneuraminic acid act as a donor for sialylation of glycoproteins. CMP-Sia is the only monosaccharide which activation occurs as a mononucleotide in the nucleus by action of *N*-acylneuraminate cytidylyltransferase enzyme(UZH 2016).

Mammalian cells use glucose transport proteins (GLUTs) to facilitate the diffusion of hexose and pentose sugars into and out the cells(Blondeel and Aucoin 2018). Monosaccharides can also be transported by energy-dependent transporters such as sodium-glucose transporters (SGLT) (Cura and Carruthers 2012, Blondeel and Aucoin 2018). For instance, glucose is taken from the gut by SGLT-1 while fructose is carried out by GLUT-5 (Varki A 2009). These two carbon sources constitute the major carbon sources that once inside the cell deliver metabolic fuel and can be used for glycoconjugate synthesis(Blondeel and Aucoin 2018). With respect to mannose, an energy-dependent transporter analogous to the SGLT for glucose and an energy independent transporter have been described in mammalian cells, which are responsible for transporting up to 80% of the mannose required for glycoprotein synthesis (Varki A 2009). Monosaccharides can also result from recycling or salvage processes carried out by lysosomal enzymes (e.g. endo- and exo-glycosidases)(Varki A 1999). The sequential degradation is dependent of the structure and steric hindrance of the protein and sugar chain. Once cleaved, monosaccharides exit the lysosome and are transported to the cytosol where biosynthetic enzymes are located (Varki A 1999). Finally, monosaccharides can be interconverted into other sugars by action of several enzymes prior to their use in glycosylation reactions. For instance, glucose

converted to conversion first to Glc-6-P by an hexokinase and then to either Fru-6-P or Glc-1-P by action of phosphoglucose isomerase or by phosphoglucomutase respectively. Glc-1-P can then react with UTP to form UDP-Glc. Similarly, Gal is first phosphorylated at C-1 to give Gal-1-P which can react with UTP to form UDP-Gal. In addition, Gal-1-P can be converted into UDP-Gal by action of uridyl transferase. Finally, UDP-Gal can also result from UDP-Glc by action of UDP-Gal-4 epimerase. In the case of mannose, it can be phosphorylated by hexokinase to render Man-6-P or can result from the conversion of Fru-6-P by action of phosphomannose isomerase which then is converted to Man-1-P by phosphomannomutase (Varki A 1999). With respect to GDP-Fuc, it is derived from GDP-Man. First, the C-6 of GDP-Man is reduced from CH_2OH to CH_3 by action of GDP-Man 4,6 dehydratase and then C-4 of GDP-Man is oxidized to a ketone and then reduced by action of GDP-keto-6-deoxymannose 3,5 epimerase/4 reductase. Finally, UDP-GlcNAc forms after three consecutive steps; first GlcN-6-P forms from Fru-6-P by transamination and then Glc-6P is *N*-acetylated via acetyl-CoA isomerized rendering GlcNAc-6-P which then isomerized to GlcNac1-P. An alternative route is the direct phosphorylation of GlcNAc to GlcNAc-6-P via kinase (Varki A 1999).

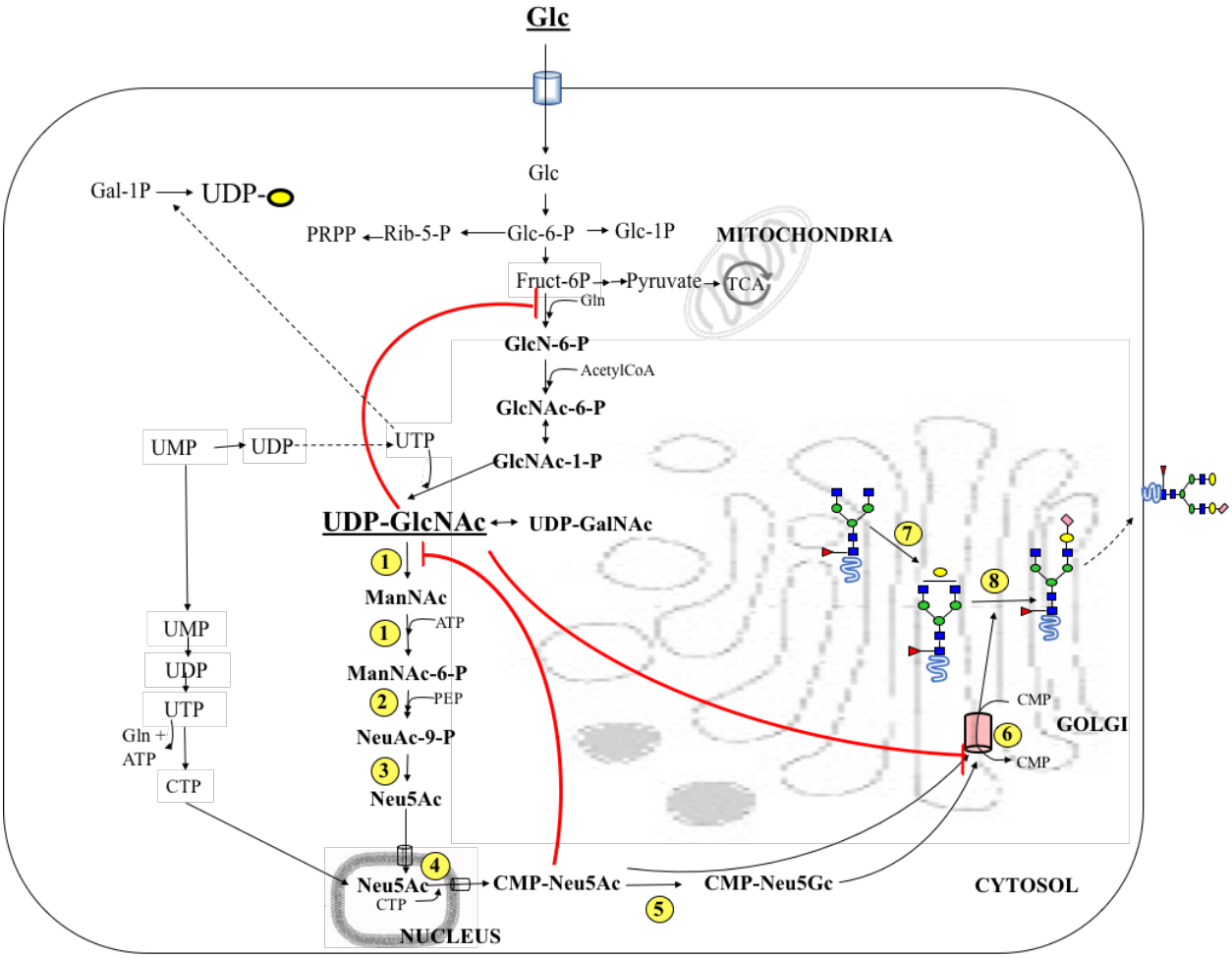
1.3.Sialylation of glycoproteins

Sias structures were elucidated after and they were released through mild acid hydrolysis of brain glycolipids and salivary mucins in the 1950s and 1960s (Varki and Schauer 2009). The family of Sias comprises approximately 50 members, all derivatives of the negatively charged 9-carbon sugar neuraminic acid (Berger M. 2011, Cao and Chen 2012). In mammalian cells, sialylation of glycoproteins starts by the conversion of UDP-*N*-acetyl-D-glucosamine (UDP-

GlcNAc) into UDP and *N*-acetyl-D-mannosamine (ManNAc) and subsequently by the phosphorylation of ManNAc at the sixth position (ManNAc-6-P) by action of UDP-GlcNAc-2-epimerase/ManNAc-6 kinase (GNE/MNK) (**Figure 1.4**)(Berger M. 2011).

Figure 1. 4 Sialic acid pathway in mammalian cells.

Enzymes and key steps of hexosamine pathways are: 1. UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE); 2. *N*-acetylneuraminic acid-9-phosphate synthase; 3. *N*-acetylneuraminic acid-9-hosphatase; 4. CMP-*N*-acetylneuraminic acid synthase; 5. CMP-*N*-acetylneuraminic acid hydroxylase; 6. Sia transporter; 7. Galactosyltransferase; 8. Sialyltransferases (trans-Golgi). Red flat arrows indicate sites of feedback inhibition.



Condensation of ManNAc-6-P with phosphoenolpyruvate (PEP) results in Neu5Ac-9-P, which after dephosphorylation by a specific phosphatase yields free Neu5Ac. Previous to its incorporation into mammalian glycoconjugates, activation of Neu5Ac occurs in the nucleus by action of CMP-sialic acid synthetase (CSS) (Gu and Wang 1998, Martinez-Duncker and Tiralongo 2013). Neu5Ac can be further modified to other Sias prior to be transferred to cell surfaces and secreted proteins and lipids in the lumen of the Golgi apparatus by sialyltransferases.

For sialylation to occur terminal Gal residues need to be available for sialyltransferases to add Sias in the *trans*-portion of the Golgi (Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001, Crowell, Grampp *et al.* 2007, Gramer, Eckblad *et al.* 2011). Excessive production of Neu5Ac can cause GNE enzyme to shut down, limiting the production of ManNAc along with a reduction in the formation of Neu5Ac(Berger M. 2011). Thus, GNE plays a key role in regulating Sia biosynthesis by binding to the downstream product CMP-Neu5Ac. This feedback inhibition is specific for the GNE enzyme epimerase activity, leaving the ManNAc kinase activity intact (Chen, Huang *et al.* 2016).

Sias are hydrophilic, large and acidic molecules that can influence the conformation of glycoconjugates they are bound to and can alter the protein's surface charge and isoelectric point (pI) which in turn has been related to prolonged circulatory lifetimes for glycoproteins (Sola and Griebenow 2010, Berger M. 2011). The latter is of great importance in biotherapeutics because the presence of Sias prevent clearance by asialyloglycoproteins receptors in the liver (Durocher and Butler 2009, Lalonde and Durocher 2017). Due to the fact that Sias occupy terminal positions on glycan chains, they can promote or prevent the biological recognition by bacterial toxins, animal

viruses, mycoplasma, plant and animal lectins (Higa and Paulson 1985). Thus, Sias can influence the life span of molecules and cells, promoting biological processes (e.g. protein binding, inflammation, immune responses) as well as pathological events (e.g. tumor metastasis) Rosenberg (1995), (Bardor, Nguyen *et al.* 2005, Du, Meledeo *et al.* 2009, Berger M. 2011, Cao and Chen 2012). The high diversity of Sias originates from **1.** Different α linkages (e.g. α2-3; α2-4; α2-6; α2-8, α2-9)(Amon, Reuven *et al.* 2014); **2.** Oligosaccharide chain position (e.g. side chain attach to Gal, GlcNAc or GalNAc or internal position linked to other sialyl residues)(Amon, Reuven *et al.* 2014); **3.** Natural modifications at the hydroxyl groups of C-4, C-7, C-8 and C-9 (e.g. *O*-acetyl, *O*-methyl, *O*-sulfate, *O*-lactyl or phosphate groups) and **4.** Unsaturated and anhydro forms of free Sia (e.g. 2-deoxy-2,3-didehydro-Neu5Ac) (Corfield A.P. 1982, Muchmore EA 1998 , Amon, Reuven *et al.* 2014). The most common Sias found in nature is Neu5Ac. Mammalian cell lines used to produce biotherapeutic glycoproteins (e.g. Chinese hamster ovary (CHO) cells, baby hamster kidney cells, murine myeloma, and murine hybridomas cell lines) also express Neu5Gc(Berger M. 2011). The latter results from the addition of a single oxygen atom to the *N*-acetyl group of Neu5Ac by CMP-*N*-acetylneuraminate monooxygenase (CMAH). Due to exon deletion in the CMAH gene, humans have lost the ability to synthesize Neu5Gc(Amon, Reuven *et al.* 2014). Nevertheless, Neu5Gc can be taken up from animal-derived culture medium materials and/or dietary sources (e.g. red meat, milk) and displayed in secreted glycoproteins from human cells (Tangvoranuntakul, Gagneux *et al.* 2003). In fact, it has been shown that during lysosomal degradation of glycoconjugates by neuraminidases, free Sias can be recycled and exported back into the cytosolic compartment where they can be reactivated by CMP-Sia synthases (Seppala, Tietze *et al.* 1991, Verheijen, Verbeek *et al.* 1999). Neu5Gc-containing therapeutic glycoproteins can cause unwanted immune responses because humans possess anti-Neu5Gc antibodies which

can reach high levels (0.1-0.2% of circulating IgG)(Yu 2016). Furthermore, Neu5Gc can accumulate in tissues causing chronic inflammation and may facilitate the progression of diseases (e.g. cancer and atherosclerosis) (Ghaderi, Taylor *et al.* 2010).

Loss of Sia has been shown to reduce glycoprotein's solubility and serum half life (Gramer, Goochee *et al.* 1995, Berger M. 2011). Low sialylation can occur under different circumstances: **1.** Limited access of CMP-Neu5Ac/Gc to the Golgi apparatus; **2.** Limited steric accessibility for sialyltransferases to reach sialylation sites; **3.** availability of sialyltransferases; **4.** Presence of sialidases which can remove previously bound Neu5Ac/Gc to the glycoprotein **5.** Low intracellular levels of CMP-Sia (Durocher and Butler 2009); presence of ammonium ions (NH^{4+}) by a pH dependent mechanism (Gawlitzek, Valley *et al.* 1998, Grammatikos, Valley *et al.* 1998, Baker, Rendall *et al.* 2001); increase in UDP-GlcNAc (Zanghi, Mendoza *et al.* 1998). The degree of sialylation can also variate depending on cell line, recombinant protein being produced and culture conditions(Hossler 2011). For instance, human cells attach Sias in both α-2,6 and α-2,3 linkages while hamster cells add Sia only in α-2,3 linkages (Durocher and Butler 2009). In addition, the levels of Neu5Gc produced in CHO cells has shown to variate between 1-15%, different from murine cell lines like NS0 which have shown levels over 60% (Baker, Rendall *et al.* 2001, Chenu, Gregoire *et al.* 2003, Lalonde and Durocher 2017).

1.4.Factors influencing glycosylation

N-linked glycosylation does not always proceed to completion and asparagines that are potential glycosylation sites may not have oligosaccharides attached. Variation in the site-

occupancy with oligosaccharides is referred to as macroheterogeneity and has been associated to changes in specific growth rates and metabolic state of the cell (Curling, Hayter *et al.* 1990, Hossler 2011), steric interference and local environment of amino acids that surround the sequon (Savage 1997). Nucleotide sugars availability constitute another key factor that strictly regulate the attachment of sugars into glycoconjugates(Blondeel and Aucoin 2018). Nucleotide sugars are made in the cytosol, except CMP-sialic acid which is generated in the nucleus, either from *de novo* biosynthesis or from the recycling of monosaccharides from proteins being degraded in the lysosome. Nucleotide sugars are then transported to the lumen of the endoplasmic reticulum and Golgi apparatus where they serve as sugar donors to glycosyltransferases (Pels Rijcken, Hooghwinkel *et al.* 1990). Failure in the synthesis of the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ needed for transfer to nascent protein has arisen due to low levels of UDP-GlcNAc and GDP-Man (Rearick, Chapman *et al.* 1981), low levels of Dol-P (Jenkins, Castro *et al.* 1994) which in turn affects the levels of lipid-linked sugar donors (i.e Man-P-Dol, Glc-P-Dol) responsible for the donation of monosaccharides. Another factors influencing the macroheterogeneity of proteins is the level and specificity of lumenal mannosyl and glucosyl-transferases (Stark and Heath 1979, Schenk, Fernandez *et al.* 2001, Jones, Tomiya *et al.* 2010, Hossler 2011), rate of folding and glucose deprivation (Stark and Heath , Chapman and Calhoun 1988). Glucose-deprived cells have shown to be unable to assembly the complete lipid-linked oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-PP-Dol, instead truncated lipid-linked mannosylated intermediates lacking glucose residues are predominant (e.g. $Man_2GlcNAc_2$-PP-Dol, $Man_5GlcNAc_2$-PP-Dol) or $Glc_3Man_5GlcNac_2$-PP-Dol (Turco 1980); (Rearick, Chapman *et al.* 1981). The latter can be transferred to protein in the lumen of the ER, resulting in abnormal glycosylated proteins.

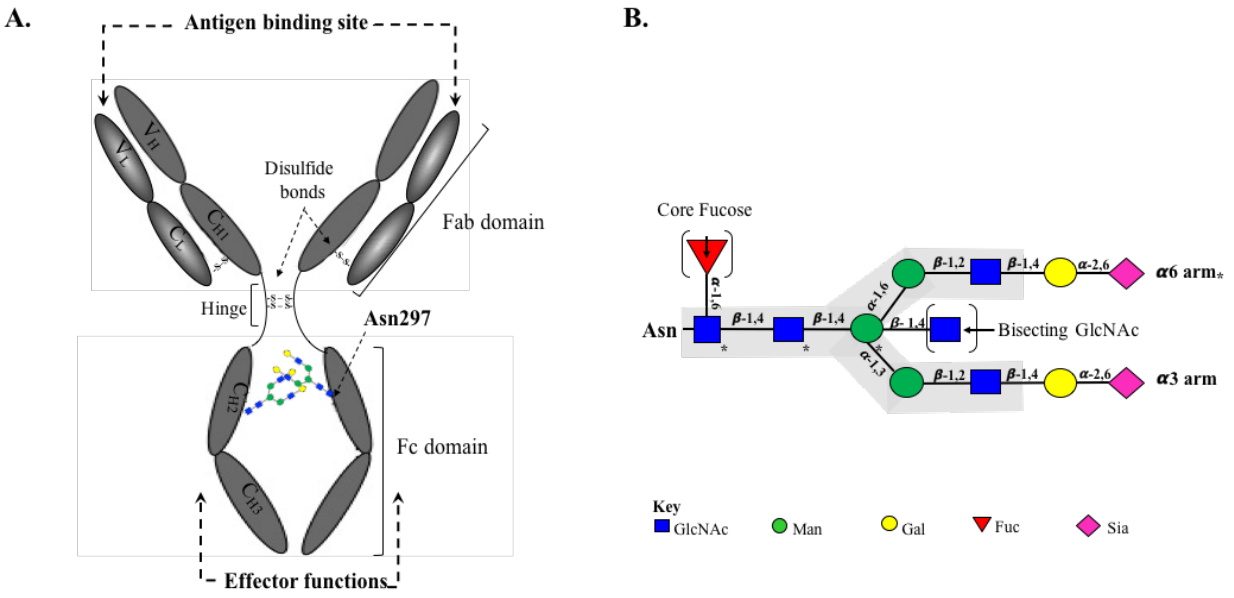
The second type of heterogeneity is called microheterogeneity which arises from the trimming sugar reactions, number of antennae, and sugar additions (i.e fucose, *N*-acetylglucosamine, Gal or sialic acid) that occur once the oligosaccharides are attached to the protein (Savage 1997). Different factors can influence the *N*-glycans diversification such as the glycoprotein conformation itself (Wright and Morrison 1997), the enzymatic machinery of the chosen expression system(Savage 1997, Taylor and Drickamer 2006, Hossler 2011), transport rates in the ER and Golgi, and various extracellular environmental variables such as nutrient limitation (Barnabe and Butler), metabolic waste accumulation Yang and Butler (2002), rate of elongation of protein synthesis (Butler), oxygen (Kunkel, Jan *et al.*), culture pH (Kunkel, Jan *et al.*), temperature (Gstraunthaler 2003), mode of culture (Hayter, Curling *et al.* , Patel, Parekh *et al.* 1992, Andersen and Goochee 1994, Majid, Butler *et al.*); and the intracellular concentrations of precursors (Nyberg, Balcarcel *et al.* , Jefferis 2002, Blondeel and Aucoin 2018). Changes in *N*-linked glycosylation have also been related to the status in diseases. For instance, the amount of IgG-G0 in healthy human serum (35%) increases in patients with autoimmune diseases (e.g. rheumatoid arthritis, Crohn's disease), infectious diseases (e.g. tuberculosis, HIV) and cancer (e.g. ovarian cancer) (Arnold, Wormald *et al.* 2007, Jefferis 2009, Jefferis 2009). These increase in non-galactosylated proteins are thought to contribute to the pathology of the disease, predisposing antibodies to associate with immune complexes and by binding to GlcNAc receptors expressed on the surface of macrophages(Patel, Parekh *et al.* 1992). In addition, the extent of IgG glycosylation has also been seen to vary depending on age, sex and pregnancy(Raju 2008, Huhn, Selman *et al.* 2009, Jefferis 2009, Jefferis 2009).

1.5. Immunoglobulins as glycoproteins

Antibodies (or immunoglobulins, Ig) are responsible for recognizing, blocking and neutralizing foreign cells and macromolecules, triggering their elimination (Huber, Deisenhofer *et al.* 1976, Amon, Reuven *et al.* 2014). Specific recognition involves the Ig-Fab (fragment antigen binding) portion which is complementary to the antigen whilst enhancement of effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) involves the Ig-Fc (fragment crystallisable) region. Ig-Fab and Fc regions are linked by a flexible hinge region allowing flexibility to bind antigens and accessibility to engage with effector ligands such as Fc receptors and C1q component of complement (Jefferis 2009). All Ig molecules are composed of two identical heavy chains and two identical light chains which are linked together by disulfide bonds. In humans, there are two types of light chains (κ and λ) and five types of Ig heavy chains (α, δ, ε, γ and μ). Secreted Igs have been grouped into five classes according to their heavy chains structure: IgG, IgM, IgA, IgD and IgE, and within human IgG and IgA four and two subclasses respectively (Arnold, Wormald *et al.* 2007, Hristodorov 2013). Among these, IgG is the most abundant immunoglobulin constituting 75% of immunoglobulins in human blood and extravascular fluids (Jefferis 2009). IgGs have a long catabolic half-life of 23 days (IgG1, IgG2 and IgG4), an attribute that has been exploited to increase the *in vivo* activity of therapeutic proteins (Jefferis 2009). The total molecular weight of IgGs is approximately 150 kDa, with two identical heavy chains (H, 50 kDa) and two identical light chains (L, 25 kDa) (Arnold, Wormald *et al.* 2007, Jefferis 2009) **(Figure 1.5A).** In addition, heavy chains contain a variable domain (V_H) and three constant domains (C_{H1}, C_{H2} and C_{H3}) whilst light chains contain one variable domain (V_L) and a single constant domain (C_L)(Hristodorov 2013). The variable domains

of heavy and light chains bear three complementarity determining regions (CDRs) responsible for the antigen specificity and affinity (Aires da Silva 2008). The hydrophobic surfaces of V_H-V_L, C_{H1}-C_L and C_{H3}-C_{H3} domains are paired together while C_{H2}-C_{H2} are not, due to the presence of a single conserved *N*-linked glycosylation site at Asn-297 (**Figure 1.5A**) (Lund, Takahashi *et al.* 1996).

Figure 1. 5 Normal human IgG-Fc domain N-glycosylation.
Representation of the asparagine 297- (Asn297) linked oligosaccharide structures are responsible for antigen binding and effector functions respectively. Heavy chains ($_H$) contain one variable domain (V_H) and three constant domains (C_{H1}, C_{H2} and C_{H3}). Light chains ($_L$) contain one variable domain (V_L) and one constant domain (C_L). B. IgG-Fc oligosaccharide is of the complex type and is constituted by a core heptasaccharide (shade) that may be further modified by the addition of GlcNAc, Fuc, Gal and Sia. *Residues involved in interactions with the surface of the protein.



Due to the presence of the oligosaccharides in the C_{H2} domains, the Fc portions acquire an open conformation (horse-show like structure) which is necessary for binding to FcγRI, FcγRII

and FcγRIII receptors and the C1q component of complement (Arnold, Wormald *et al.* 2007, Nimmerjahn and Ravetch 2007, Aires da Silva 2008, Huhn, Selman *et al.* 2009, Jefferis 2009) (Hossler 2011, Hristodorov 2013). X-ray crystallography has shown that a single IgG molecule can contain different glycoforms at each of the Asparagine 297 (Asn-297) motifs (Sutton and Phillips 1983).

Human IgG Fc glycans are mainly complex bi-antennary structures constituted by a common core containing two *N*-acetylglucosamine (GlcNAc) residues linked to Asn-297 and three mannose (Man) residues. Further modifications can occur by the addition of bisecting GlcNAc, core Fuc, Gal and Sia (Jefferis 2002, Quast, Keller *et al.* 2015) **(Figure 1.5B).** The largest variations occur in the content of Gal with glycan chains containing zero (G0), one (G1) or two (G2) Gal residues(Peng, Patil *et al.* 2018). Among the residues, the core GlcNAc, first mannose and the 1-6 branch of each oligosaccharide chain interact with the surface of C_{H2} domain through hydrophobic interactions (H-bonds) with amino acid residues (Sutton and Phillips 1983). These non-covalent interactions restrict the movement of the glycan (Sutton and Phillips 1983, Krapp, Mimura *et al.* 2003). Furthermore, the opposite Fc-oligossacharide chains can also interact with each other, contributing to the atypical disposition of the C_{H2} domains (Lund, Takahashi *et al.* 1996, Krapp, Mimura *et al.* 2003). Fc glycans can make up to 2%-3% of the molecular weight of IgG molecule (150 kDa) and are responsible for stablishing hydrophobic and hydrophilic interactions with amino acid from the C_{H2} domains (Arnold, Wormald *et al.* 2007). These interactions are important not only to maintain a proper conformation but also because minor variations in terminal sugars can perturb interactions with immune complexes (Scallon, Tam *et al.* 2007). For example, aglycosylated Mabs have shown a closed conformation, which destabilizes

Igs molecules and perturbs the binding regions for C1q component of complement and Fc receptors (Lund, Takahashi *et al.* 1996, Wright and Morrison 1997, Krapp, Mimura *et al.* 2003, Jefferis 2009, Harris, Chin *et al.* 2010). In the same manner, truncated oligosaccharides lacking terminal GlcNAc and mannose residues also show a closer conformation of the C_{H2} domains which results in a reduced affinity for FcγRIIb (Shields, Lai *et al.* 2002, Krapp, Mimura *et al.* 2003, Raju 2008, Jefferis 2009). In contrast, high mannose structures ($Man_{5-9}GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$) are cleared at a faster rate than complex glycans, thus reaching very low levels in humans(Raju 2008). The presence of Gal residues has shown to enhance complement-dependent cytotoxicity (CDC) but have no effect on FcR mediated effector functions (Jefferis 2009, Harris, Chin *et al.* 2010). Without Gal, GlcNAc residues are recognized by lectin molecules such as mannan binding lectin (MBL) and mannose receptor (MR), which results in the activation of the complement cascade and reduced serum half-life. The presence of bisecting GlcNAc / absence of fucose which have been related to enhanced FcγRIII-mediated ADCC activity (Umana, Jean-Mairet *et al.* 1999, Harris, Chin *et al.* 2010, Peng, Patil *et al.* 2018). With respect to Sia, less than 10% of the total IgG molecules have sialylated glycans, possibly because of sialyltransferases' limited accessibility to the Fc portion of IgGs and not because of deficits in the processing Sia machinery(Jefferis 2009). Sia has been shown to have anti-inflammatory properties which results from a reduced affinity for FcRIIIa observed in highly sialylated IgG (Scallon, Tam *et al.* 2007, Raju 2008, Jefferis 2009). In addition, glycans increase protein resistance to proteases thanks to steric and electrostatic repulsions(Hristodorov 2013). These proteolytic stabilizations depend on the number of glycans, their length and branching, and charges of terminal end glycans. Thus, glycans can influence protein's surface charge and isoelectric point (pI) which can prolong recombinant proteins *in vivo* lifetime (Sola and Griebenow 2010).

Fab glycosylation has been observed because of random hypermutations occurring randomly in both variable domains of light and heavy chains (V_H and V_L). The somatic mutations are believed to occur to influence on antigen binding (Jefferis 2009). There are no consensus sequences for *N*-linked glycosylation in either of the constant chains of heavy and light chains (C_{H1}, C_{H3} and C_L) (Jefferis 2009). As a result, up to 30% serum IgGs can contain the glycosylation motif Asn-Xaa-Ser/Thr in one or both V domains (Raju 2008, Hristodorov 2013). Human monoclonal Fab glycosylation is characterized by complex bi-antennary structures that are highly galactosylated and sialylated in contrast to oligosaccharides present in the Fc region (Jefferis 2009).

1.6.Monoclonal antibodies

In 1975, Köhler and Milstein developed the mouse hybridoma technology where an antibody producing B lymphocytes were fused with immortal myeloma cells. The resultant hybrid cells retain the immortal characteristics and are able produce large amounts of a single antibody (Delves and Roitt 1998, Walsh 2003). However, murine monoclonal antibodies had a limited use as therapeutics in humans because they triggered a human anti-mouse-antibody response (HAMA). In an attempt to reduce the immunogenicity of mouse antibodies, recombinant DNA technology was used to manipulate monoclonal antibodies (Mabs) leading to more humanized variants. To reduce Mab's immunogenicity, mouse variable regions were linked to human constant domains of the human IgG molecule which lead to the development of chimeric antibodies. However, chimeric antibodies still posed risk of eliciting immune responses. Thus, another attempt to further reduce

the immunogenicity of murine elements, was the development of humanized antibodies which were produced by associating mouse antigen-binding sequences (CDRs) with human framework regions. Nevertheless, even fully human Mabs, which are 100% human in sequence, were also able to induce minimal therapeutic side effects (Gomord, Chamberlain *et al.* 2005). In 1989, 11% of all biologic products approved corresponded to monoclonal antibodies-based products, with a prevalence for humanized over chimeric Mabs (Walsh 2014). Furthermore, their high specificity has made monoclonal antibody technology extremely valuable with a steady increase of up to 24% in Mabs approvals within the period of 2010-2014. Thus, Mabs have become the most lucrative single product class with $69.8 billion in global sales in 2013 (Zhang, Woen *et al.* 2016).

Mabs have been used as therapeutics to protect against pathological conditions by preventing interaction of growth factors, cytokines or other soluble mediators with their receptors(Jefferis 2009) and to diagnose and treat a wide variety of illnesses (e.g. cancer, many auto-immune disorders) (Delves and Roitt 1998, Brekke and Sandlie 2003, Jefferis 2009, Jefferis 2009, Walsh 2014). Mabs mode of action and efficacy will depend on the kind of oligosaccharide that is attached to a single site on each of the IgG heavy chains. Variations in the sugar composition of glycan chains can result in subtle changes in structure, stability, solubility, immunogenicity, and serum half-life. Thus, glycosylation is now considered as a Critical Quality Atribute (CQA) that must be monitored to ensure safety, potency and efficacy of any biopharmaceutical product before regulatory approval (Zhang, Woen *et al.* 2016, Peng, Patil *et al.* 2018). So far, most rMabs approved are of the human IgG isotype and are produced in mammalian cells, mainly Chinese hamster ovary (CHO) cells or mouse myeloma-derived cells (e.g SP2/0 and NS0 cells) because of their human-like glycosylation (Durocher and Butler 2009, Lalonde and Durocher 2017).

1.7.Expression systems

One of the key steps during a bioprocess is selecting a high-producing cell line while achieving consistency during manufacturing (Hossler 2011, Lalonde and Durocher 2017). Mammalian cell lines are the preferred expression system chosen for therapeutic protein production because they have been extensively characterized and genetically modified to produce high yields of recombinant protein therapeutics with human-like glycosylation (Lai, Yang *et al.* 2013). The most widely used include Chinese hamster ovary cells (CHO), baby hamster kidney (BHK21) cells and murine myeloma cells (NS0 and Sp2/0)(El Mai, Donadio-Andrei *et al.* 2013, Yu 2016, Lalonde and Durocher 2017). However, several minor differences exist between mammalian and human cells glycosylation machineries. For example, mammalian cells (e.g. CHO, BHK) lack enzymes such as α-2,6 sialyltransferase, α-1-3/4 fucosyltransferase and bisecting *N*-acetylglucosamine transferase which are present in human cells **(Figure 1.6)** (Raju 2003, Durocher and Butler 2009, Hossler 2011, Hossler 2011, Lalonde and Durocher 2017). Furthermore, mouse-derived cell lines contain an additional enzyme referred as α1,3 galactosyltransferase which gene is not functional in humans and higher primates (Amon, Reuven *et al.* 2014, Yu 2016). The alpha-Gal epitope has also been found to a lesser extent in hamster cell lines such as BHK-21 and CHO cells (Bosques, Collins *et al.* 2010, Flickinger 2013). The presence of alpha-Gal in non-primate mammals and New World monkeys, not only constitutes a barrier for organ transplantation but also limits the use of murine cell lines used to produce biologics such as Mabs (Steinke, Platts-Mills *et al.* 2015, Lalonde and Durocher 2017).

In addition, mouse and hamster cell lines produce oligosaccharides containing Neu5Gc in addition to Neu5Ac, which differ by the presence of only an additional oxygen atom in NGNA(Raju 2003, Hossler 2011, Lalonde and Durocher 2017) **(Figure 1.6)**. The levels of Neu5Gc produced in CHO cells has shown to variate between 1-15%, different from murine cell lines like NS0 which have shown levels over 60% (Baker, Rendall *et al.* 2001, Chenu, Gregoire *et al.* 2003, Lalonde and Durocher 2017). Neu5Gc is immunogenic to humans due to an irreversible mutation in the CMP-*N*-acetylneuraminic acid hydroxylase enzyme (CMAH) responsible for the formation of Neu5Gc from Neu5Ac(Amon, Reuven *et al.* 2014). Unlike Neu5Gc, alpha-gal residues are normally absent from human tissues and cannot be incorporated from dietary sources. In addition, CHO and human cells contain a higher proportion of core fucosylated glycans (~95%) in comparison to mouse cell lines (10-40%) (Raju 2003). The presence of immunogenic epitopes can reduce the efficiency of a given therapeutic due to rapid clearance by the immune system(Lalonde and Durocher 2017). In addition, immune reactions can arise after re-exposure to non-human epitopes (Durocher and Butler 2009, Amon, Reuven *et al.* 2014). Human *N*-glycosylation structures such as α2-6 Neu5Ac, Neu5α2-8-Neu5Ac, bisecting GlcNAc and Le^x and sLe^x are not found in hamster cells(Flickinger 2013). Similarly, mouse cell lines lack some glycan structures present in human cells such as Le^x, sLe^x, Fucα1,2-Gal, Galβ1-3GlcNAc and GalNAcβ1-4-GlcNAc (Flickinger 2013). However, mouse and humans express the enzymes α2-6 ST and α2-3 ST (Ozturk and Hu 2005, Hossler 2011).

Human cells-based expression systems (i.e. HEK-293, HT-1080, PER.C6) can produce recombinant proteins with post-translational modifications that closely resemble their *in vivo* counterparts. However, the use of human cells imposes the risk of transfer of human adventitious agents due to the lack of a species barrier. Furthermore, the use of human cell lines does not

guarantee the absence of Neu5Gc as human cells can metabolically incorporate this immunogenic residue from animal-derived products present in culture media (Swiech, Picanco-Castro *et al.* 2012). Another disadvantage is that human cells are not very well characterized as CHO cells.

Non-mammalian host expression systems such as insects, plants and yeasts also produce glycoforms distinct from those observed in human cells(Hossler 2011). With respect to insect cells, their glycoproteins include hybrid, high-mannose or pauci-mannose glycans as well as non-human sugars such as α-1,3 fucose. In the case of plant cell lines, glycoproteins contain immunogenic sugars such as α1-3 fucose and xylose and lacking Gal and Sias while yeast cells produce hypermannosylated glycans (Subramanian 2012). These non-human glycosylation patterns can make a glycoprotein less potent, more immunogenic and thus less effective when used as an biotherapeutic. Thus, special concern has been directed towards minimizing/eliminating the addition of immunogenic epitopes or identifying clones with an acceptable glycan profile (Dumont, Euwart *et al.* 2016).

Knowing that complex proteins require post-translational modifications for their biological activity and that the complexity and type of glycosylation can be tissue- and cell-type specific within a given species, different strategies have focused on attaining a final product that closely resembles the native protein (Goochee and Monica 1990, Raju 2003, Hossler 2011, Dumont, Euwart *et al.* 2016). These strategies include: **a.** genetic engineering of host cells to enhance (e.g. α2,6-ST in CHO, BHK; GnTIII in CHO, NS0) or decrease the activity of specific glycosyltransferases (e.g. FUT gene knockout in CHO; α1,3-GalT gene knockout in NS0 and CHO) (Hossler 2011, Subramanian 2012)(Blondeel and Aucoin 2018)**; b**. prevention of final product degradation by glycosidase enzymes (Gramer and Goochee 1993, Kaneko, Sato *et al.*

2010); **c.** feeding sugars or other substrates required for oligosaccharides glycosylation (Gramer, Eckblad *et al.* 2011, Blondeel and Aucoin 2018); **d.** use of exoglycosidases and glycosyltransferases to remodel glycoforms (Zhang, Woen *et al.* 2016, Blondeel and Aucoin 2018). These strategies can reduce the production of unfavorable glycan structures (i.e. pauci-mannose, high mannose, non-galactosylated, non-sialylated) and immunogenic residues (i.e. Neu5Gc, alpha-Gal, xylose)(Hossler 2011).

1.7.1 NS0, DP12 and CHO EG2 cells

Murine NS0 cells are non-immunoglobulin secreting myeloma cells originated from a mineral-oil-induced plasmacytoma (MOPC-21) in BALB/c mouse. After sequential cloning, the non-secreting clone P3-NSI/1Ag4.1 (NS1) was isolated and constituted the origin of NS0 cells over 20 years ago (Whitford 2003). NS0 cell are cholesterol auxotrophs which require the presence of cholesterol; however, some cholesterol-independent cells have been established (Li, Vijayasankaran *et al.* 2010). The presence of immunogenic residues such as Neu5Gc acid and alpha-Gal epitope have limited the use of these cell lines to produce therapeutic antibodies(El Mai, Donadio-Andrei *et al.* 2013). Chinese hamster ovary cell line DP12 (ATCC clone #1934) produces an anti-interleukin 8 (IL-8)-antibody that weights 150 kDa. DP12 cells are reported to produce up to 250 mg/L of recombinant IgG1which neutralizes IL-8 by binding to human neutrophils (Zhang, Liu *et al.* 2009, Bell, Wang *et al.* 2010). Chinese hamster ovary cell line (CHO DUXB, CHO-DG44 clone 1A7), also referred as EG2 cells, produces a chimeric camelid:human/anti-hu-epidermal growth factor receptor Mab against the epidermal growth factor receptor (EGFR) overexpressed in tumor cells. The produced camelid antibody is devoid of light chains and consist

of only a variable heavy (V_H), and two constant heavy domains (C_H2, C_H3); with two antigen binding sites and a molecular weight of ~80kDa (without consideration of glycosylation) (**Figure 1.7**) (Zhang, Liu *et al.* 2009, Bell, Wang *et al.* 2010). In here, CHO-DG44 cell line will be referred as EG2 cells.

1.8.Culture media

Mammalian cell culture media has around 60 individual components that will provide the cells with functional proteins, growth factors, sources of carbon and trace elements for cell metabolism, growth and proliferation (Hodge 2005). Thus, culture medium supplies biosynthetic precursors for cell anabolism; catabolic substrates for energy metabolism; vitamins and trace elements for primarily catalytic functions; and inorganic ions for both catalytic and physiological functions (Brunner, Frank *et al.* 2010, Lalonde and Durocher 2017). In addition, culture media may contain uncharacterized or semi-characterized components that can result in lot-to-lot inconsistencies. This is the case of serum which has been widely used as a supplement for *in vitro* cell culture because it is rich in transport proteins, cytokines, hormones, co-factors, essential minerals, trace elements and growth factors, all of which promote animal cell proliferation (Gstraunthaler 2003, Hossler 2011). However, serum's undefined and variable composition makes it difficult to conserve a comparable quality of the final product between batches. In addition, serum can be a source of possible contamination with bacterial, fungal, prions and viruses (Hodge 2005, Brunner, Frank *et al.* 2010). Furthermore, serum is a source of contamination with proteins that contain Neu5Gc which can be incorporated by cultured cells which can result in immunogenic reactions in humans (Corfield A.P. 1982).

Figure 1. 6 Schematic comparison of typical N-linked glycosylation features in human, mouse and hamster cell lines.

Structures presented in brackets are characteristic of each cell line. The expression of immunogenic residues such as alpha-Gal and Neu5Gc variates in mouse vs. hamster cell lines. Hamster cell lines lack the enzyme α2-6 ST present in mouse and human cells. Human cells containing bisecting GlcNAc opposite to mouse and cell lines. Key shows the symbol corresponding to each monosaccharide

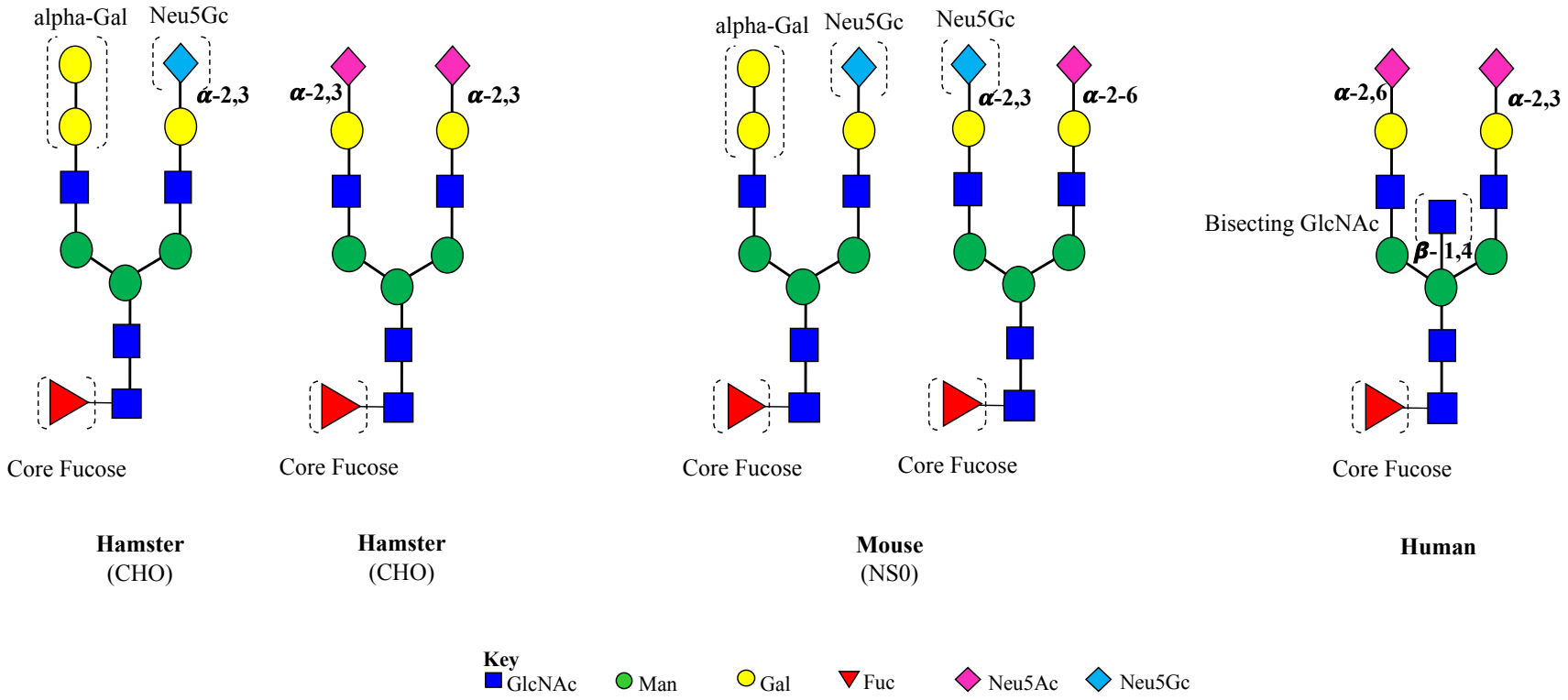
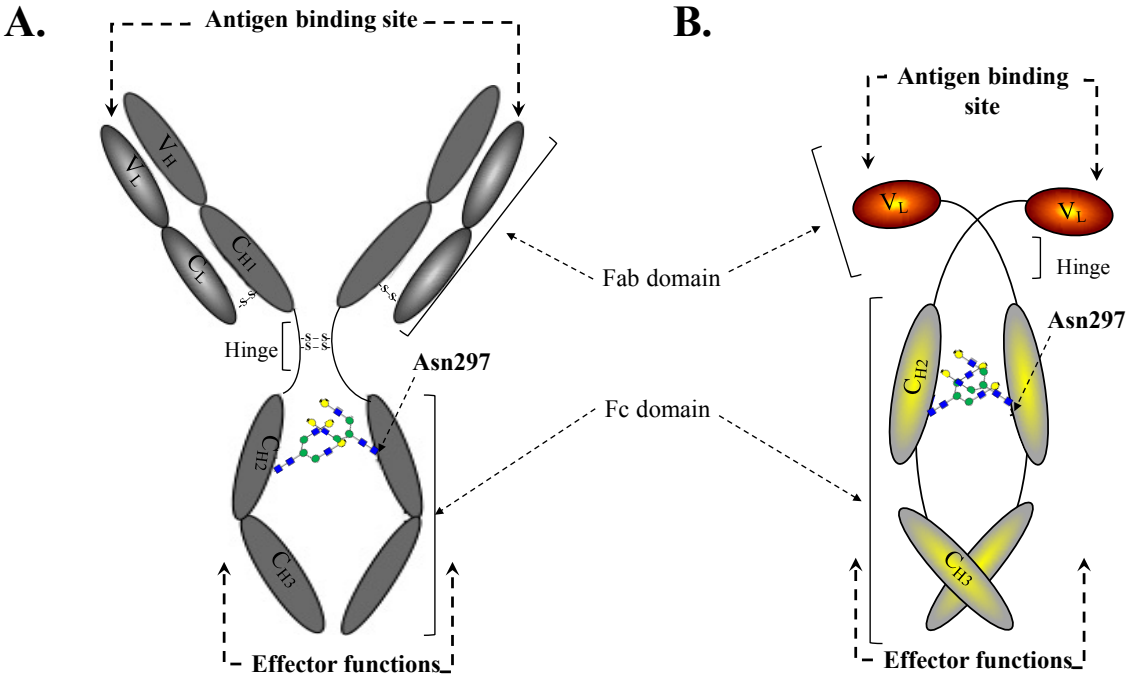


Figure 1. 7 Schematic comparison of IgG1 and EG2-hFc Mab

A. Typical mammalian immunoglobulin G (IgG) produced by NS0 and DP12 cells and **B.** camelid heavy chain (EG2-hFc Mab) detailing the antigen binding Fab region and the Fc portion involved in effector functions. The molecular weights of the presented antibodies are 150 kDa and 80 kDa for IgG and EG2-hFc Mabs respectively. The antigen binding and effector functions sites are indicated by arrows



Another source of batch to batch variation is the usage of protein hydrolysates (e.g. yeast, soy, wheat, and other plant sources) that have an undefined nature (Butler 2004, Hossler 2011). Different strategies have been implemented to replace the components present in serum-based media due to animal-free components may or may not provide the same performance as animal-derived components. Thus, key serum proteins (i.e. bovine serum albumin, transferrin, and insulin) have been incorporated into the formulation of serum-basal media (Butler 2004). Thus, eliminating both animal-derived materials (i.e. serum) and complex and undefined components (i.e. protein

hydrolysates) has led to the development of chemically-defined media. Chemically defined media formulation provides a more defined composition, reduced degree of microbial contamination, a lower cost, a more consistent performance and an easier purification and downstream processing(Hossler 2011).

1.9.Thesis objectives

Monoclonal antibodies have become the largest and fastest growing class of therapeutic pharmaceuticals used for the treatment of a large number of diseases (e.g. cancer, inflammatory and autoimmune diseases). In 2015, the global monoclonal antibody market accounted for up to $85.4 billion and it is expected to continuously grow by up to 5.7%. Thus, pharmaceutical manufacturers need to meet the high demand for Mabs by increasing yields while maintaining the quality of the product. This thesis focuses on the effect that nutrient limitations and feeding strategies have on cell growth, metabolism, specific cell productivity and particularly on the *N*-glycosylation of monoclonal antibodies.

Section A focused on a well-studied parameter in cell culture bioprocesses which is limited cell exposure to glucose. The latter has been used as an strategy to lower cells' specific glucose consumption and as a consequence to reduce cell's specific lactate production. Many literature studies have investigated the effect of glucose limitation on cells' metabolism, productivity and recombinant proteins' glycosylation. However, there is limited information on the effect that nutrient limitation and cell inocula have on Mabs, particularly on single chain antibodies. Therefore, the following constitute the objectives of this section: **a)** To investigate the

effect that glucose starvation had on both, cell metabolism as well as in Mab's *N*-glycosylation in mammalian cell cultures inoculated at low and high inocula; **b**) To determine if glucose starvation has the same effect in two different types of Mabs, DP12-IgG1 and CHO EG2-hFc single chain antibody; **c)** To assess the differences found in *N*-glycosylation of Mabs produced by starved cells via analysis of intracellular levels of nucleotide and nucleotide sugar precursors.

Section B and C focused on implementing feeding strategies to improve *N*-glycosylation in Mabs produced by murine and mouse cell lines. Since glycans have a profound impact on effector functions, *in vivo* half-life and immunogenicity of proteins, glycosylation has become a critical quality attribute (CQA) required to ensure safety and potency of Mabs and other biopharmaceutical drugs. In an attempt to improve Mabs *N*-glycosylation, the *N*-glycosylation pathways were used as a reference to design sugar and nucleotide precursor feeding strategies. In addition, particular attention was directed to non-human glycans commonly added by mammalian cells (e.g alpha-Gal, Neu5Gc) which can be responsible for immunogenic reactions. Finally, NS0 and EG2 cell's abitity to incorporate Neu5Gc from external sources was also evaluated in IgG1 and EG2-hFc Mabs. Thus, the main objectives of these two sections were as follow: **a)** To establish the effect of galactosylation precursors (e.g. galactose (Gal), manganese chloride (M) and uridine (Urd) and sialylation precursors (e.g peracetylated mannosamine, Ac_4ManNAc; peracetylated glucosamine, Ac_4GlcNAc; cytidine, Cyt; uridine, Urd; free Sias, Neu5Gc and Neu5Ac; ferric citrate and ferrous sulfate) on mammalian cell growth, viability and productivity; **b)** To enhance galactosylation and sialylation by supplementing culture media with the aforementioned galactosylation and sialylation precursors; **c)** To determine the effect of galactosylation precursors on the relative abundance of the immunogenic residues alpha-Gal and Neu5Gc in IgG1 Mab

produced by NS0 cells; **d)** To investigate the effect of sialylation precursors on the amount of Neu5Gc with respect to Neu5Ac in IgG1 Mab produced by NS0 cells; **e)** To explore the ability of using much lower concentrations of the Sia precursors analogs (Ac_4ManNAc, Ac_4GlcNAc)to improve sialylation of Mabs; **f)** Investigate FBS's influence on sialylation, particularly on the content of Neu5Gc in antibodies produced by two different mammalian cell lines, NS0 and EG2, known to have different initial Neu5Gc content.

Chapter 2

Materials and Methods[1]

2.1 Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Media supplements were cell culture grade or of the highest purity available. Milli-Q water used for solution preparation was filtered (Millipore filtration system) by reverse osmosis. Cell culture procedures were performed inside a laminar flow cabinet.

2.2 Cell culture

2.2.1 Cell lines

EG2 cells correspond to a transfected CHO (DUXB) cell line (CHO-DG44 clone 1A7) expressing a chimeric human-llama (EG2-hFc) monoclonal antibody targeting epidermal growth factor receptor (EGFR) was provided by Yves Durocher from the NRC, Canada (Zhang, Liu *et al.* 2009). The EG2-hFc Mab has a size of ~80 kDa without consideration of glycosylation (Zhang, Liu *et al.* 2009, Bell, Wang *et al.* 2010). The second cell line (CHO DP12 clone #1934) was acquired from the ATCC (CRL-12445^{TM}). cells are reported to produce up to 250 mg/L of recombinant IgG1 against IL-8 in batch culture. Finally, a proprietary NS0 (non-secreting null) cell line (HU1D10) was provided by Abbvie (Redwood City, CA, USA). This cell line was

[1] Partial content of this chapter was included in the following publications:
- Villacres, C., et al. "Low Glucose Depletes Glycan Precursors, Reduces Site Occupancy and Galactosylation of a Monoclonal Antibody in Cho Cell Culture." *Biotechnol J* 10.7 (2015): 1051-66.
- Braasch, K., Villacres, C., and Butler, M. (2015), Evaluation of quenching and extraction methods for nucleotide / nucleotide sugar analysis: in Glyco-Engineering: Methods and Protocols (ed. Alexandra Castilho), Methods in Molecular Biology. Humana Press.

transfected with a gene for recombinant humanized IgG1 having 90% human and 10% murine amino acid sequence.

2.2.2 Media Formulation and culture maintenance

Cell cultures were harvested at mid-exponential phase where viability was over 95%. Cells were cultured in 250 mL shaker flasks with a culture volume of 80 mL of Biogro™ media containing different culture supplements. Shake flasks were shaken at 120 rpm in a shaker platform inside humidified incubators at 37°C and 10% CO_2.

CHO EG2 and DP12 cells were sub-cultured every three to four days in Biogro™ serum-free media (SFM) (Biogro™ Technologies, Winnipeg, MB.) supplemented with 0.5 g/L of yeast extract (BD, Sparks, MD). Biogro™ medium was developed at the Butler's lab and its formulation is proprietary. Biogro™ media contains 25g/L of glucose, a starting pH of 0.5 g/L and an osmolality of 285-300 Osm/kg. NS0 cells were adapted to grow in suspension in media lacking cholesterol. The media defined as PFBM-1 was a proprietary chemically defined protein free basal medium from Abbvie. This media has 15 g/L glucose, starting pH of 7.2±0.1 and osmolality of 300 mOsm/kg H_2O. NS0 cells were then adapted to grow in the proprietary serum-free media Biogro™ supplemented with 0.5 g/L yeast extract. Continuous passaging was performed every 3-4 days with an initial cell density of $2.5x10^5$ cells/mL into 125 mL vented shake flasks (VWR international) in humidified incubators at 37°C and 10% carbon dioxide.

2.3 Cell counting

Cell density and viability were determined using the trypan blue dye-exclusion method using a Cedex XS Analyzer (Roche, Indianapolis, IN, USA). This dye-exclusion test is based on the principle that live cells have intact membranes which exclude certain dyes, such as trypan blue, eosin or propidium whereas dead cells absorb the dye due to the loss of integrity of their membranes and therefore stain blue. Daily supernatant samples of 900 µL were taken from shake flasks to determine cell density and viability. A 50 µL of cell culture supernatant was mixed with 50 µL 0.4% Trypan blue solution (Gibco^{TM}, Fisher Scientific, USA) and loaded onto a Cedex XS slide (Roche). Slides were then entered the Cedex Analyzer for image analysis where information such as cell density, viability, cell diameter and cell aggregation was obtained. Supernatants were then spun down at 1600 rpm for 5 minutes in a microcentrifuge for metabolite analysis and protein production determination. The viable cell concentration was used to calculate the specific growth rate (µ), defined as the number of cells produced per cell per hour after exponential growth using:

$$\mu = \frac{lnX_{v2} - lnX_{v1}}{t_2 - t_1} \qquad \textbf{Eq. 1}$$

where X= viable cell concentration (cells/mL), t= time (h), μ= specific growth rate (h^{-1})

2.4 Sample preparation prior to analysis

Cell culture supernatant was spun down to get rid of cells and debris at 1500 rpm for five minutes in megafuge. The resultant supernatant was collected, filtered using a 50 mL 0.2 µm filter

and concentrated 10-fold using 30 kDa Amicon MWCO (Sigma. Catalog # Z71785). Using the same Amicon filters, supernatant was washed with previously filtered Dulbecco's Phosphate-Buffered Saline (PBS, Gibco). Samples were frozen at -20°C before further analysis.

2.5 Analysis of media components

Daily supernatant samples of 900 μL were taken from shake flasks to determine cell density and viability. Supernatants were then spun down at 1600 rpm for 5 minutes in a microcentrifuge for metabolite analysis and protein production determination. CHO EG2 and DP12 cells were harvested on day four and six respectively. Cell culture was spun down, and supernatant was filtered, concentrated and stored as

2.6 Glucose and lactate concentration

Glucose and lactate concentrations were measured simultaneously using the YSI 2700 biochemical analyzer (Yellow Springs Instruments). YSI technology employs an enzyme membrane that is coupled with an electrochemical probe and housed in a chamber where the sample is delivered. The substrate will diffuse through a thin polycarbonate membrane where encounters an oxidase enzyme where one or more catalyzed reactions will result in the production of hydrogen peroxide (H_2O_2). Hydrogen peroxide will then be oxidized producing a signal current at an electrochemical probe. For the determination of glucose and lactate concentrations, a 50 μL of previously spun down supernatant was injected and the following reaction occurs:

$$Dextrose + O_2 \xrightarrow[Glucose\ oxidase]{} H_2O_2 \text{ + D-Glucono-δ-Lactone}$$

$$L - Lactate + O_2 \xrightarrow[L-Lactate\ oxidase]{} H_2O_2 + \text{Pyruvate}$$

2.7 Specific consumption and production rates

Specific substrate consumption rate (q_s) and specific production rate (q_{mab}) were calculated from:

$$\boldsymbol{q_{Mab}} = \frac{C_{protein}\,(t) - C_{protein}\,(t_0)}{\int_0^t XV\,(t)dt} \qquad \boldsymbol{Eq.2}$$

where C =concentration (μg/l), q = specific production rate (pg/cell/day), X = viable cell, ∫ = viability index (x10^6 cell-day/mL).

2.8 Isolation and purification of Mab

On day 4 or 6, cell supernatant was collected after centrifugation at 1500rpm for 5 minutes and filtered with 0.22 μm membrane filter to remove cell debris remaining in the media. Samples were concentrated from 5X to 10X using multi weight cut off filters and the media was exchanged with Dulbecco's Phosphate-Buffered Saline (PBS, Gibco). Previously concentrated cell culture supernatant containing the Mab was purified using Protein A HP SpinTrap™ columns (GE Healthcare, Pittsburgh, PA, USA). Protein A columns were prepared by removing the storage solution by centrifugation. All centrifugation steps were performed for one minute at 1600 rpm. Protein A media was then re-suspended in 600 μL of binding buffer (PBS, pH 7.0). Columns were

spun down to remove the binding buffer and up to 600 μL of culture supernatant were loaded. Tubes were mixed gently for about 4 minutes to allow antibody binding, and then the contents were spun down. Several sample applications were made consequently until reaching an amount of up to 50 -100 μg/mL. Two consecutive washes with 600 μL of binding buffer were performed with the filtrate being discarded every time. For antibody elution, 400 μL of elution buffer (0.1M Glycine, pH 2.7) were added to the protein A columns in two consecutive rounds. The eluant was collected in a 2 mL microfuge tube containing 30 μL neutralizing buffer (1M Tris-HCl, pH 9.0). Purified samples were run on reduced SDS-PAGE gel to validate the purity of the samples before proceeding to further analysis. EG2-hFc Mab appeared as a band at 40 kDa and IgG1 as two bands one at 25 kDa and another at 50 kDa.

2.9 Antibody concentration

2.9.1 ELISA

EG2-hFc and IgG1 Mab concentrations were determined by enzyme-linked immunosorbent (ELISA) assay following the MabNet SOP# MNSOP009. Briefly, a sandwich ELISA was performed using a goat anti-human IgG (Fc-specific) antibody (Sigma, St. Louis, MO, I2136) as the plate-coating antibody overnight at 37 °C. After blocking plate for an hour at room temperature, previously purified culture supernatant and standard were diluted and added into the 96-well plate where incubation took place for an hour to allow binding of antibody. A highly concentrated and pre-purified CHO-EG2 supernatant was used as a standard. A horseradish peroxidase (HRP)-conjugated goat antihuman IgG (Fc-specific) antibody (Sigma, St. Louis, MO, A0170) was added as the detection antibody. After an hour, 3,3′, 5,5′-Tetramethylbenzidine digydrochloride (TMB) substrate solution was added to react with the peroxidase for 20-25

minutes at room temperature, with the plate wrapped in tinfoil. After the substrate solution reaction, plate was read at 630 nm to assess the assay prior to stop the reaction with H_2SO_4. 50 µL /well of 2 M H_2SO_4 were added, changing the color from blue to yellow. Absorbance was measured at a wavelength of 450 nm. Optical densities (OD) provided from samples and blanks were used to calculate sample concentrations by comparing their OD with respect to the standard curve.

2.9.2 A280 protein quantitation by Nanodrop 2000

Antibody concentration was also measured using NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA). First, 2 µL of elution buffer (0.1M glycine, pH 2.7 containing 1M tris-HCl, pH 9.0) were placed on the pedestal to blank the instrument. After wiping off the buffer, 2 µL of previously purified antibody were placed on the pedestal. The absorbance readings obtained at A280 nm were used to calculate antibody concentrations based on the Beer-Lambert equation:

$$A = \varepsilon * c * L \qquad \textbf{Eq. 3}$$

Where A corresponds to absorbance, ε is the wavelength-dependent absorptivity coefficient (or extinction coefficient) with units of liter/mol-cm, L is the length of solution the light passes through in centimeters and c is the analyte concentration in moles per liter. Solving the expression of Beer's Law for concentration yields the following equation:

$$\boldsymbol{c} = \frac{A}{\varepsilon\, L} \qquad \textbf{Eq. 4}$$

The molar extinction coefficients used for these calculations are presented in **Table 2.1**

Table 2. 1 Molar extinction coefficients and molecular weights for IgG1 and EG2-hFc used to calculate Mab concentration in solution by A280 in Nanodrop 2000.

Mab	Molecular Weight (kDa)	Molar extinction coefficient ($M^{-1}cm^{-1}$)
IgG1 (NS0, DP-12)	150	210,000
EG2-hFc (CHO)	80	114,805

2.10 SDS-PAGE electrophoresis

Concentrated and purified protein-containing samples were diluted (1:1) in Laemli sample buffer containing beta-mercaptoethanol, bromophenol blue and 10% SDS and denatured by heating at 95°C for 5 minutes. The samples were separated according to their molecular weight by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on 8-16% Criterion™ TGX Precast Gels (Bio-Rad, Mississauga, ON, Canada) using a Tris/SDS/Glycine pH 8.3 running buffer. The Precision Plus Protein™ Dual Color Standard was used as a protein marker (10-250 kDa, Bio-Rad #161-0374) which was added to either end of the gel to determine the molecular weights of the sample proteins. Gels were placed in a Bio-Rad Criterion gel box and run at 200V for about one hour using Bio-Rad power supply (Model 1000/500). Gels were stained with Coomasie blue for at least 3 hours and then were submerged in Destain solution 1 containing 50% (v/v) methanol, 7% (v/v) acetic acid, 43% water for about 30 minutes and later in Destain solution 2 containing 5% (v/v) methanol and 7% (v/v) acetic acid, 88% water until bands were clearly visible and the background was almost clear.

2.11 Gel Analysis

2.11.1 Molecular Weight Determination and Densitometry

An AlphaImager (ProteinSimple, Santa Clara) was used to capture images of gels before further gel processing. Images were taken using white light translumination. The AlphaEaseFc software was used to calculate molecular weight and densitometry on bands. A molecular weight marker (The Precision Plus Protein™ Dual Color Standard) added to either end of the gel was used to determine the molecular weights of unknown bands. For this purpose, the known molecular weights for markers were entered manually. Unknown bands were selected manually and molecular weight was automatically calculated by software based on the known marker bands.

For densitometry analysis, regions of interest were selected manually by using a rectangle tool. The object was drawn so that it enclosed the band of interest, but did not included an excessive background area. Any background signal was subtracted by using the multi-regional background correction option where a background region was manually selected closely to each band. This to account for differences in background levels across the gel. The band analysis results were displayed as the sum of all pixel values after background correction.

2.11.2 *N*- glycan release, extraction and labeling

2.11.2.1 In-gel

In-gel glycan analysis was performed following Royle *et al.* protocol (Royle, Radcliffe *et al.* 2006). After separation using SDS-PAGE, band corresponding to Mab were cut and frozen. Dye was removed from the gel pieces using a series of washing steps using a mixture 1:1 of acetonitrile and 20mM $NAHCO_3$ pH 7.0 and with $NAHCO_3$ alone, each wash last 30 minutes on

a mixer. A final wash was done with pure acetonitrile and then tubes were placed on speed vacuum (Thermo, Waltham, MA, USA) until gel pieces were completely dry. *N*-glycans release was accomplished by incubation with 6 μL of 1000 U/mL PNGase-F (Roche, Mannheim, Germany) and 54 μL of wash buffer (20mM $NAHCO_3$ pH 7.0) overnight at 37 °C. Extensive washing and sonication of the treated gel pieces was done to remove the released glycans. Sonication rounds were done using water followed by acetonitrile, pipetting the liquid containing glycans out into a 1.5 mL microcentrifuge tube. The final extract was dried down in speed vacuum overnight and resuspended in 500 μL milliQ water. Desalting of glycan-containing samples was done with Dowex-50W (Sigma 21751-4) with milliQ water. Once Dowex solution settled down, liquid was recovered and filtered through a 0.45 μm syringe-driven filter unit (Millipore, Billerica, MA) into a fresh microcentrifuge tube and dried down in speed vacuum prior to 2-AB labelling.

2.11.2.2 In-solution

Protein A HP SpinTrap™ columns (GE Healthcare, Pittsburgh, PA, USA) were used to perform glycan analysis following Venkata *et al* protocol (Tayi 2015). Storage solution from protein A columns was removed by centrifugation followed by two consecutive washes with 600 μL of 20mM phosphate buffer (80% 20mM Na_2HPO_4 + 20% 20mM NaH_2PO_4). Protein A columns were plugged at the bottom and previously concentrated and purified Mab samples were loaded. Incubation at room temperature took place for 10 minutes on an orbital shaker to allow antibody binding. Columns content was spun down and if necessary samples (50-100 μg/mL) were reloaded into the columns. Columns were washed three times with 600 μL of phosphate buffer. For glycan extraction, columns were plugged at the bottom and 150 μL of phosphate buffer and 10-20 units

of PNGase F (Roche, Mannheim, Germany) (1-2 μL) were added. Samples were incubated for 24 hours at 37 °C on a rotary shaker. Cleaved glycans were collected in a 1.5 mL tube and columns were washed with 200 μL of milliQ water to recover any glycans remaining in the columns. Bound antibody was collected separately and discarded. Glycans were 0.45 μm filtered (Millipore, Billerica, MA) and dried down in speed vacuum. Samples were kept at -20 °C prior to 2-AB labelling.

2.12 2-Aminobenzamide (2-AB) Labeling and clean-up

The released N-glycans were subsequently labeled with 2-aminobenzoic acid (2- AB) labeling solution which was freshly prepared by subsequently dissolving 10 mg 2-AB and 6.2 mg sodium cyanoborohydride ($NaBH_3(CN)$) in a 200 μL mixture of 350 μL DMSO with 150 μL glacial acetic acid. Labelin solution (5 μL of 2-AB) was added to the tubes containing the dry glycans followed by incubation at 65°C for two hours. 2-AB labeled glycans were stored at –20°C. Excessive label was removed using HyperSep™ Diol cartridges (Thermo Fisher Scientific). Columns were prepared by washing the cartridges with 1 mL of ultrapure water and then by conditioning with 4 mL of acetonitrile (1 mL each time). Labeled samples were re-suspended into 40 μL of acetonitrile, mixed well and then transferred on to the top of the cartridge. After sitting for 15 minutes allowing glycans to bind, cartridges were washed with 6 mL of acetonitrile (1 mL each time). Finally, glycans were eluted with 3 x 400 μL of ultrapure water into a 1.5 mL micro-centrifuge tube. Samples were dried in speed vacuum overnight. A dextran calibration standard was prepared by mixing 5 μL of 5ug/ml standard solution (Oxford GlycoSystems). Dextran sample was labeled with 2-AB, cleaned up and dried down as described above.

2.13 HPLC profiling of 2-AB-labeled N-glycans

The 2-AB glycan pools were analyzed by hydrophilic interaction liquid chromatography (HILIC) using a Water HPLC system equipped with a 1525 μ binary pump, 2707 auto-sampler and 2475 multi-wavelength fluorescent detector. The Waters X-Bridge amide column (3.5μm; 4.6 x 250 mm) was housed in an Eppendorf CH-30 column heater box where temperature was kept at 30 °C. For fluorescence detector settings see **Table 2.2**. Data acquisition and analysis was performed using Empower 2.0 software. The flow rates of all HPLC elutions were carried out at 0.86mL/min using a gradient as shown in **Table 2.3**. Running buffer (buffer A) was 50 mM ammonium formate brought to pH 4. 4 with ammonia hydroxide. Buffer B was acetonitrile. HPLC gradient buffers were filter through a 0.2 μm filter and degassed using an in-line degasser attached to HPLC system.

Table 2. 2 Settings for 2475 multi-wavelength fluorescent detector.

Parameter	Set value
Mode	2D channel
Excitation	330 nm
Emission	420 nm
EUFS	10000
Gain	1
Time constant	1.5

Samples were re-suspended in 15 μL of milliQ water and mixed well. HPLC vials were prepared by adding 24 μL of acetonitrile and 6 μL of sample into a HPLC auto-sampler tube. Each batch of samples included a dextran ladder followed by a blank sample with the injection volume set to 25 μL. Dextran calibration standard was reconstituted in 100 μL of milliQ water. From this solution, 1 μL of standard was mixed up with 5 μL of milliQ water and 24 μL of acetonitrile.

Dextran ladder chromatogram was integrated with empower software, obtaining the retention times of at least 13 glucose unit (GU) polymers.

Table 2. 3 Flow rate and HPLC gradient for 1525 μ Binary Pump

Time	Flow	Buffer A	Buffer B	Curve
	0.86 mL/min	20	80	6
48	0.86 mL/min	50	50	6
49	0.86 mL/min	100	0	6
53	0.86 mL/min	100	0	6
55	0.86 mL/min	20	80	6
63	0.86 mL/min	20	80	6
64	0 mL/min	20	80	6

GU values were fit as a function of the retention time (t) of dextran standard with a polynomial equation:

$$\boldsymbol{GU} = a \times t + b \times t^2 + c \times t^3 \qquad \boldsymbol{Eq.5}$$

The retention times of samples were inserted into the same fifth order polynomial equation to obtain GU values corresponding to individual peaks by reference to a dextran ladder (**Figure 2.1**). The GU value obtained is glycan-specific and can be used to predict structures that will differed on the number and linkage of monosaccharides present. Samples' GU values, retention times and peak areas were recorded for further analysis using the NIBRT Glycobase database (https://glycobase.nibrt.ie) (Dublin, Ireland). GU values were matched to known GU values in the database, making possible a preliminary assignment of glycan structures. When the glycan structures shared the same GU value, an exoglycosidase digest was performed to confirm the identity of the structures.

Figure 2. 1 An example of dextran ladder calibration curve used to define the relative retention time of 2-AB-glycans in terms of GU values.

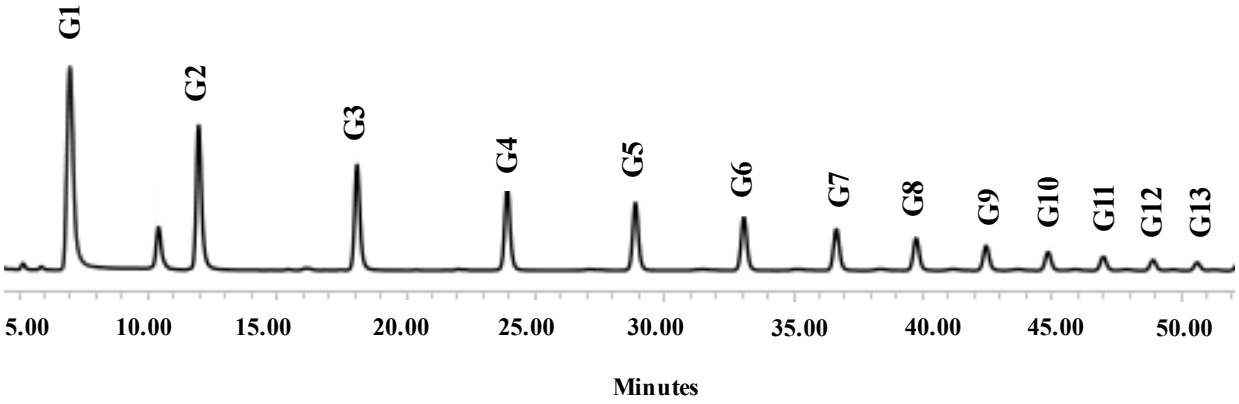
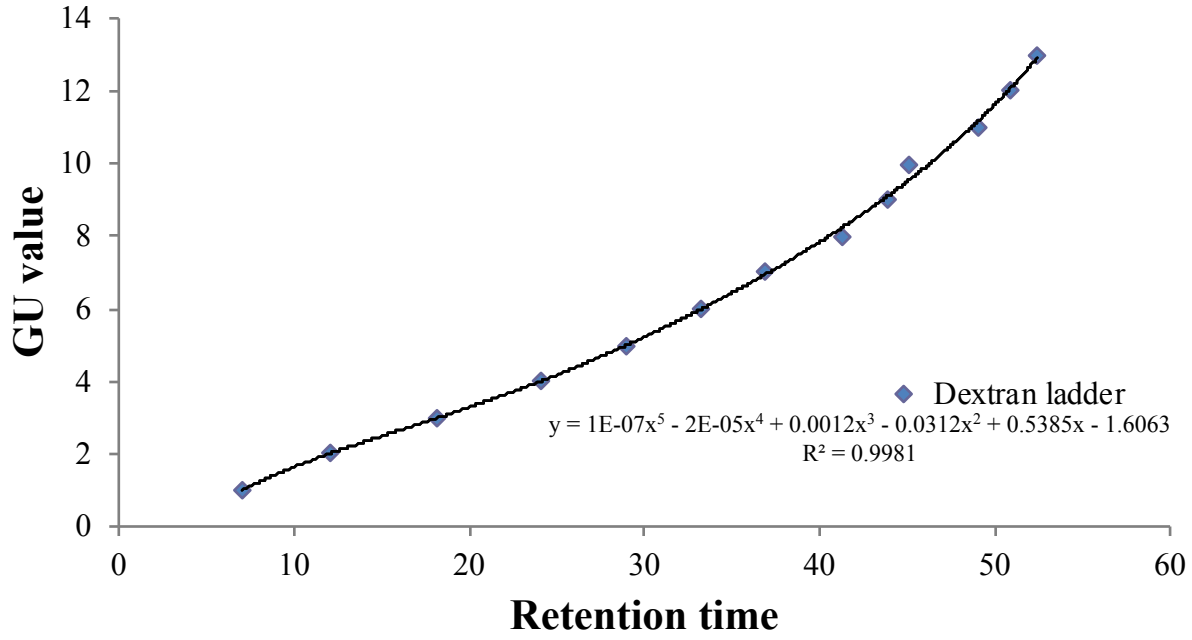


Figure 2. 2 Chromatogram of a 2-AB-labeled dextran ladder run on a 3.5 μm Waters X-Bridge amide column and analyzed by HILIC chromatography



2.14 Exoglycosidase digestion

Exoglycosidase digestions were performed to elucidate glycans structures. The array of exoglycosidase enzymes used are listed in **Table 2.4.**

Table 2. 4. Exoglycosidase enzymes for glycan analysis.

Exoglycosidase	Source	Specificity
A. α Neuraminidase (BioLabs, P0720S)	*Clostridium perfringens*	Neu α2-3,6,8
α Neuraminidase (BioLabs P0728)	*Salmonella typhimurium*	Neu α2-3>,6,8
B. β Galactosidase (Glyko®, GKX-5014)	*Streptococcus pneumoniae*	Gal β 1-4
C. α Galactosidase (Glyko®, GKX-5007)	Green coffee bean	Gal α1-3,4,6
D. β *N*-Acetylhexosaminidase (Glyko®, GKX-5003)	Jack bean	GlcNAc/GalNAc β1-2,3,4,6
E. α Fucosidase (Glyko®, GKX-5006)	Bovine Kidney	Fuc α1-6>2>3,4
F. α Mannosidase (Glyko®, GKX-5010)	Jack bean	Man α1-2,6, >3

Aliquots of 2-AB labeled samples were pipetted into 5 to 6 tubes depending on the number of digests to be performed and dried down in speed vacuum centrifuge (Thermo, Waltham, MA, USA). Samples were reconstituted with 4 μL of 5X manufacturer's buffer, the required enzyme or array of enzymes and water to make up to 22.5 μL. The 5X buffer at the optimal pH are usually provided with individual enzymes. For mixed enzyme incubations, sialidase 5X buffer was used. An example of a exoglycosidase digestion array is shown in **Table 2.5**.

Table 2. 5 Exoglycosidase digestion array used in previously 2-AB- labeled glycan pools. enzymes used for exoglycosidase treatment used: A. αNeuraminidase; B. β Galactosidase; C. αGalactosidase; D. β N-Acetylhexosaminidase; E. α Fucosidase; and F. α Mannosidase

Exoglycosidase treatment	5X Buffer	milliQ water	Exoglycosidase
CONTROL	4 μL	18.5 μL	0 μL
A	4 μL	16 μL	2.5 μL
A+C	4 μL	13.5 μL	(2x) 2.5 μL each
A+C+D	4 μL	11 μL	(3x) 2.5 μL each
A+C+D+E	4 μL	8.5 μL	(4x) 2.5 μL each
A+C+D+E+F	4 μL	6 μL	(5x) 2.5 μL each

Incubation took place overnight at 37°C. The digested glycan samples were then filtered through a previously washed 10000 Da MWCO filter (Millipore, Billerica, MA) to remove enzymes. The digestion tube was washed with 200 μL of milliQ water to recover any glycans left behind in the tube. Samples were dried down and re-suspended into 6 μL of milliQ water for HPLC analysis using the HILIC method mentioned above. HILIC exoglycosidase digestion profiles were interpreted by comparison with the control sample. Any peak loss will correspond to a loss of an individual monosaccharide at a specific linkage, reducing a given amount to the GU value of the original glycan. By comparing the successive enzyme digestions is possible to allocate all glycan structures (**Table 2.5)**.

2.15 Galactosylation Index

Galactosylation index (GI) was determined based on the abundance of terminal Gal residues present in the Mab as previously described by the equation (Majid, Butler *et al.* 2007):

$$GI = \frac{G2 + 0.5 * G1}{G0 + G1 + G2} \qquad \textbf{Eq. 6}$$

where G0, G1 and G2 correspond to the relative areas for the non-galactosylated (G0), monogalactosylated (G1) and digalactosylated (G2) glycans respectively.

2.16 Mass Spectrometry

2.16.1 Purification of intact Mab for MS analysis

A sample (100 μL) of protein A purified Mab (1 mg/mL) was desalted using a C18 cartridge previously conditioned with acetonitrile and water. The column was washed with 5 x 1 mL of deionized water and the protein was eluted with 2 mL of acetonitrile/water (1:1) with 0.1% trifluoroacetic acid (TFA). Eluents were evaporated and analyzed by MS.

2.16.2 In-gel trypsin digestion

Excised gel bands of interest were chopped into small cubes (~1 mm2), placed into microcentrifuge tubes and washed with water, following ACN (four times). Shrunk gel pieces were dried in a vacuum centrifuge, rehydrated in 25 mM NH_4HCO_3 and incubated with trypsin (Sigma, St. Louis, MO) at 37ºC for 18 h. After the incubation, the supernatant was removed and kept frozen until fractionation.

2.16.3 RP-HPLC fractionation

HPLC fractionation was performed on a Waters system 1525 equipped with a photodiode array (PDA) detector (Mississauga, Ontario) and coupled with a Vydac 218 TP54 C18 Protein & Peptide analytical column (300-Å pore size, 0.46 x 25 cm, Separation Group, Hesperia, CA, USA). Solvent A was 5% ACN in water with 0.1% (TFA) and solvent B was 90% ACN with 0.1% TFA. The flow rate was 0.8 mL/min and the elution gradient applied was from 10 to 70% of solvent B over 60 min. Fractions of 1 mL were collected manually and then evaporated prior to characterization by MS. This step was necessary for the isolation and MS analysis of glycopeptides. Otherwise, other tryptic peptides cause interference due mass overlap and competitive ionization (Bodnar and Perreault 2013).

2.16.4 Mass spectrometry (MS)

Fractionated samples were reconstituted in 10 µL of deionized water and 1 µL of each was spotted onto partially dried matrix of 2,5-dihydroxybenzoic acid (DHB; Sigma, St. Louis, MO) on the surface of a MALDI target, and were air-dried. MALDI-MS analysis was carried out on the UltraFleXtreme (Bruker, Billerica, MA) or Manitoba/Sciex prototype Qq-TOF mass spectrometer (University of Manitoba, Physics Department and Sciex, Mississauga, Canada) operated in the positive ion mode with a mass resolving power of ~10 000 FWHM, and accuracy of 10 ppm (Loboda, Krutchinsky *et al.* 2000). Data were analyzed by using FlexControl^{TM} software. The instruments were calibrated externally using a mixture of standard peptides with masses ranging from *m/z* 600–8000. Individual parent ions were manually selected for MS/MS

experiments. The collision energy was set between 50 and 140 V as a function of the mass ion when using Qq-TOF instrument. The peptide identities were first assigned manually using a partial *de novo* approach, and then verified using the online version of Mascot search engine (www.matrixscience.com). CID spectra of peptides in figures were annotated according nomenclature proposed by Roepstorf and Fohlman (Roepstorff and Fohlman 1984). The assignment of asparagine-linked oligosaccharides was performed after deglycosylation of tryptic glycopeptides following derivatization of glycans with phenylhydrazine (Lattová and Perreault 2009). To distinguish between isomeric glycan structures, general rules described previously were applied (Lattova, Perreault *et al.* 2004).

2.17 Nucleotide and nucleotide sugar analysis

2.17.1 Quenching

For intracellular nucleotide and nucleotide sugar analysis, cells were grown until day four and then quenched following a modified protocol from Sellick *et al.*(Sellick, Hansen *et al.* 2009). A supernatant sample containing 1 x 10^7 cells was quenched with 5 volumes of quenching solution kept at -70°C and gently mixed. The quenching solution used was 60% methanol with 0.85% (w/v) ammonium bicarbonate (AMBIC) pH 7.4 (Sigma: Catalog # A6141). Cell mixture was then centrifuged at 4000 rpm in megafuge at -9°C for one minute and the quenching solution was removed carefully to avoid disturbance of the cell pellet. Tubes containing cells were fast frozen in liquid nitrogen and then stored at -70°C prior to metabolite extraction.

2.17.2 Nucleotide and nucleotide sugar extraction.

The extraction of nucleotide and nucleotide sugars was performed following a modified protocol from Rabina *et al.* (Rabina, Maki *et al.* 2001). All buffers were kept in ice prior to use. Prior to use, Supelclean^{TM} ENVI^{TM}-Carb SPE Tubes (Supelco Inc., Bellafonte, PA: Catalog # 57088) were conditioned using 3 mL of 80% acetonitrile (v/v) (Fisher: Catalog #A998) in 0.1% (v/v) trifluoroacetic acid (Fisher: Catalog #0-4901). When all contents dripped through completely by gravity, 3 mL of milliQ water were added to the column. Flash frozen cell pellet sample was re-suspended in 500 μL of PBS containing 40 μM internal standard Guanosine 5'-diphosphoglucose (GDP-Glc, Sigma: Catalog #103301-72-0) that serve as an internal standard. This mixture was sonicated for 3 x15 seconds on ice at level 7 (Qsonica, LLC XL-2000 Series) and then cell debris was removed by centrifugation at 4000 rpm for 10 mins. Supernatant was removed and diluted up to 1 mL with 10 mM NH_4HCO_3 pH 7.0 and applied to previously conditioned Ervi-Carb column. To remove salts, detergents and impurities, SPE tubes were washed with 3 mL of milliQ water, 3 mL of 25% (v/v) acetonitrile and 3 mL of 1M triethylammonium acetate buffer (TEAA, Fluka, Buchs, Switzerland: Catalog #90357) pH 7.0. Nucleotide and nucleotide sugars were eluted with 2 mL of 25% (v/v) acetonitrile containing 50 mM TEAA buffer (pH 7.0) into a 1.5 mL tube that was previously treated with methanol. Collected samples were then dry completely in speed vacuum overnight. Samples were re-dissolved in 40 μL of water before ion-pair RP-HPLC.

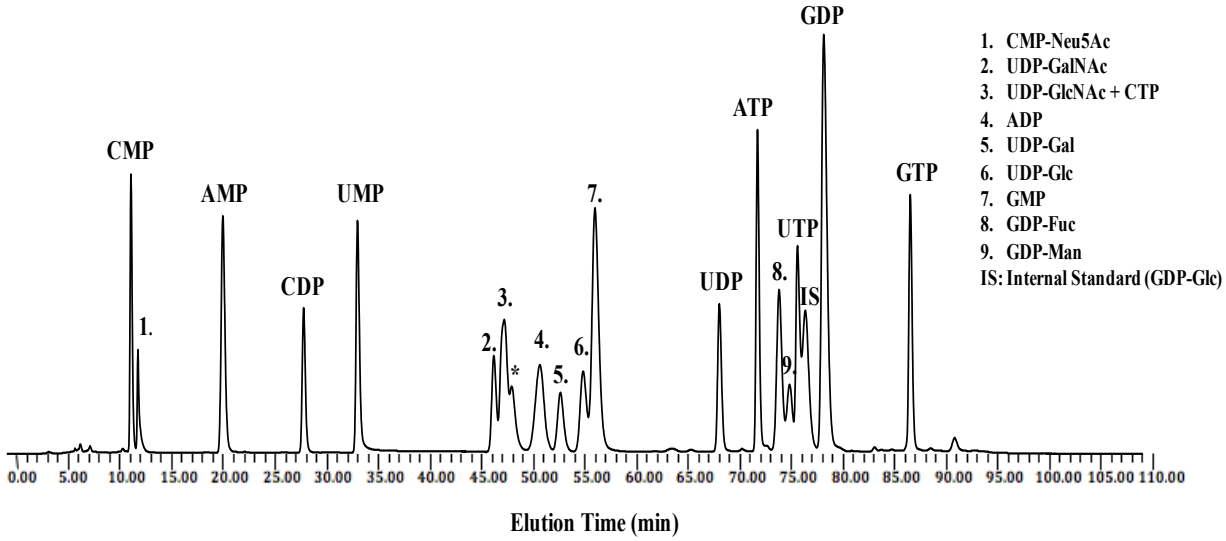
2.17.3 Separation of metabolites by ion-pair RP-HPLC.

A Waters HPLC system consisting of a binary pump 1525 with a 717 plus autosampler and a linear UVIS 200 UV detector were used. Separation of nucleotide and nucleotide sugars was performed at 27°C on a Dionex CarboPac® Pa1 column (4 x 250 mm) in combination with a guard column (4 x 50 nm) and UV detection at 260 nm. Buffers were prepared and filtrated right before use. Two separate runs were performed to obtain the separation of all nucleotide and nucleotide sugars with a flow rate of 0.6 mL/min unless otherwise specified. Elution gradient A used 2 mM sodium hydroxide (buffer A) and 1 M sodium acetate in 1 mM sodium hydroxide (buffer B) as eluents. The elution gradient A is shown in **Table 2.6.** The second elution gradient B used 2 mM sodium hydroxide (buffer A) and 1 M sodium acetate in 2 mM sodium hydroxide (buffer B) (pH adjusted to 10) with a HPLC gradient as shown in **Table 2.7**.

Table 2. 6. HPLC gradient A used for nucleotide and nucleotide sugar separation.

Time	Flow	Buffer A	Buffer B	Curve
0	0.6 mL/min	80	20	6
10	0.6 mL/min	70	30	6
20	0.6 mL/min	60	40	6
30	0.6 mL/min	45	55	6
35	0.6 mL/min	45	55	6
36	0.1 mL/min	0	100	6
44	0.1 mL/min	20	100	6
45	0.6 mL/min	45	55	6
60	0.6 mL/min	45	55	6
65	0.6 mL/min	20	80	6
75	0.6 mL/min	20	80	6
80	0.6 mL/min	0	100	6
89	0.6 mL/min	0	100	6

Figure 2. 3 Nucleotide and nucleotide sugar analysis.
HPAEC separations are shown of nucleotide and nucleotide sugars standard mixture used for the identification of metabolites. The elution gradient A used sodium hydroxide (E_1) and 1 M sodium acetate in 1 mM sodium hydroxide (E_2) as eluents and allowed the separation of 19 out of 20 standard nucleotide and nucleotide. Elution gradient was as follows for E_2:T_0 = 20% v/v; T_{10} = 30% v/v; T_{20} = 40% v/v; T_{30} = 55% v/v; T_{35} = 55% v/v; T_{36} = 100% v/v (0.1 mL/min); T_{44} = 100% v/v (0.1 mL/min); T_{45} = 55% v/v; T_{60} = 55% v/v; T_{65} = 80% v/v; T_{75} = 80% v/v; T_{80} = 100% v/v; T_{89} = 100% v/v. Metabolites were detected by absorbance at 260 nm. The peak shown with an asterisk is unknown. **1.** CMP-Neu5Ac, **2.** UDP-GalNAc, **3.** UDP-GlcNAc+CTP, **4.** ADP, **5.** UDP-Gal, **6.** UDP-Glc, **7.** GMP, **8.** GDP-Fuc, **9.** GDP-Man. GDP-Glc was used as internal standard (IS).



Prior to injection, 36 µL of 2 mM sodium hydroxide, 9 µL of 1 M sodium acetate in 1 mM sodium hydroxide and 15 µL of previously re-constituted sample were added into a plastic vial. Peaks present on samples were identified and quantified by comparing their retention times with standards run alone or in a standard mixture. The mix contained 20 standards (2 µl of 5 mM each standard) which were added to a plastic vial containing 16 µl 2 mM sodium hydroxide and 4 µL of 1 M sodium acetate in 1 mM sodium hydroxide (**Figure 2.3**). Standards were from Sigma (St. Louis, MO).

Table 2. 7 HPLC gradient B used for nucleotide and nucleotide sugar separation

Time	Flow	Buffer A	Buffer B	Curve
0	0.6 mL/min	80	20	6
20	0.6 mL/min	70	40	6
26	0.6 mL/min	60	50	6
30	0.6 mL/min	45	60	6
38	0.6 mL/min	45	70	6
43	0.1 mL/min	0	80	6
50	0.1 mL/min	20	95	6
53	0.6 mL/min	45	100	6
60	0.6 mL/min	45	100	6

Individual group mix of 5 standards were also made to facilitate the identification of each peak by adding 2 μL of each standard at a concentration of 5 mM into a vial containing 40 μL of 2 mM sodium hydroxide and 10 μL of 1 M sodium acetate in 1 mM sodium hydroxide. Group 1 included: Cytidine-5'-monophospho- *N*-acetylneuraminic acid sodium salt (CMP-NeuAc), Uridine-5'-diphospho-*N*-acetylglucosamine sodium salt (UDP-GlcNAc), Uridine-5'diphosphogalactose disodium salt (UDP-Gal), Uridine 5'-triphosphate (UTP) and Guanosine-5'diphosphate sodium salt (GDP). Group 2 contained: Adenosine-5'-monophosphate disodium salt (AMP), Uridine-5'-diphospho-*N*-acetylgalactosamine disodium salt (UDP-GalNAc), Guanosine-5'-monophosphate disodium salt (GMP), Guanosine-5'-diphospho-β-L-fucose (GDP-Fuc) and Guanosine-5'-triphosphate sodium salt (GTP). Group 3 included: Cytidine 5'-diphosphate sodium salt (CDP), Cytidin-5'-triphosphate disodium salt (CTP), Adenosine-5'-diphosphate sodium salt (ADP), Uridine 5'diphosphate disodium salt (UDP) and Guanosine-5'-diphospho-D-mannose sodium salt (GDP-Man). Group 4 contained: Cytidine 5'monophosphate disodium salt (CMP), Uridine-5'-monophosphate (UMP), Uridine-5'-diphosphoglucose disodium salt (UDP-Glc), Adenosine-5'triphosphate disodium salt (ATP) and Guanosine-5'-diphosphoglucose sodium salt (GDP-Glc). GDP-Glc was the only standard added at 7.5 mM into the mix to facilitate its recognition. The injection volume for standards and sample was 30 μL.

Peak areas of nucleotides and nucleotide sugars were used to determine their intracellular concentrations using their relative molar response and expressed in fmoles/cell.

$$\boldsymbol{molar\ response} = \frac{moles\ (std)}{area\ (std)} \qquad \boldsymbol{Eq.7}$$

The adenylate energy charge (AEC) was determined as an index based on the measurement of the intracellular concentrations of Ade nucleotides following the equation:

$$\boldsymbol{AEC} = \frac{[ATP] + 0.5 * [ADP]}{[ATP] + [ADP] + [AMP]} \qquad \boldsymbol{Eq.8}$$

2.18 Sialic acid analysis

2.18.1 Acetic Acid Hydrolysis of proteins

Sia release was performed on previously purified Mab by preparing aliquots containing up to 50-100 μg in 0.6 μl tubes. Samples were dried in speed vacuum overnight and re-suspended with 25 μL of milliQ water and 25 μL of 4 M acetic acid. Samples were mixed and centrifuged briefly to ensure contents reached the bottom of the tube. An incubation at 80°C for 2 hours allowed the release of Sias. Neu5Ac and Neu5Gc (Sigma, St. Louis, MO) were used as standards for Sia identification and quantification. Standards were prepared by mixing 25 uL of standard stock solution and 25 uL of 4M acetic acid. It was important that samples and standards conditions matched, that is using same reagents to avoid systematic error du to different derivatization efficiency.

2.18.2 Derivatization of samples and standards

DMB solution was prepared by mixing 1.5 mL of milliQ water, 172 μL of glacial acetic acid, 4.9 mg of sodium hydrosulfite. To this solution, 3.5 mg of DMB hydrochloride diluted with 200 μL of milliQ water were added and mixed well. DMB solution was cover with thin foil to avoid light exposure. The DMB labelling solution was added to the samples and standards in a proportion of 1:1 and then incubated at 50°C for 2.5 hours in the dark. After incubation, samples were frozen at -70°C to slow the reaction.

2.18.3 DMB-labelled samples analysis by fluorescence HPLC

The DMB labeled Sias were analyzed by reverse phase chromatography using a Kinetex 5 μ C18 100A column (150 x 4.6 mm, Phenomenex) along with a SecurityGuard ULTRA holder and cartridges for C18 columns all kept inside a column heater box. Waters HPLC system counted with a binary pump 1525, a Waters 2707 autosampler and a linear UVIS 200 UV detector. An isocratic elution of 0.5 mL/min was performed for 30 minutes using Methanol: CH3CN: Water (7:8:85 v/v) as a solvent. The volume of injection was 10 μL. The settings for fluorescent detector are shown in **Table 2.8.** Preparation of serial dilutions of previously DMB-derivatized Neu5Ac and Neu5Gc standards enable the quantitative analysis of Sias by reference to standard curves shown in **Figure 2.4 and Table 2.9.**

Table 2. 8 Settings for 2475 multi-wavelength fluorescent detector for sialic analysis.

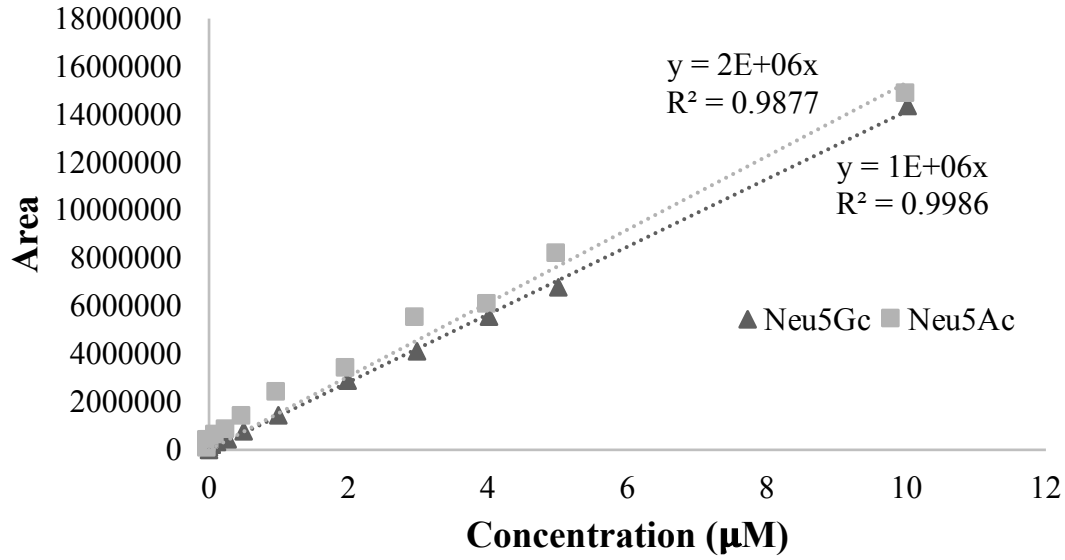
Parameter	Set value
Excitation	373 nm
Emission	448 nm

EUFS	3000
Gain	1

Table 2. 9 Sialic Acid Standards concentration and area obtained after HPLC analysis.

Neu5Gc Concentration (μM)	Neu5Gc Area	Neu5Ac Concentration (μM)	Neu5Ac Area
10	14297779	**10**	14746902
5	6819305	**5**	8087307
4	5586315	**4**	5996171
3	4069524	**3**	5418492
2	2925863	**2**	3280853
1	1453147	**1**	2277138
0.5	779984	**0.5**	1374389
0.25	451479	**0.25**	824893
0.1	341872	**0.1**	507028
0.05	254798	**0.05**	378289
0.025	187155	**0.025**	290420
0.01	153541	**0.01**	271599
0.005	182391	**0.005**	336441
0	0	**0**	0

Figure 2. 4 Calibration curves prepared from serial dilutions of Neu5Ac and Neu5Gc standards.



Section A

Glucose starvation compromises *N*-linked glycosylation of Mabs produced by mammalian cells

Mammalian cells are used for the production of therapeutic proteins because of their capacity for post-translational modification of proteins. In particular, *N*-glycosylation is critical for the pharmacokinetic properties of glycoproteins including bioactivity (Arnold, Wormald *et al.* 2007), secretion (Gala and Morrison), *in vivo* clearance (Abès and Teillaud), solubility (Gu and Wang), thermal stability (Mimura, Church *et al.*) and antigenicity (Schauer). The assembly of *N*-glycans results from two distinct events. The first stage occurs in the endoplasmic reticulum (ER), where the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ is synthesized on a dolicholpyrophosphoryl carrier (Dol-P) and *en bloc* transferred to a polypeptide chain (Taylor and Drickamer). In the second stage, the newly glycoprotein travels through the different Golgi compartments where further step-wise addition of sugars takes place through the action of glycosyltransferases (Spiro , Berger M. 2011). Rapid cessation of the synthesis of the usual $Glc_3Man_9GlcNAc_2$ lipid-linked oligosaccharide has been observed when mammalian cells are cultured in media devoid of glucose. The latter has become evident during industrial bioprocesses where carbon sources have been kept at low concentrations or have been replaced with more slowly metabolized carbon sources with the aim of reducing deleterious levels of lactate and ammonia (Glacken, Fleischaker *et al.* 1986, Hayter, Curling *et al.*). Thus, the effect that feeding strategies can have on the glycosylation profile of the secreted protein needs to be constantly monitored to ensure not only appropriate cell growth and high productivity, but to guarantee the

final product's quality, function and stability. Glucose-deprived cells have shown to produce truncated lipid-linked mannosylated intermediates lacking glucose residues (e.g. $Man_2GlcNAc_2$-PP-Dol, $Man_5GlcNAc_2$-PP-Dol or $Glc_3Man_5GlcNac_2$-PP-Dol) (Turco 1980, Rearick, Chapman *et al.* 1981, Liu, Spearman *et al.* 2014). Chapman and Calhoun showed that a severe depletion of the intracellular nucleotide-sugar, GDP-mannose, occurred in glucose- starved CHO cells and was responsible for aberrant glycosylation observed from a $Man_5GlcNAc_2$-PP-Dol precursor(Chapman and Calhoun 1988). In this section, the impact of low glucose concentrations and high cell density inocula on CHO cell growth, metabolism and productivity will be described along with their influence on the *N*-glycosylation quality and heterogeneity.

A.1 Hypothesis of Research.

A decrease in the availability of nucleotide sugar precursors can be the source responsible for the synthesis of non-glycosylated proteins or with decreased glycosylation previously observed in cultures experiencing glucose starvation (Chapter 3). It is suspected that the longer the time of glucose starvation the more detrimental is the effect in glycosylation of glycoproteins (e.g. high inoculum experiments, Chapter 4). Decrease in glucose availability will result on reduced glycosylation no matter the cell line used, as glucose is considered as the main source for energy, metabolism and glycoconjugate synthesis.

Chapter 3

Effects of low glucose on CHO and DP12 cells' growth, metabolism, antibody production and *N*- glycosylation[2]

3.1. Introduction

The microenvironment of cells in culture needs to be tightly controlled in a way that closely mimics physiological conditions *in vivo.* Furthermore, understanding cells' metabolic behaviour can lead to the development of different formulations and feeding strategies to maintain cells in a viable and productive state as long as possible. Several on-line and off-line methods have been applied to monitor cell physiology and metabolism at the development and at the production scale including: cell number and viability, amino acid consumption and oxygen uptake (Grammatikos, Tobien *et al.* 1999). In addition, special attention has been directed towards avoiding nutrient depletion and waste product accumulation often seen in batch culture and fed-batch systems. Glucose and glutamine are both important nutrients that are normally added to culture media. Glucose acts as the major carbon and energy source for cells and provides precursors for ribose formation through the pentose phosphate pathway (PPP) for nucleic acid synthesis (Fitzpatrick, Jenkins *et al.* 1993). On the other side, glutamine also acts as a carbon and energy source, supplying the necessary intermediates to keep the TCA cycle operating. The excess

[2] Partial content of this chapter was included in the following publication:
Villacres, C., et al. "Low Glucose Depletes Glycan Precursors, Reduces Site Occupancy and Galactosylation of a Monoclonal Antibody in Cho Cell Culture." *Biotechnol J* 10.7 (2015): 1051-66.

of nitrogen from this amino acid is either eliminated as ammonium or used in the production alanine or asparagine (Vriezen, Romein *et al.* 1997).

Under normal conditions, cells will consume large amounts of glucose, nearly 90% of which is converted to lactate during aerobic glycolysis (Schenk, Fernandez *et al.* 2001). High concentrations of lactate have shown to adversely affect cell growth and recombinant protein production. Thus, low glucose feeding strategies have been implemented to increase cell concentration and productivity while reducing lactate production. However, glucose limitation has shown to affect the assembly of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ due to a decrease in the levels of lipid-mediated mannosyl- and glucosyl-transferases (Schenk, Fernandez *et al.* 2001, Jones, Tomiya *et al.* 2010), Dol-P (Jenkins, Castro *et al.* 1994) and lipid-linked sugar donors (i.e. Man-P-Dol, Glc-P-Dol) (Sefton 1977, Stark and Heath 1979, Turco 1980, Rearick, Chapman *et al.* 1981, Chapman and Calhoun 1988, Nyberg, Agudo *et al.* 1998, Wagner 2011). Nutrient limitation has also shown to affect protein's microheterogeneity, that is the series of trimming sugar reactions and sugar additions that occur once the oligosaccharides are attached to nascent proteins (Barnabe and Butler 2000). In addition to nutrient limitation, there are another parameters responsible for non-fully glycosylated proteins such as: the glycoprotein conformation itself (Wright and Morrison 1997), the enzymatic machinery of the chosen expression system (Taylor and Drickamer 2006) and various extracellular environmental variables such metabolic waste accumulation (Yang and Butler 2002), rate of elongation of protein synthesis (Butler 2009), oxygen (Kunkel, Jan *et al.* 1998), culture pH (Glacken, Fleischaker *et al.* 1986, Borys, Linzer *et al.* 1993, Chee Furng Wong, Tin Kam Wong *et al.* 2005) and mode of culture (Hayter, Curling *et al.* 1992, Majid, Butler *et al.* 2007). In this Chapter, the effect of low glucose concentrations on

growth and metabolism as well as on Mab production and *N*-glycosylation were evaluated on two mammalian cell lines: CHO-EG2 cells expressing the chimeric human-llama Mab (EG2-hFc) and CHO-DP12 expressing an IgG1 Mab.

3.2. Objectives

Nutrient limitation has had a negative impact on the glycosylation profile of secreted glycoproteins, the following experiments focused on:

- Evaluating cell's growth, metabolism, productivity and particularly *N*-linked glycosylation of the single chain Mab EG2-hFc and the equivalent to human IgG1, DP12-anti-IL6 produced by CHO and DP12 cells experiencing glucose deprivation
- Analyze the relationship between changes in cells' metabolism and *N*-linked glycosylation with respect to the intracellular nucleotide and nucleotide sugars in glucose-deprived CHO cells.

3.3. Experimental Set Up

CHO EG2 and DP12 cultures were transferred into 250 mL shaker flasks with a culture volume of 80 mL of Biogro™ media containing 0.5 mM glucose for about 17 hours. Cells were then spun down and re-seeded at a cell density of 2.5 x 10^5 cells/mL in Biogro™ media containing: **a)** 3 mM, 5.5 mM, 11 mM, 17.5 mM and 25 mM glucose with 0 mM glutamine or **b)** 3 mM glucose with 0 mM, 1 mM, 2 mM, 3 mM, 4 mM glutamine. Control culture consisted of culture media

containing 4 mM glutamine and 25 mM glucose. Each experimental condition was run in duplicates and results obtained were confirmed by repeating experiment three times, giving a total of 6 replicates. Cell culture was terminated on day four and on day six for EG2- and DP12- cells respectively. A much longer cell culture period was required for DP12 cells because of their slower growth rate. Daily samples were taken to determine cell density, viability, metabolite analysis and protein production determination. Treatment of previously protein A purified CHO EG2-hFc and DP12-IgG1 Mabs with PNGase-F allowed the release of *N*-glycans prior to 2AB-labelling and HPLC analysis (**See Chapter 2, Section 2.11**). Additional supernatant sampling (containing 1 x 10^7 cells) was used for nucleotide and nucleotide sugar analysis. For this purpose, cell metabolism was stopped on day four and metabolites were extracted prior to RP-HPLC (**See Chapter 2, Section 2.11.8**).

3.4. Results

3.4.1 Glucose availability affects growth and metabolism of CHO and DP12 cells.

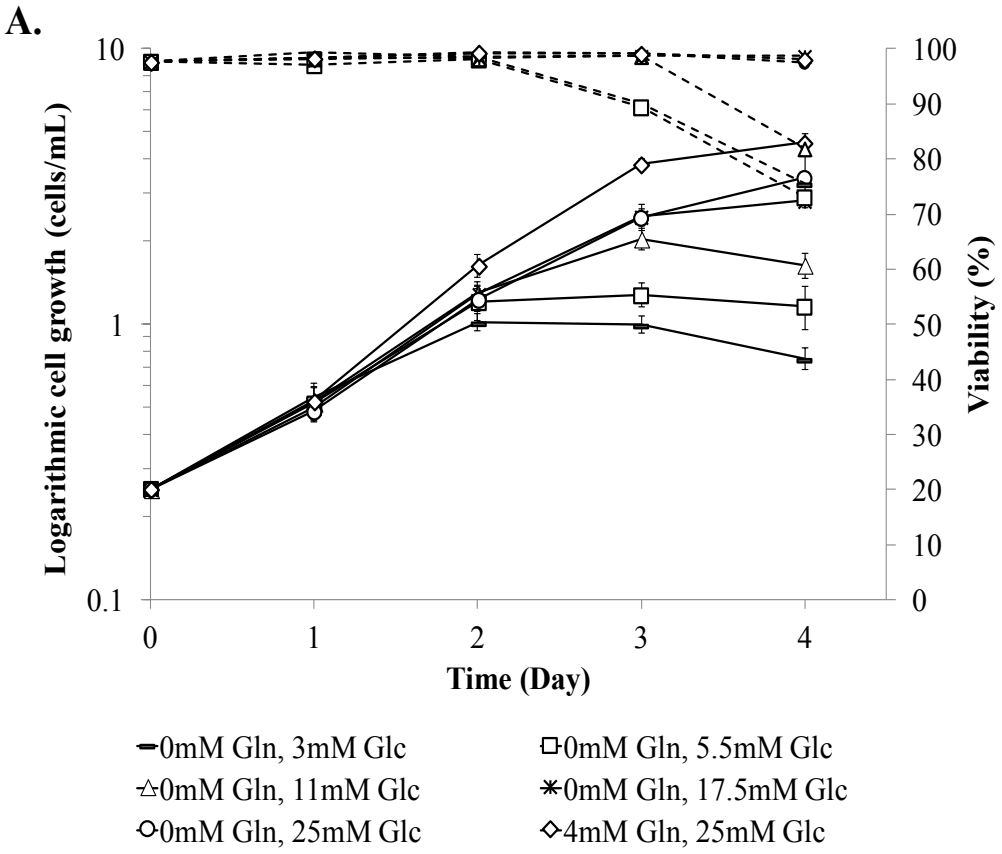
The initial goal of the present work was to study the effect of glucose deprivation on the metabolism, productivity and particularly *N*-linked glycosylation of EG2-hFc and IgG1 Mabs produced by CHO cells and DP12 cells respectively. For this purpose, batch cultures with varying concentrations of glucose and no glutamine were inoculated with previously starved cells. **Figure 3.1A** shows the effect of culture media containing 3 mM, 5.5 mM, 11 mM, 17.5 mM or 25 mM glucose with 0 mM glutamine on CHO EG2 cells' growth over four days. Similar growth rates (0.04 h^{-1}) were observed in each culture over the first 48 h, after which cell yield and viability depended upon the initial glucose concentration. There was a gradual loss of overall viability of

the cell population after two days in the presence of a glucose concentration <5.5 mM from a high value of 98%. This high viability was maintained over four days in cultures supplemented with an initial glucose concentration >17.5 mM. A cell yield of 4.58 x 10^6 cells/mL was observed on day four in the control culture (25 mM glucose and 4 mM glutamine) compared to a lower cell yield of 1.02 x 10^6 cells/mL on day two in the culture containing 3 mM glucose. The absence of glutamine significantly reduced the cell yield of the 25 mM glucose cultures to 74% of the control (p-Value < 0.001).

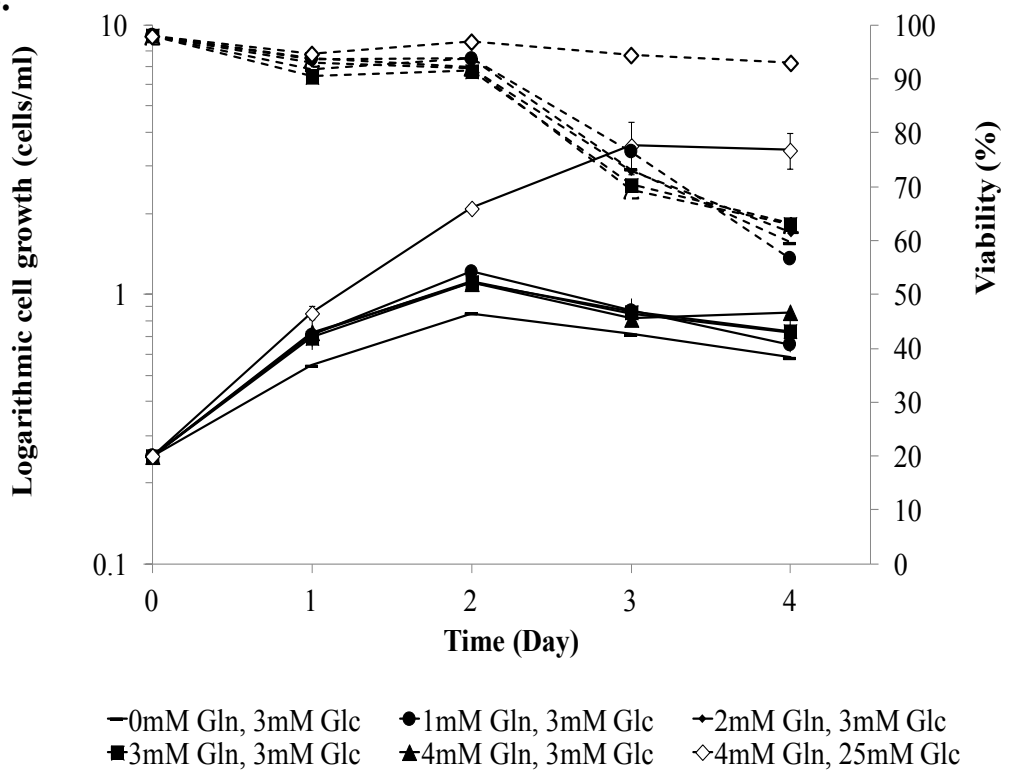
To determine the effect of glutamine concentration on growth, cells were cultured in media containing 3 mM glucose with variable glutamine (0–4 mM). **Figure 3.1B** shows the change in cell concentration and overall viability over a four-day incubation period. This shows that lower cell yield observed in all cultures at low glucose (3 mM) could not be substantially improved by the addition of glutamine up to 4 mM. In the presence of low glucose there was a substantial decrease in cell viability from 90% (day two) to 57% (day four) with a significantly lower final cell yield of 0.58 x 10^6 cells/mL (p-Value < 0.0001). **Figure 3.1C** shows the effect of glucose concentration on DP12 cells growth and viability. DP12 cells grew at a slower rate since day one with a μ as low as < 0.01h^{-1} in comparison to EG2 cells with 0.03 h^{-1}. For this reason, culture period was extended until day 6 where most of cell culture conditions reached their MCDs (**Table 3.2**). Low glucose concentrations (< 11 mM) also affected DP12 cells' growth with a decreased of up to 57% in MCDs when compared with control culture with 25 mM glucose. However, DP12 cells reached lower MCDs on day 6 when compared to EG2 cells on day 4 **(Table 3.1.)**. A decrease of about 15% in viable cells was observed in DP12 cells on day six as was for EG2 cells were viability dropped by 23% at the end of culture.

Figure 3. 1 Effect of increasing glucose concentration on the growth and viability of A. and B. CHO EG2 and C. DP12 cells.

CHO EG2 cells and DP12 cells were cultured in media containing: 3 mM, 5.5 mM, 11 mM, 17.5 mM and 25 mM glucose without glutamine or 3 mM glucose plus 1 mM, 2mM, 3mM and 4 mM glutamine. Control culture media contained 25 mM glucose and 4 mM glutamine. The viable cell densities (solid lines) and viability (dashed lines) were determined in each culture at daily intervals over four days (EG2) and six days (DP12) by a trypan blue exclusion assay using a computer-based image analyzer. Data points represent mean ±SD of duplicates for each condition for one of three independent experiments.



B.


Logarithmic cell growth (cells/ml)
Viability (%)
Time (Day)
10
1
0.1
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
0mM Gln, 3mM Glc
1mM Gln, 3mM Glc
2mM Gln, 3mM Glc
3mM Gln, 3mM Glc
4mM Gln, 3mM Glc
4mM Gln, 25mM Glc

C.

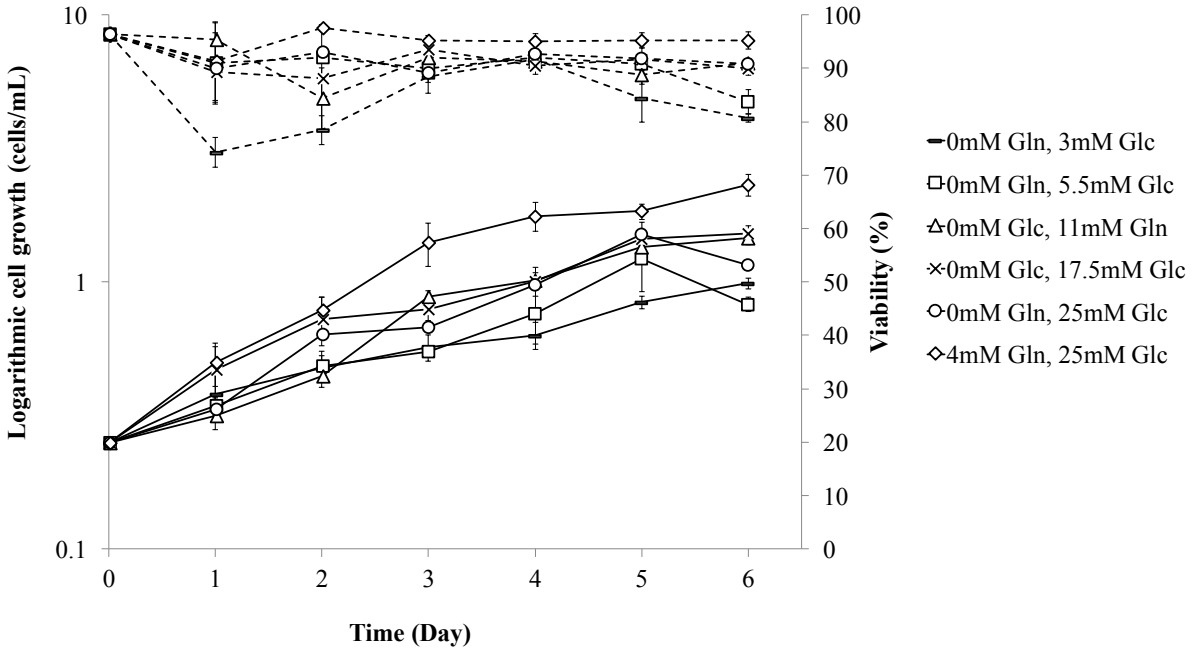

Logarithmic cell growth (cells/mL)
Viability (%)
Time (Day)
10
1
0.1
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
0mM Gln, 3mM Glc
0mM Gln, 5.5mM Glc
0mM Glc, 11mM Gln
0mM Glc, 17.5mM Glc
0mM Gln, 25mM Glc
4mM Gln, 25mM Glc

Table 3. 1 Culture parameters of CHO-EG2 cells inoculated at 2.5 x 10^5 cells/mL in increasing glucose concentrations.

Culture condition		Maximum Viable Cell Density	Viability[a)]	Mab Yield[a)]	*qMab*[c)]	Glucose depletion	q_{Glc}[b)]	q_{Lac}[b)]	*GI*[a)]	$Y_{Lac/Glc}$
Glc	Gln									
(mM)	(mM)	(x10^6 cells/mL)	(%)	(μg/mL)	(μg/10^6 cell.day)	(Day)	(μmol/10^6 cell.day)	(μmol/10^6 cell.day)		(mol/mol)
3	1	1.10	63.0	3.22 ± 1.50	2.65 ± 1.21	<2	-1.09 ± 0.23*	0.80 ± 0.13*	0.48 ± 0.07*	1.44 ± 0.19
3	2	1.20	63.3	3.40 ± 1.50	2.69 ± 1.32	<2	-1.06 ± 0.18*	0.81 ± 0.17*	0.57 ± 0.05	1.51 ± 0.04
3	3	1.22	81.6	3.57 ± 1.68	2.48 ± 1.20	<2	-1.04 ± 0.08*	0.85 ± 0.11*	0.54 ± 0.04*	1.45 ± 0.14
3	4	1.24	80.0	3.40 ± 1.57	2.69 ± 1.34	<2	-0.80 ± 0.32*	0.66 ± 0.44*	0.54 ± 0.10*	1.52 ± 0.07
3	0	0.85	75.5	3.40 ± 1.02	2.76 ± 0.87	<2	-0.88 ± 0.19*	0.65 ± 0.31*	0.48 ± 0.03*	1.60 ± 0.10
5.5	0	1.16	73.1	5.01 ± 1.53	2.84 ± 0.26	2	-2.86 ± 0.43*	4.38 ± 0.34	0.48 ± 0.04*	1.82 ± 0.11
11	0	1.81	82.0	8.70 ± 2.51	2.71 ± 1.12	2	-6.18 ± 0.47	10.7 ± 1.72	0.57 ± 0.07*	1.74 ± 0.11
17.5	0	2.56	98.7	12.8 ± 6.16	2.97 ± 1.07	3	-6.09 ± 0.80	11.5 ± 0.55	0.62 ± 0.04	1.68 ± 0.09
25	0	2.52	97.6	13.7 ± 7.18	3.09 ± 0.88	4	-6.27 ± 0.04	12.0 ± 0.50	0.64 ± 0.05	1.72 ± 0.07
25	4[e)]	4.51	98.1	21.4 ± 10.8	3.04 ± 1.15	4	-6.77 ± 0.58	11.6 ± 0.78	0.68 ± 0.05	1.47 ± 0.10

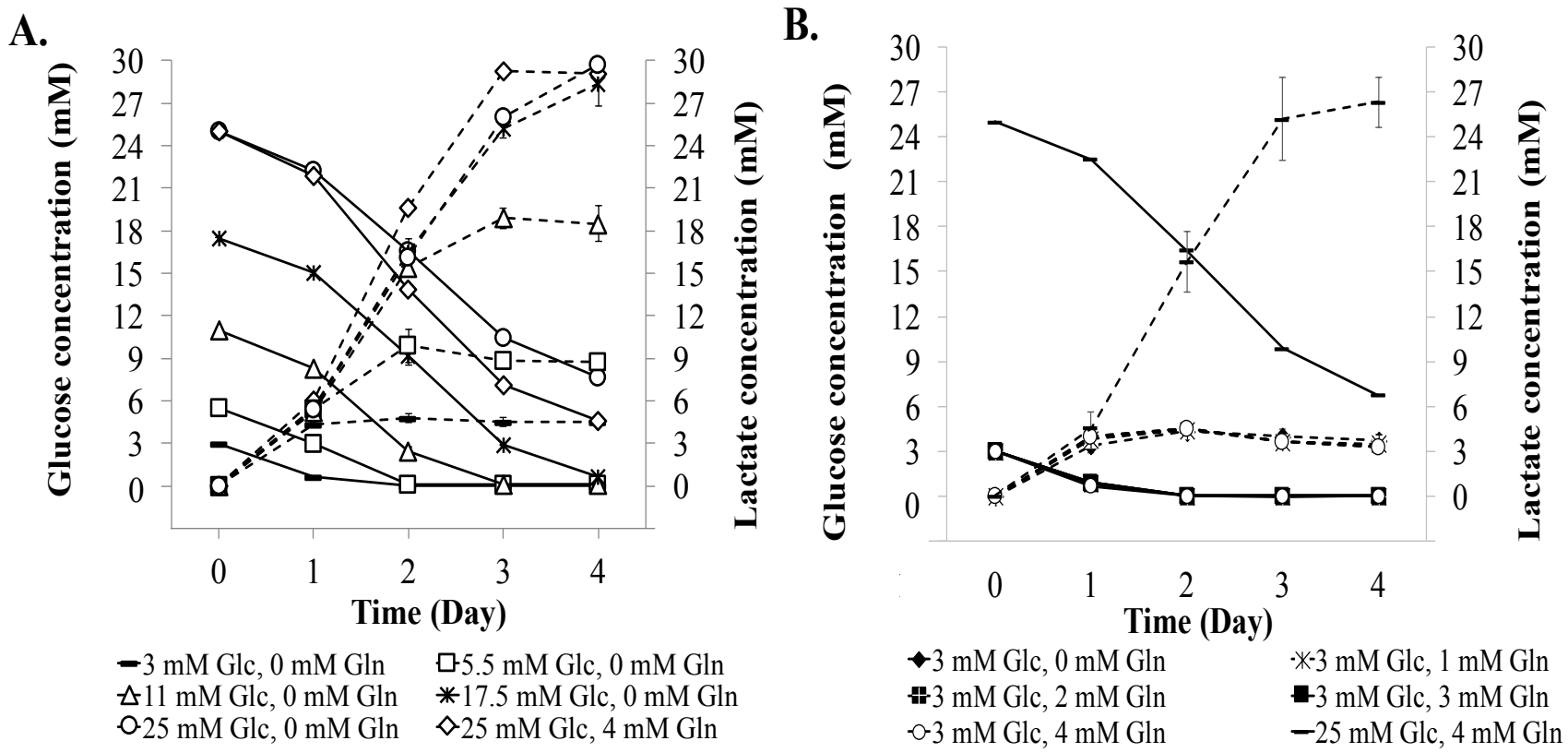
a) Measured on day 4.
b) Measured on day 2.
c) Over the period of time where glucose was still available.
d) Pairs of means ±SD significantly different ($P \leq 0.01$) with respect to control culture. Data corresponds to replicates of three independent experiments (n = 6).
e) Control culture

Table 3. 2 Culture parameters of DP12 cells inoculated at 2.5 x 10^5 cells/mL in increasing glucose concentrations.

Culture conditions		Maximum Viable Cell Density	Viability [a]	Mab yield [a]	q_{Mab} [c]	Glucose depletion	q_{Glc} [b]	q_{Lac} [b]	GI [a]	$Y_{Lac/Glc}$
Glc (mM)	Gln (mM)	(x10^6 cells/mL)	(%)	(μg/mL)	(μg/10^6 cell.day)	(Day)	(μmol/10^6 cell.day)	(μmol/10^6 cell.day)		(mol/mol)
3	0	0.99 ± 0.05	80.7	5.08 ± 0.51	2.34 ± 0.16	2	-3.11 ± 0.03*	4.57 ± 0.01*	0.47 ± 0.05	1.43 ± 0.00*
5.5	0	0.82 ± 0.05	83.8	8.31 ± 0.36	3.00 ± 0.23	3	-5.14 ± 0.28	8.74 ± 0.43	0.46 ± 0.04	1.51 ± 0.05*
11	0	1.46 ± 0.08	91.0	9.19 ± 0.59	2.44 ± 0.59	>6	-5.75 ± 0.01	8.89 ± 0.40	0.46 ± 0.03	1.21 ± 0.02
17.5	0	1.52 ± 0.10	90.0	10.7 ± 0.59	2.12 ± 0.59	>6	-4.42 ± 0.07	6.34 ± 0.08	0.45 ± 0.00	1.20 ± 0.01
25	0	1.15 ± 0.00	90.9	9.84 ± 0.67	2.04 ± 0.67	>6	-5.39 ± 0.71	10.1 ± 0.07	0.45 ± 0.00	1.11 ± 0.01
25	4 [e]	2.31 ± 0.22	95.3	11.5 ± 0.53	3.19 ± 0.53	>6	-5.63 ± 0.42	7.98 ± 0.15	0.49 ± 0.01	1.31 ± 0.00

a) Measured on day 4.
b) Measured on day 2.
c) Over the period of time where glucose was still available.
d) Pairs of means ±SD significantly different ($P \leq 0.0001$) with respect to control culture. Data corresponds to duplicates (n = 2)
e) Control culture

Figure 3. 2. Effect on the glucose consumption and lactate production for A. and B. CHO EG2 and C. DP12 cells
Cells were starved by incubation in media containing 0.5 mM glucose for 17 hours prior to the experiment. Cells (2.5 x 10^5 cells/mL) were inoculated into 75 mL of media in 250mL shake flasks with **A.** and **C.** Increasing glucose concentrations without glutamine or **B.** increasing glutamine concentrations with 3 mM glucose. The control culture media contained 25 mM glucose and 4 mM glutamine. Glucose (solid lanes) and lactate (dashed lanes) concentrations were determined simultaneously using a biochemical analyzer. Data points represent mean ±SD of duplicates for each condition for one of three independent experiment



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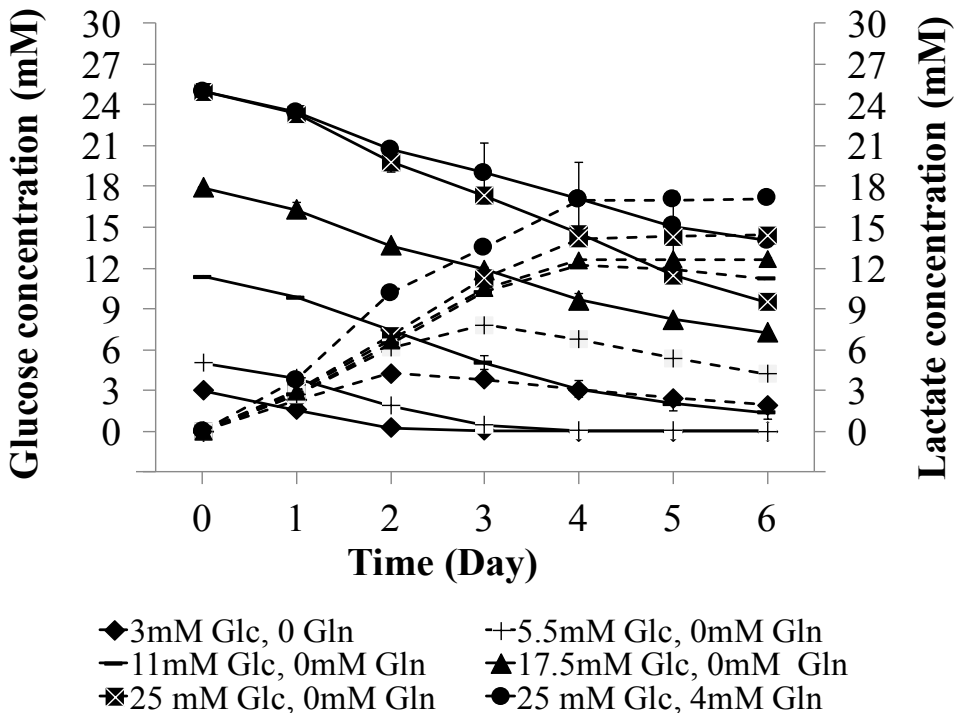


These data indicated the importance of glucose as the primary media carbon source to enable cell growth and maintenance of viability in both cell lines. However, EG2 and DP12 cells behave differently when cultured in increasing glucose concentrations. A significant decrease of up to 81%, 74%, 60% and 43 % in MCDs was observed in EG2 cultures containing 3 mM, 5.5 mM, 11 mM and 17. 5 mM respectively. In addition, 25 mM glucose-containing cultures without glutamine showed a significant reduction of up to 44% in MCDs in comparison to the control having 25 mM glucose and 4 mM glutamine. The lower growth rates and high viabilities observed in DP12 may indicate that cells were still in exponential phase. In addition, MCDs for DP12 cells were not as high at the end of culture as the ones observed in EG2 cells on day four. Finally, glutamine did not improve cell growth in cultures containing low glucose concentrations.

3.4.2 Effect of glucose concentrations on CHO EG2 and DP12 cultures on glucose consumption, lactate production and productivity.

Figure 3.2 shows the profile of glucose consumption and lactate production for CHO cultures. The depletion of glucose on day two (<5.5 mM initial glucose) and day three (11 mM glucose) limited the cell yield in these cultures. Significantly lower glucose consumption rates (q_{Glc}) (0.8 ± 0.32 to 2.86 ±0.43 μmol/10^6 cells/day) were observed at <11 mM glucose in comparison to the control (6.77 ±0.58 μmol/10^6 cells/day) on day two (p-Value < 0.0001; **Table 3.1**). In contrast, DP12 cells cultured in media containing 3 mM and 5 mM glucose also experienced glucose depletion at day two and three respectively (**Figure 3.2C)**. However, only cultures containing 3 mM glucose showed a significantly lower q_{Glc} (3.11 ± 0.03) with respect to the control (5.63 ± 0.42) (p-Value < 0.0001; **Table 3.2**). Both, EG2 and DP12 cultures with initial glucose <11 mM showed a shift towards lactate consumption once glucose was depleted in the media (**Figure 3.2A**, **B and C**). Under control condition (25 mM Glc/4 mM Gln), EG2 cells released lactate into the medium concomitant with the utilization of glucose giving a slightly but significantly lower (p-Value < 0.01) metabolic coefficient ($Y_{Lac/Glc}$) of 1.47 ±0.10 in comparison to 1.60 ±0.10 in cultures with 3 mM glucose (**Table 3.1**). The $Y_{Lac/Glc}$ was also lower in DP12 control cultures with 1.31 ± 0.00 in comparison to low glucose cultures which shown metabolic coefficients of up to 1.51 ± 0.05 (p-value ≤0.0008) (**Table 3.2**). The cell-specific productivities of antibodies (q_{Mab}) of CHO EG2 and DP12 cells were determined at varying glucose concentrations. The q_{Mab} was determined from the slope of the plot of EG2-hFc and anti-IL8 Mab concentrations and the IVCD values. **Table 3.1** and **3.2** show the calculated q_{Mab} during the period where glucose was still available in the media for EG2 cells and DP12 cells respectively. Both cell lines did not

show significant differences in lower glucose concentrations with respect to the control cultures. However, volumetric productivity decreased significantly by up to 85% and 56% in low glucose-containing EG2 and DP12 cells respectively, where glucose was depleted in less than three days. These results indicate that glucose was the key nutrient responsible for robust growth of both cell lines reflected by the maximal cell densities and viability achieved at high glucose. Furthermore, glucose consumption (by up to 58- 88%) and lactate production rates (by up to 62- 94%) were markedly reduced at < 11 mM glucose, showing dependence on glucose availability.

3.4.3 Initial low glucose influences site-occupancy in EG2-hFc and anti-IL8 Mabs.

The effect of glucose concentration on glycan site-occupancy (macroheterogeneity) was analyzed in protein A purified antibodies from four-day (EG2-hFc Mab) or six-day (IgG1 Mab) batch cultures. **Figure 3.3** and **3.4** shows the analysis of reduced SDS-PAGE gels from EG2-hFc and anti-IL-8 Mabs respectively. **Figure 3.3A, B** and **C** showed a strong band at 40 kDa corresponding to single heavy chains of EG2-hFc Mab, followed by a secondary band of lower molecular weight in cultures containing 3 mM and 5 mM/ no glutamine (**Figure 3.3A,** lane 2 and 3) and in 3 mM glucose/ 0-4 mM glutamine (**Figure 3.3B** lane 2-6). This lower band was assigned as a non-glycosylated Mab based upon the time-dependent appearance of a lower band following treatment of a Mab sample (25 mM glucose, 4 mM glutamine) with PNGase F (**Figure 3.3C,** lane 3 and 4**)** and mass spectrometry evidence as explained below. Increasing glutamine concentrations (0 to 4 mM) did not decrease the amount of non-glycosylated antibody (**Figure 3.3B**). With respect to DP12 cells, visible Mab bands appeared at 50 kDa corresponding to single heavy chains in all glucose concentrations. As EG2 cells, DP12 Mabs also showed a more fainted band below the HC band in cultures with <11 mM glucose (**Figure 3.4**, lane 2 and 3). Glycosylated and non-

glycosylated Mabs were distinguished by a molecular weight difference of 3.3 kDa on SDS-PAGE gels (AlphaView Software). In addition, bands intensities were used to determine the relative abundance of glycosylated vs. non-glycosylated Mab with ~ 38.49% and a 35.10% of non-glycosylated Mab under 3mM and 5mM glucose respectively.

Figures 3.5A and B show the MALDI-MS spectra of intact Mabs in solution produced in 3 mM and 25 mM glucose respectively. **Figure 3.5A** shows a major peak at *m/z* 82660 that can be assigned as glycosylated Mab. The additional peak at *m/z* 79340 was ascribed to the non-glycosylated Mab. The Mab sample from the 25 mM glucose culture produced a similar single peak at *m/z* 82650 (**Fig. 3.5B**). The existence of non-glycosylated Mab was verified by mass spectrometry using two approaches: analysis of tryptic digestion products excised from bands, and analysis of intact Mab de-glycosylated using PNGase F. **Figures 3.6A and B** show the MALDI-MS spectra of trypsin digested upper bands (glycosylated Mab) of lanes 2 and 7 of **Figure 3.3A** produced in 3 mM and 25 mM glucose, respectively. The spectra show evidence of the presence of a range of glycopeptides (*m/z* range 2200–3600). Glycopeptide peaks were assigned in mass spectra based on the observation of typical patterns featuring *m/z* intervals of 146 (Fuc), 162 (Hex), 203 (GlcNAc), and 291 (NeuAc) between peaks. The structures were confirmed using tandem MS (MS/MS) with assessment of the fragmentation patterns. An example is in **Figure 3.8** which shows the fragmentation pattern of the peak at *m/z* 2796.09 derived from the spectrum in **Figure 3.6A** allowing a structural assignment of a G1 glycopeptide. In this MS/MS spectrum, ions at *m/z* 1272.55 correspond to characteristic [peptide+84]$^+$ fragments, which allow for calculation of the mass of the bare peptide (Krokhin, Ens *et al.* 2004). The residual mass (2796-1188) corresponds to the glycan G1F. **Figure 3.6A** (3 mM glucose) shows a higher proportion of non-galactosylated

structures (*m/z* at 2634.1) in comparison to **Figure 3.6B** (25 mM glucose), which indicates a degree of microheterogeneity supported by the HPLC data of isolated glycans. **Figures 3.7A and 3.7B** show the MS/MS spectra of precursor ions at *m/z* 1189.51 and 1190.49 respectively. In **Figure 3.7A,** it was possible to sequence the peptide **EEQYNSTYR**, which includes the glycosylation site at asparagine 297 of the Mab. This peptide was present in the non-glycosylated band in lane 2 of the gel in **Figure 3.3A**. In **Figure 3.7B**, the sequence found by MS/MS is **EEQYDSTYR,** corresponding to the de-glycosylated Mab produced at high glucose concentration (**Figure 3.3C**). The action of PNGase F converts glycosylated asparagine (N) into aspartic acid (D). The MS spectrum showing the parent ions of **Figures 3.7A and 3.7B** are shown in **Figures 3.9 A** and **B** respectively. None of the glycosylated peptides were found in the tryptic digest of lower band (lane 2, **Figure 3.3A**). Instead, an intense peak was observed at *m/z* 1189.5 (**Figure 3.9A**). This peak corresponded to a non-glycosylated peptide with the amino-acid sequence EEQYNSTYR (**Figure 3.7B**) and thus confirming the assignment of the lower band (lane 2, **Figure 3.3A**) as non-glycosylated. These results indicate that glucose starved cells produced significant amounts of non-glycosylated antibody in cultures with <11 mM glucose. The data represent the accumulated antibody over the culture period and extracted on day four.

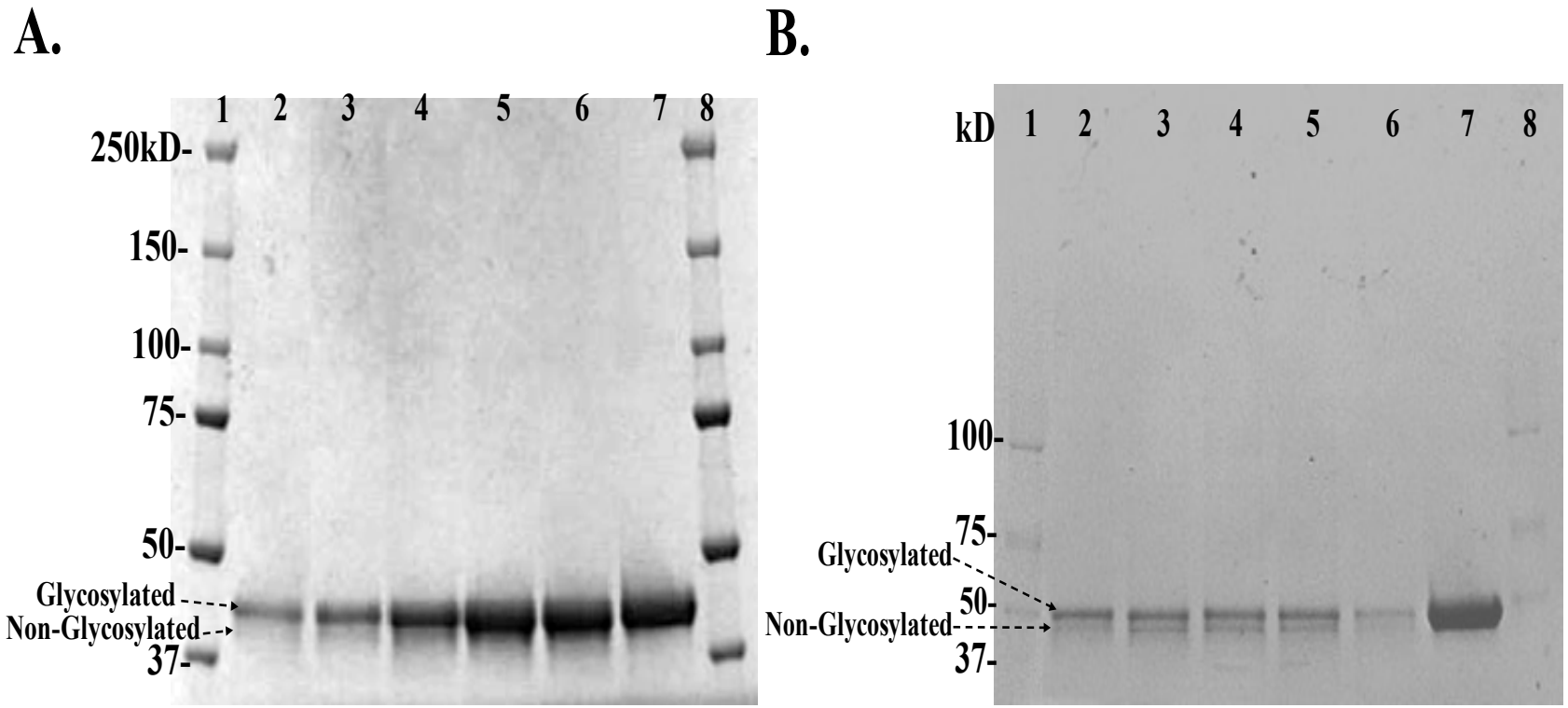
3.4.4 Initial low glucose effect on the microheterogeneity of EG2-hFc and anti-IL8 Mabs.

The effect of glucose availability on microheterogeneity of EG2-hFc Mabs was further analyzed by HPLC. The glycosylation profiles correspond to the cumulative production from the start of the culture up to day four where supernatants were collected. The predominant peaks were

assigned based on comparison with GU values previously recorded on the NIBRT Glycobase 3.2. The structures identified corresponded to core-fucosylated bi-antennary glycans containing different amounts of terminal galactose and Sia. Control cultures showed a higher proportion of galactosylated glycans containing two or one galactose residues (G2= 52% ± 6.15; G1= 30% ± 5.41) followed by a smaller amount of non-galactosylated glycans (G0= 15% ± 5.71). These structural assignments were confirmed by MALDI-MS spectra of tryptic digests of glycopeptides at *m/z* 2634.1, 2796.1 and 2958.2 respectively (**Figures 3.6A** and **3.6B**). In addition, smaller amounts of glycans containing one or two Sias (13.0% ± 2.71; F6A2G2S1, F6A2G2S2) were also observed in control cultures and confirmed by MALDI-MS spectra at *m/z* 3087.2, 3249.3 and 3540. **Figure 3.10** shows the relative abundance of glycans at different glucose concentrations. A significant decline of up to 39% in G2 glycans was observed as glucose concentrations decreased from 25 mM to 3 mM (p-Value ≤0.01). At the same time, a significant increase of about 108% in G0 glycans was observed in cultures with ≤11 mM (p-Value ≤0.03). The galactosylation indices (GI) were calculated according to ***Eq. 6*** previously defined in Section 2.11.6. As expected, a significant increase in GI (from 0.48 ± 0.03 to 0.64 ± 0.05) was observed as initial glucose concentration increased from 3 mM to 25 mM, showing a correlation between the availability of glucose and the content of galactosylated glycans (R^2= 0.94*)*. In addition, as more terminal Gal became available at higher glucose concentrations, more sialylated glycoforms were observed at 25 mM (13.0%) than at 3 mM glucose (4.74%) (p-Value <0.01) (**Figure 3.10**). On the other hand, increasing the initial glutamine concentration from 0 to 4 mM did not significantly improve the galactosylation or sialylation observed in 3 mM glucose cultures (GI=<0.57) in comparison to the control (GI=0.68) (**Table 3.2, Figure 3.11**). With respect to DP12 cells, anti-IL-8 IgG1 Mabs showed a significantly different glycosylation profile when compared to EG2-hFc Mabs (**Figure**

3.12). The predominant structures identified in DP12 control cultures were also the core-fucosylated bi-antennary glycans F6A2G0, F6A2G1 and F6A2G2. However, the previously seen EG2-hFc Mabs distribution (G0<G1<G2) differed by a higher content of G0 glycans (51.2% ± 1.02) followed by G1 (40.43% ± 0.46) and G2 glycans (8.38% ± 0.57) (**Figure 3.13)**. This represents a lower GI value of 0.34 ± 0.02 than the previously shown for EG2 cells (GI = 0.68 ± 0.03). DP12 cultures showed the following distribution regardless of glucose concentration (3 mM-25 mM glucose). Furthermore, sialylated glycans were only 1.49% ± 0.22 of the total glycan pool.

Figure 3. 3 Effect of increasing glucose concentration on the macroheterogeneity of EG2-hFc Mab.
A. EG2-hFc Mabs harvested on day four from media containing 3 mM, 5 mM, 11 mM, 17.5 mM and 25 mM glucose (lanes 2, 3, 4, 5, 6) without glutamine. **B.** 0 mM, 1 mM, 2 mM, 3 mM and 4 mM glutamine with 3 mM glucose (lanes 2, 3, 4, 5, 6) **C.** Protein-A purified EG2-hFc antibody was produced in control culture (same as Fig. 3.2A and B, lane 7) after PNGase F digestion over a period of 0, 30 and 60 min (lanes 2, 3 and 4 respectively). Protein samples loaded into gel where 16-fold the original supernatant concentration. Proteins were separated under reducing conditions through 8 to 16% by SDS-PAGE gel and stained with coomasie blue. Lanes 1 and 8 show marker proteins. Non-glycosylated, deglycosylated and glycosylated forms of Mab are indicated by arrows



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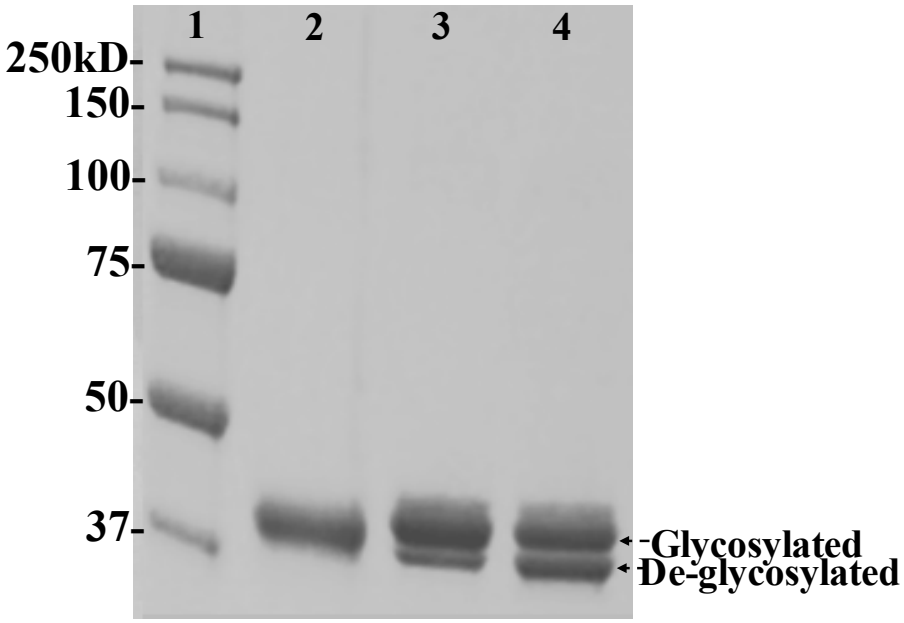


These data indicate that the initial glucose concentration Mab *N*-linked glycosylation. For instance, a significant increase of up to 130% and 4% in G0- and G1- glycans along with a significant decrease of up to 39% in G2- glycans were observed in cultures with <11 mM glucose. Furthermore, the levels of sialylated glycans were also affected by glucose depletion in cultures containing ≤11 mM initial glucose. In contrast, decreasing glucose concentrations from 25mM to 3 mM did not have an impact of anti-IL-8 Mab glycosylation probably because G0 glycans were already the most abundant structures (51.2%) under normal conditions. The latter also explains why DP12 cells produce Mabs with lower sialylation due to the absence of terminal Gal.

Figure 3. 4 Effect of increasing glucose concentrations on the macroheterogeneity of IgG1 produced by DP12 cells .

Mabs harvested on day six from media containing **A.** 3 mM, 5 mM, 11 mM, 17.5 mM and 25 mM glucose (lanes 2, 3, 4, 5, 6) without glutamine. **B.** 0 mM, 1 mM, 2 mM, 3 mM and 4 mM glutamine with 3 mM glucose (lanes 2, 3, 4, 5, 6). Lane 7 shows Mab produced in control culture media (25 mM glucose, 4 mM glutamine). Protein samples loaded into gel where 16-fold the original supernatant concentration. Proteins were separated under reducing conditions through 8 to 16% by SDS-PAGE gel and stained with coomasie blue. Lane 1 shows marker proteins. Non-glycosylated and glycosylated forms of Mab are indicated by arrows

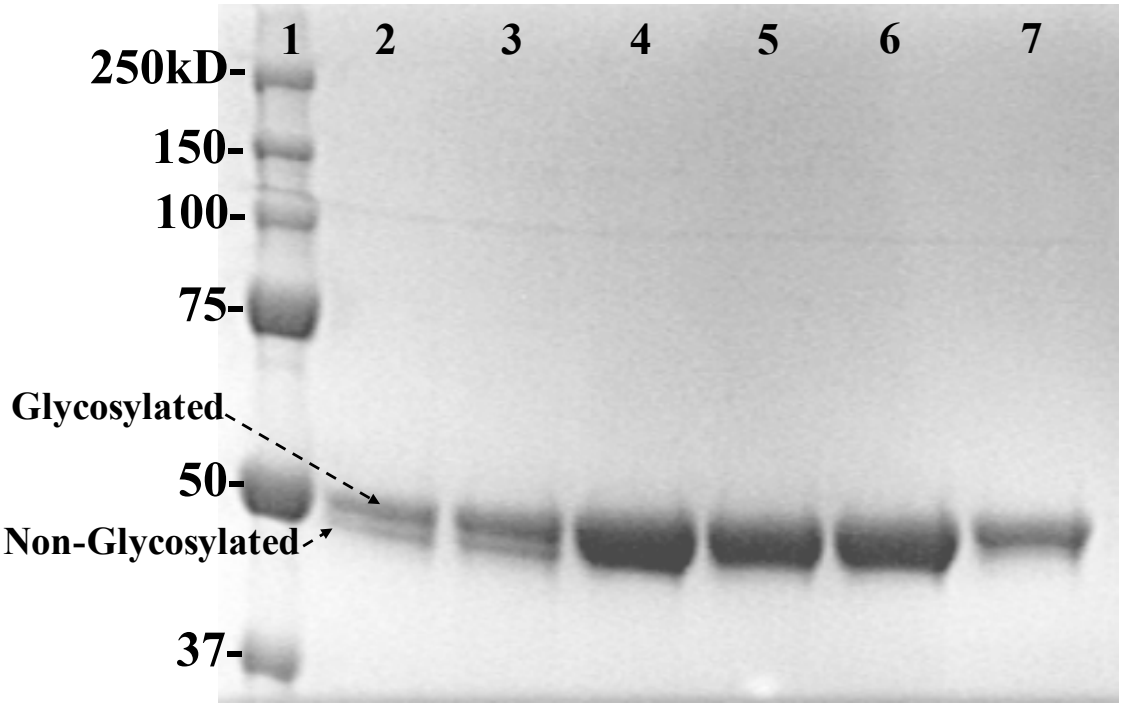


Figure 3. 5 Mass spectrometry of CHO EG2-hFc Mab.

MALDI-MS spectra were recorded with an UltrafleXtreme (Bruker) instrument in the linear mode from intact Mabs in solution produced in: **A.** 3 mM glucose, 2.5 x 10^5 cells/mL inoculum and **B**. 25 mM glucose, 2.5 x 10^5 cells/mL inoculum.

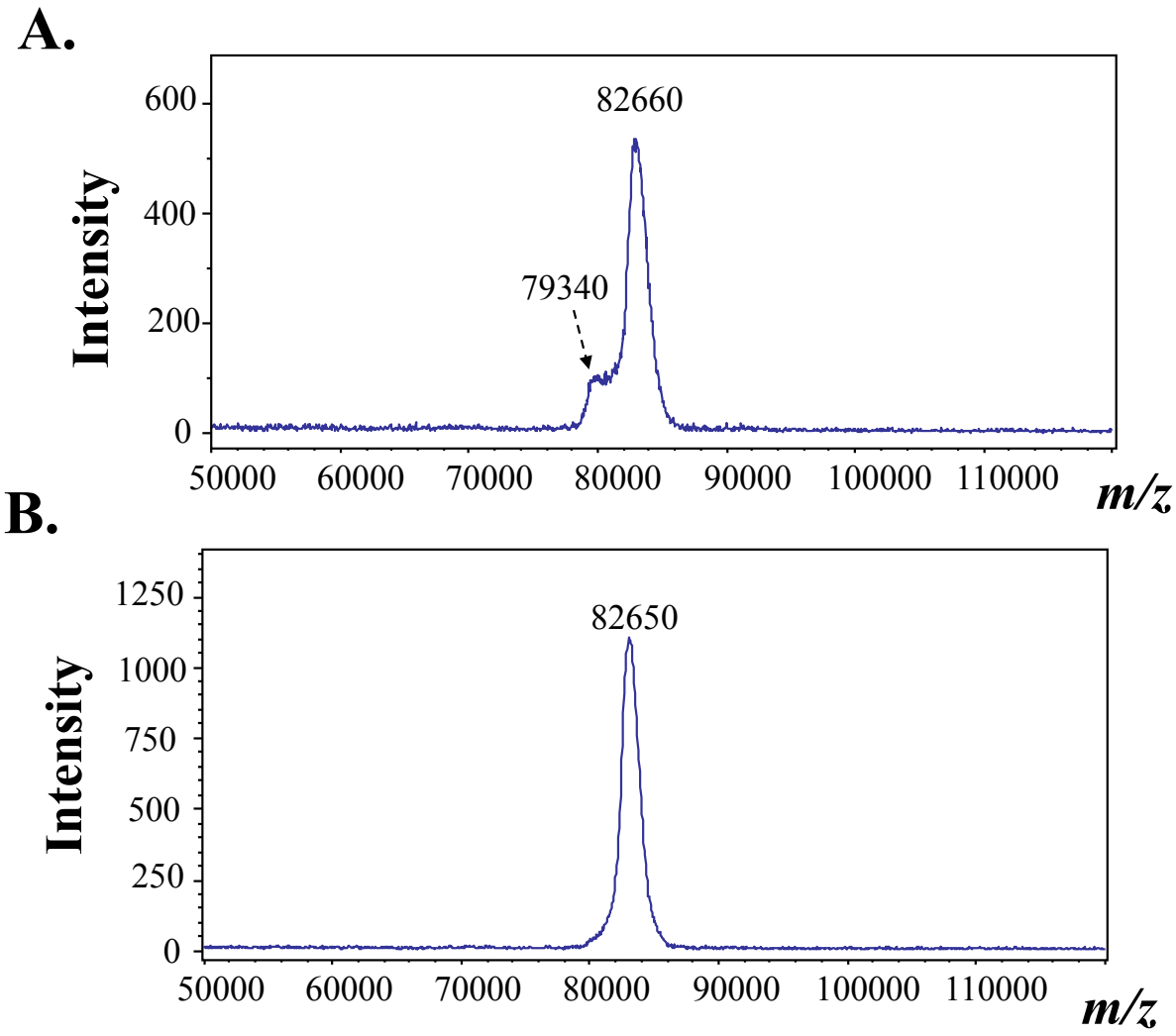


Figure 3. 6 Mass spectrometry of CHO EG2-hFc Mab.

Individual protein bands were extracted for MALDI-MS analysis of glycopeptides recorded in the positive mode with Qq-TOF (Manitoba Sciex) from RP-HPLC fractions of gel trypsin digested: A. Upper band of lane 2 (3 mM glucose) and B. lane 7 (25 mM glucose) in Figure 3.3A and 3.3B respectively. All glycopeptides were detected as [M+H]+ and all illustrated glycans are attached at asparagine in the amino acid sequence of a composition EEQYNSTYR (*m/z* 1189.5).

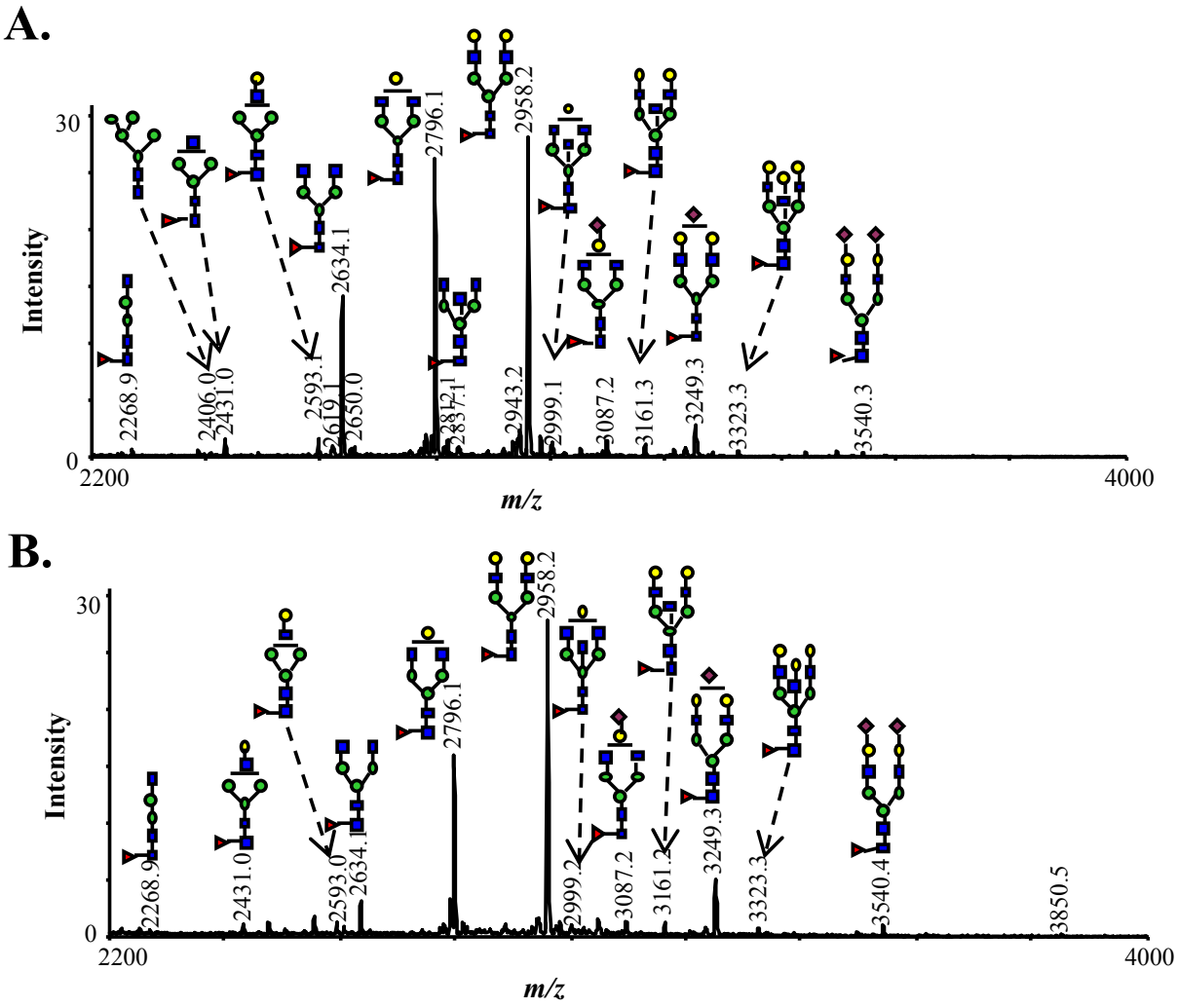


Figure 3. 7 MALDI-MS/MS spectra analysis of CHO EG2-hFc Mab.

MALDI-MS/MS of peptides with precursor ions at *m/z*: **A.** 1189.511, corresponding to upper band of lane 2 (3 mM glucose) and **B.** 1190.499 of lane 7 (25 mM glucose, after PNGase treatment) in Figure 3.3A and 3.3B respectively. All glycopeptides were detected as $[M+H]^+$ and all illustrated glycans are attached at asparagine

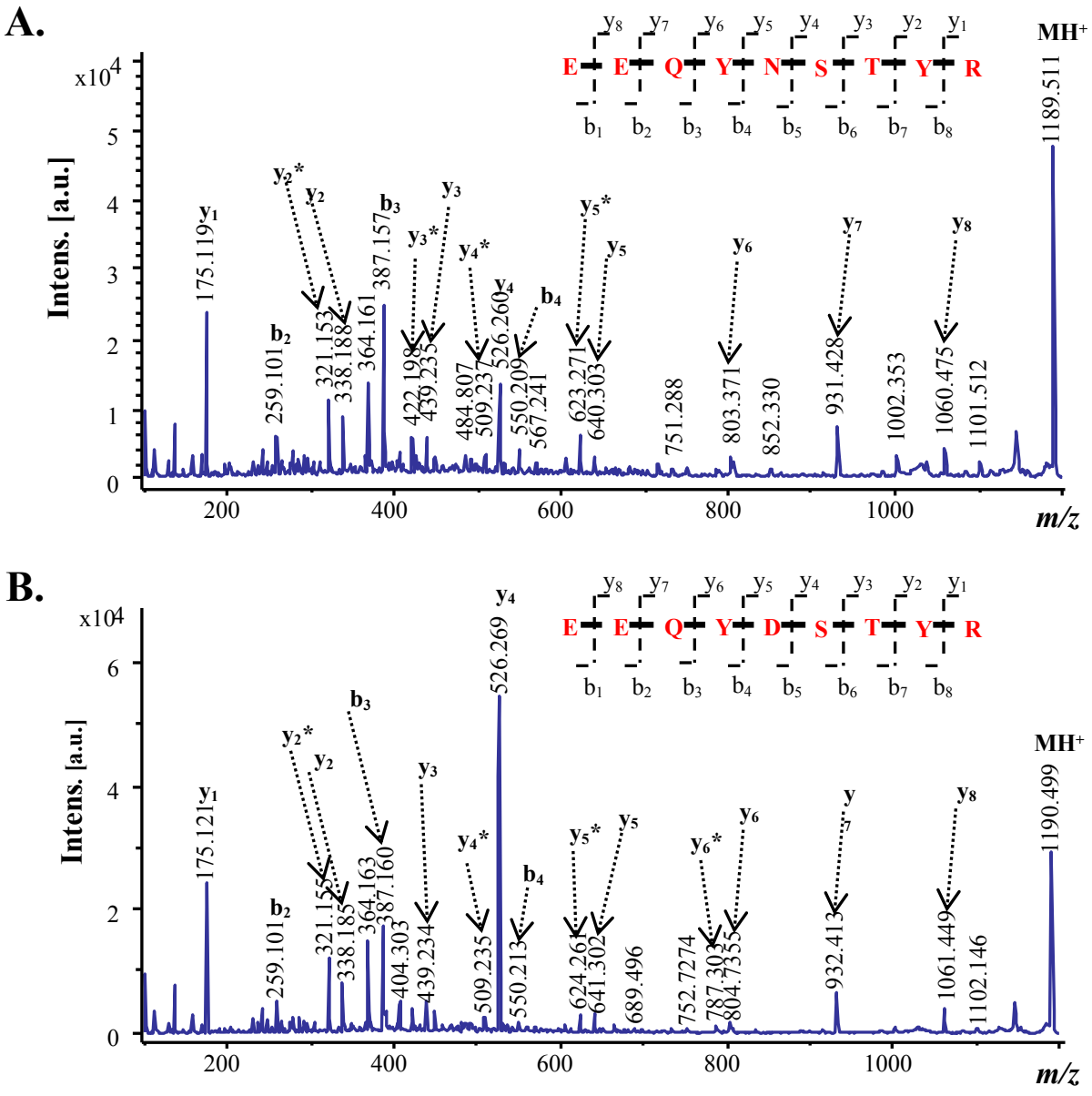
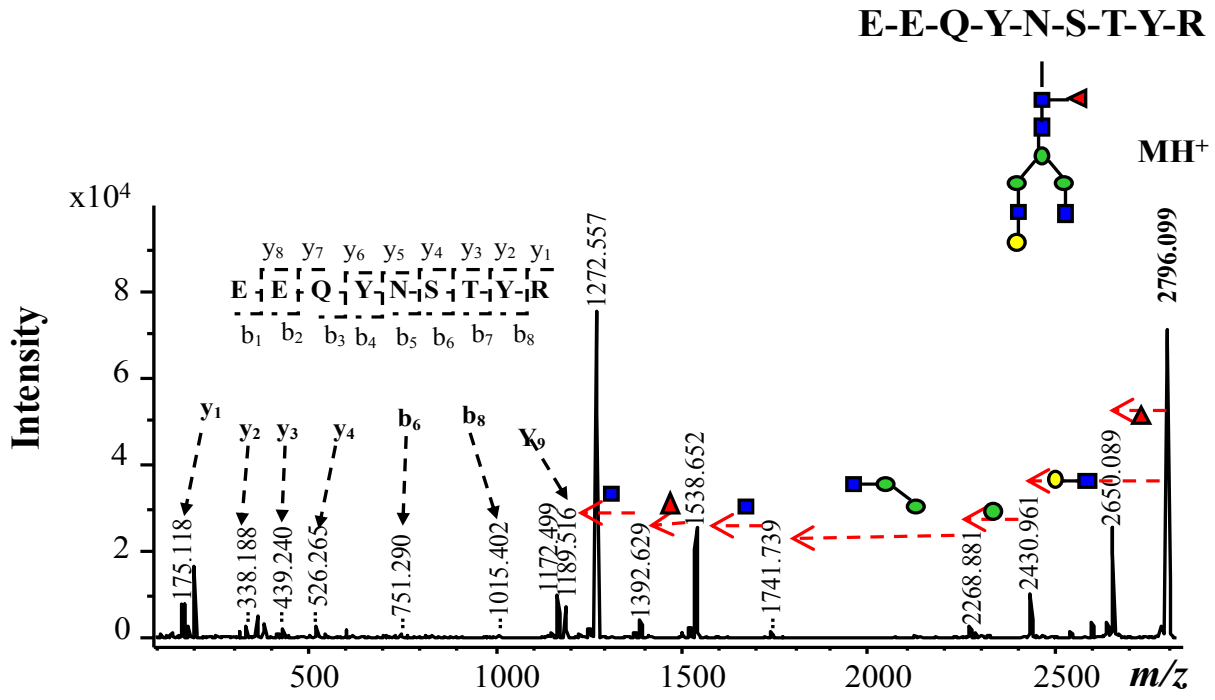


Figure 3. 8 Selected MALDI-MS/MS spectrum and fragmentation schemes of EG2-hFc Mab glycopeptide.

For **A.** Precursor ions at *m/z* 2796.09 ($M+H^+$). Peaks were originated from tryptic digests of sample corresponding to Figure 3.6A. Illustrated glycans are attached at asparagine in the amino acid sequence of a composition EEQYNSTYR (*m/z* 1189.5).



3.4.5 Glucose depletion causes a decrease in energy charge and intracellular levels of nucleotide sugars.

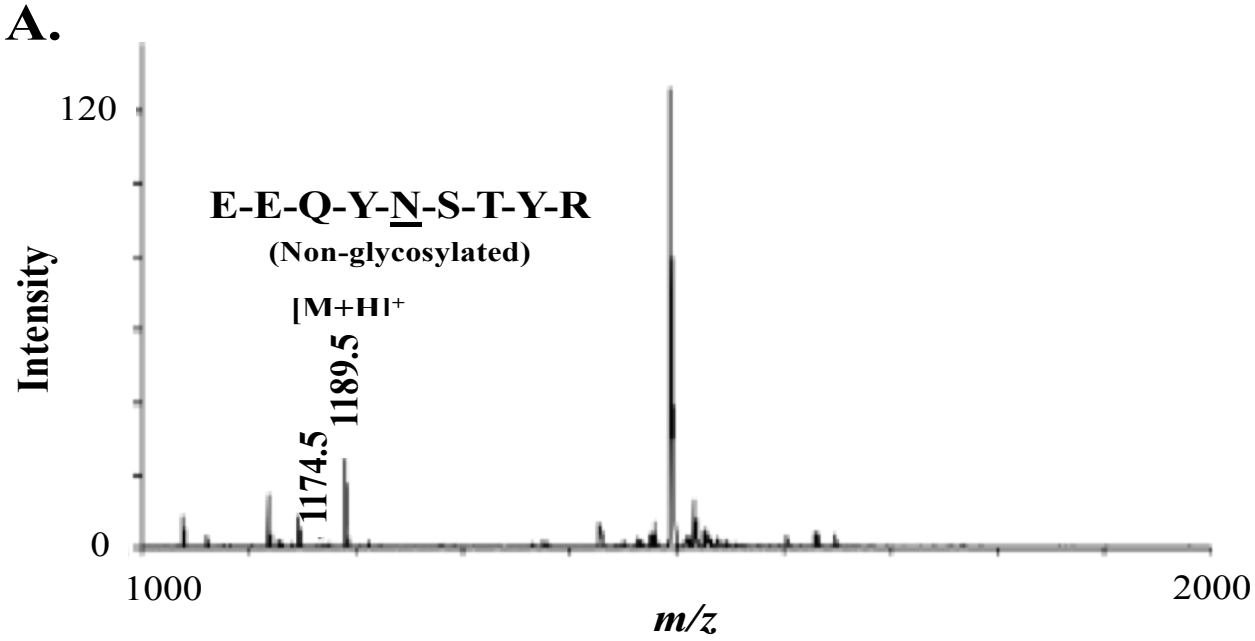
A reduced pool of intracellular nucleotide and nucleotide sugars could be responsible for the changes observed in both, macro- and micro-heterogeneity in glucose-depleted cells. To explore this hypothesis, previously starved EG2 cells were seeded at 2.5 x 10^5 cells/mL in media with increasing glucose concentrations without glutamine or media with low glucose and increasing glutamine concentrations. **Figure 3.14** shows the separation of nucleotides and nucleotide sugars from cell extracts taken at day four of culture in 25 mM and 3 mM glucose

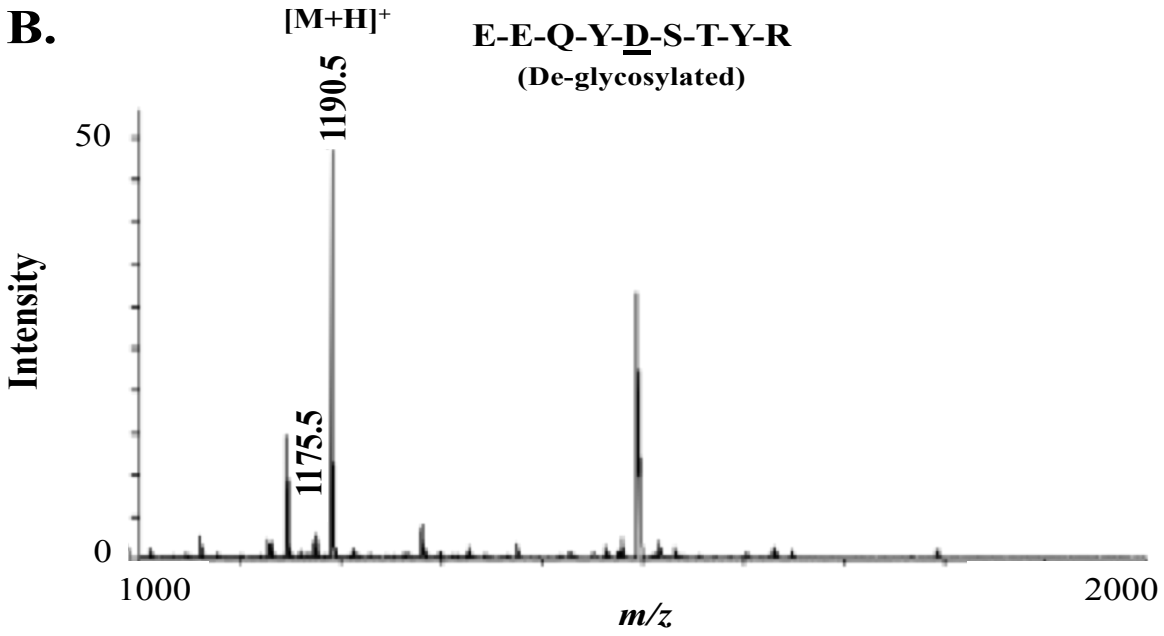
(**Figures 3.14A.** and **B.** respectively). These are representative analyses of a series of extracts taken from cultures with a range of initial glucose concentration from 3 to 25 mM. The structural assignment of the peaks was based upon an equivalent chromatographic run of 20 standard molecules. Intracellular concentrations of each of these metabolites was obtained by quantitative analysis of the peak areas in relation to GDP-Glc used as internal standard (IS).

There were significant differences in the intracellular profile of nucleotides and nucleotide sugars from cell extracts analyzed from cultures with differing glucose concentrations.

Figure 3. 9 Selected MALDI-MS spectra of non-glycosylated and glycosylated EG2-hFc Mabs.

RP-HPLC fractions of tryptic peptides obtained from A. lower band of lane 2 in Figure 3.3A (3mM glucose) and from B. Mab produced in control culture (25mM glucose) after PNGase F deglycosylation. Peaks without assigned *m/z* values corresponded to the tryptic peptides from Mab or peptides originated from trypsin.





For the cell extract taken from the 25 mM glucose culture the major peaks corresponded to ATP (6.07 ± 0.13 fmoles/cell) followed by GTP (2.67 ± 0.48 fmoles/cell), ADP (2.18 ± 0.21 fmoles/cell) and AMP (0.80 ± 0.25 fmoles/cell).In 3 mM glucose cultures, the major peaks corresponded to monophosphate nucleotides such as CMP (0.06 ± 0.03 fmoles/cell), AMP (0.12 ± 0.04 fmoles/cell) and the nucleotide sugars UDP-Gal (0.14 ± 0.05 fmoles/cell) and UDP-Glc (0.33 ± 0.19 fmoles/cell). The extract from low glucose cultures also had very low levels of nucleotide tri- and diphosphates as well as the GDP-sugars (GDP-Fuc + GDPMan).

Figure 3. 10 Effect of glucose concentrations on the N-linked glycosylation of EG2-hFc Mab.

Relative abundance of glycans containing zero, one and/or two Gal residues (G0, G1 and G2) Mabs. Values correspond to the mean ± SD of duplicates of three independent experiments (n = 6). Pairs of means ± SD significantly different (p-Value <0.01*; <0.001**; <0.0001***) with respect to the control culture

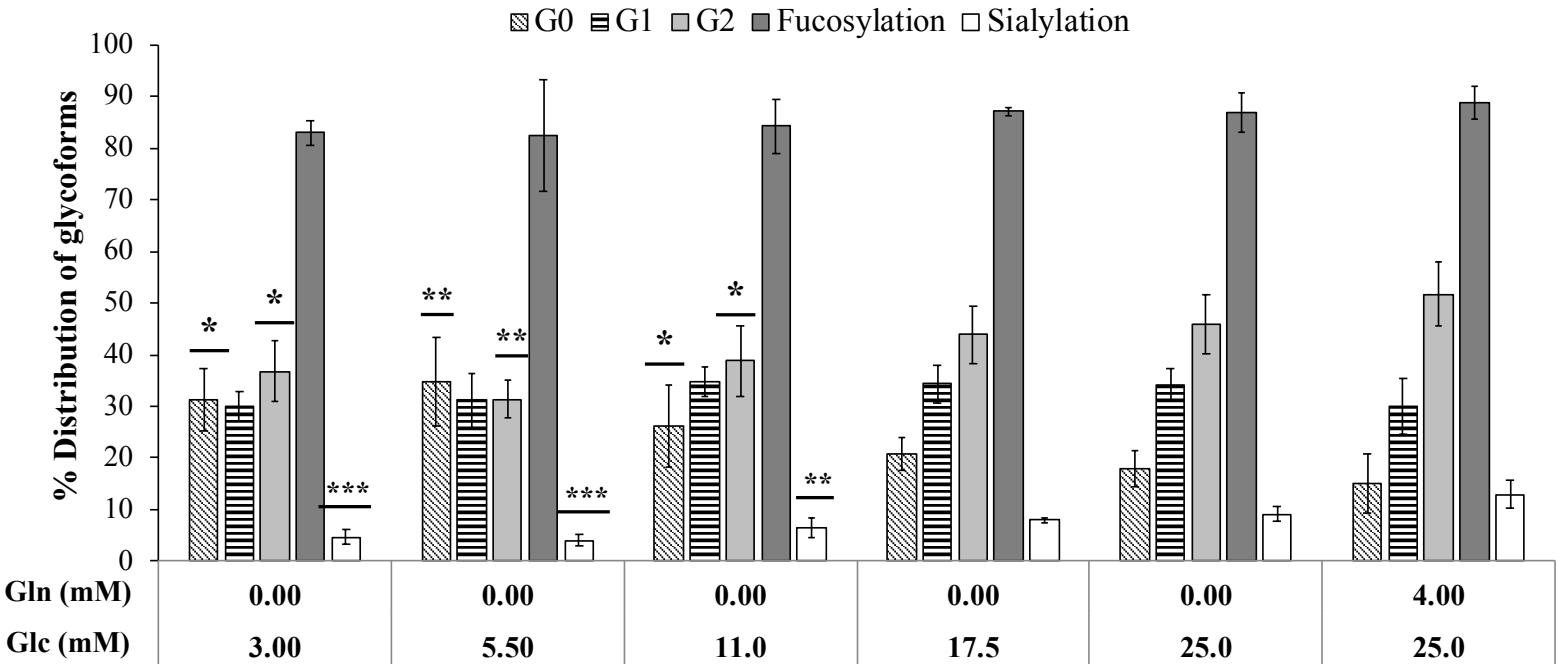


Figure 3. 11 Distribution of glycoforms (%) of EG2-hFc Mabs produced in 3 mM glucose and increasing glutamine concentrations.

A. Relative abundance of glycans containing zero, one and/or two Gal residues (G0, G1 and G2) and **B.** Sias from EG2-hFc Mabs cultured in media containing 3 mM glucose and increasing glutamine concentrations (0 mM- 4 mM). Control culture contained 25 mM glucose and 4 mM glutamine. Values correspond to the mean ± SD of duplicates of three independent experiments (n = 6). Pairs of means ± SD significantly different (p-Value <0.05*; <0.001**) with respect to the control culture.

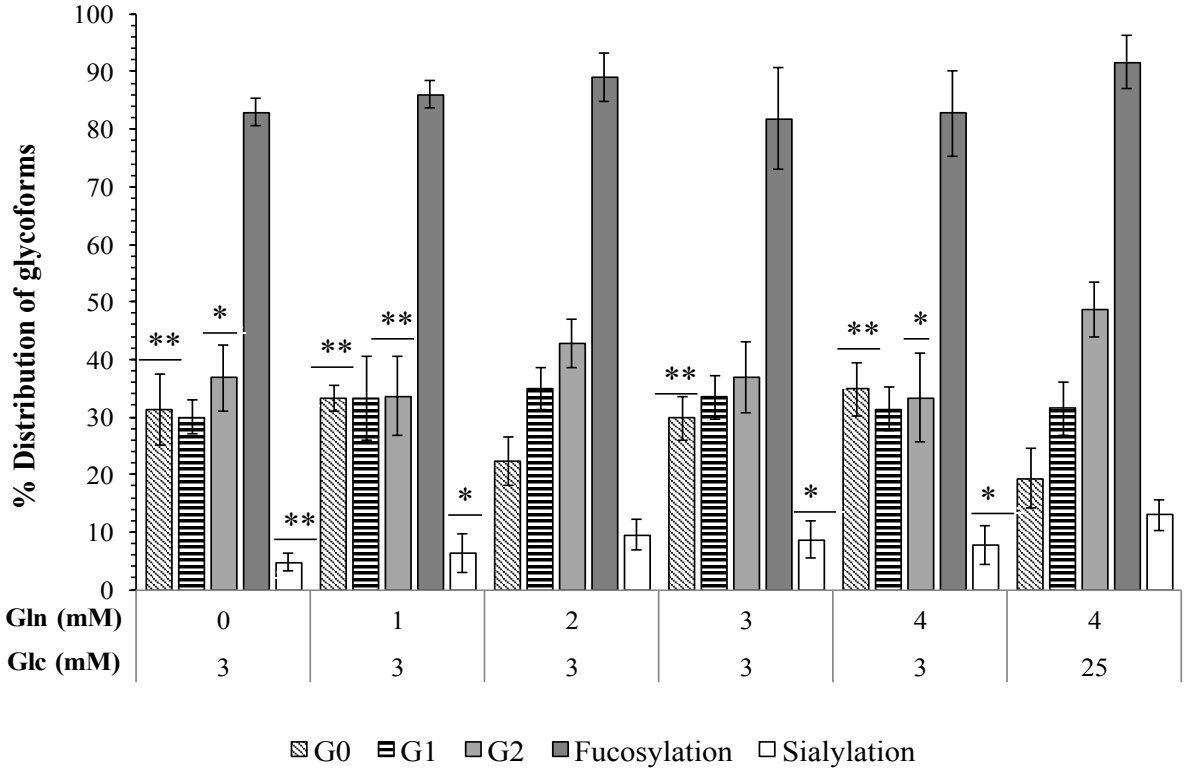


Table 3.3 shows the intracellular nucleotide and nucleotide sugar concentrations determined at day four for a series of cell extracts taken from cultures with differing glucose concentrations. The data show a gradual increase in the intracellular levels of nucleotide and nucleotide sugars as the initial glucose concentration increases from 3 to 25 mM. The concentration of intracellular pools of uridine (Urd), guanosine (Gua), adenosine (Ade) and

cytidine (Cyt) were significantly lower in cultures with ≤11 mM initial glucose. For example, at 3 mM glucose cultures the Urd, Gua, Ade and Cyt pools were 5.87, 0.86, 1.46 and 8.15% respectively for that of the control ($P \leq 0.01$). UDP-hexoses concentration (UDP-Gal + UDP-Glc) was within the range 0.47–0.99 fmoles/cell, and were not significantly different with respect to the control (0.66 fmoles/cell). The level of UDP-hexosamines (UDP-GlcNAc + UDP-GalNAc) was below 0.49 fmoles/cell in cultures with ≤11 mM glucose, equivalent to a 98.8% decrease in 3 mM glucose cultures ($P < 0.0001$). All cultures maintained CMP-NeuAc concentrations between 0.05 and 0.25 fmoles/cell indicating that CMP-NeuAc concentrations may be independent of the initial glucose concentration despite the higher sialylation observed at higher glucose concentrations. The GDP-sugars (GDP-Fuc + GDP-Man) concentrations in cultures with ≤11 mM initial glucose were below 0.04 fmoles/mL and above 0.11 fmoles/mL in cultures with ≥17.5 mM glucose.

Table 3.3 shows the adenylate energy charge (AEC) calculated as the ratio of adenylate nucleotides (AMP, ADP and ATP) for cell extracts taken from each culture. The values were determined from the HPAEC chromatographic runs of each cell extract. Extracts of actively growing cells (≥17.5 mM glucose) showed an AEC value ≥0.78, reflecting a high-energy state, whereas AEC values <0.57 were determined in glucose- depleted cultures (≤11 mM glucose). This initial concentration of 11 mM appeared to be a threshold value below which the AEC declined sharply. Thus, the cellular AEC of our benchmark values for low glucose (3 mM) and high glucose (25 mM) were 0.01 ± 0.02 and 0.81 ± 0.05 respectively (**Table 3.3**). In contrast, increasing glutamine concentrations (0 to 4 mM) at low glucose (3 mM) did not increase the AEC value above 0.03 ± 0.05 (**Table 3.4**). This was observed with a significantly low (p-Value < 0.01) Ade nucleotide pool (0.31 fmoles/cell) in comparison to the control (9.05 fmoles/cell). In addition,

significantly lower concentrations of Gua (<0.1 fmoles/cell; p-Value <0.01), Urd (<0.6 fmoles/cell; p-Value < 0.001) and Cyt (<0.06 fmoles/cell; p-Value < 0.001) pools were observed at low glucose irrespective of the glutamine concentration. CMP-NeuAc increased 78% (0.05–0.21 fmoles/cell) as glutamine increased to 4 mM in media containing low glucose. UDP-hexoses pool (0.28–0.76 fmoles/cell) and UDP-hexosamine pool (0.01–0.03 fmoles/cell) concentrations were not influenced by increasing glutamine concentrations (**Table 3.4**). These data show that glucose depletion (cultures with <11 mM initial glucose) caused a drastic decrease in overall intracellular nucleotide and nucleotide sugar levels as well as nucleotide triphosphate. Increased glutamine concentrations in the media did not enhance nucleotide and nucleotide sugar levels when glucose was low (3 mM).

Opposite to EG2 cells, glucose availability impacted only the site-occupancy of anti-IL8 and not the original distribution of glycoforms observed in control cultures. To further explain the changes observed in macro-heterogeneity, the intracellular levels of nucleotides and nucleotide sugars were extracted on day six and analyzed by ion pair RP-HPLC (**Table 3.5**). As the initial concentration decreased from 25 mM to 3 mM, a strong decrease in nucleotide and nucleotide sugars were observed. For instance, cultures that experienced starvation (<11 mM) showed a significant decrease of up to 102%, 97.7%, 100% and 95.6% in nucleotide triphosphates levels (CTP, ATP, UTP and GTP) with respect to the control culture. On day six, the total intracellular concentration of Urd, Gua, Ade and Cyt pools were also significantly lower in ≤17.5 mM (**Table 3.5**). For instance, adenylate nucleotides (AMP + ADP + ATP) decreased from 10.45 fmol/cell to 0.53 fmol/cell as the initial glucose decreased from 25 to 3 mM respectively (**Table 3.5**) (p-Value <0.05). As a result, a decrease of up to 53% was observed in the AEC ratio, defined as (ATP +

0.5ADP)/(AMP+ADP+AMP) in 3 mM glucose-containing cultures. AEC ratio tends to be around 0.9 for exponential growing cells and declines as the cellular physiological status worsens. The AEC values observed for DP12 cells were higher than that observed on CHO EG2 cells on day 4 in ≤5.5mM cultures. This correlated to the higher viability observed on day six in glucose starved DP12 cells. The significant decrease of about 87% in Urd pools in ≤5.5 mM cultures (p-Value <0.001) may be the responsible of the significant decline in UDP-Hex concentrations observed in the control (1.65 fmole/cell) to as low as 0.46 fmol/cell. In addition, a 91% decrease was observed in UDP-GNAc concentrations in 3 mM cultures when compared to the control (p-Value 0.02). Gua pools were significantly lower by up to 93% in ≤17.5mM cultures which could be responsible for the decline of up to 67% in GDP-sugar concentrations in these cultures (p-Value 0.01). Finally, Cyt concentrations were significantly lower in ≤5.5 Mm cultures by 90 % when compared to the control (p-Value <0.001). As in EG2 cells, CMP-NeuAc concentrations were not influenced by the initial glucose concentration. These results show that regardless of the cell line used, nucleotide and nucleotide sugar intracellular concentrations declined significantly in cultures that experienced glucose starvation

Figure 3. 12 Comparison of N-linked glycosylation profiles produced by HPLC chromatography of previously purified and 2-AB labeled Mabs produced by EG2 and DP12 cells.

EG2-hFc and DP12-anti-IL-8 Mabs were harvested on day four and day six respectively from control cultures containing 25 mM glucose and 4 mM glutamine.

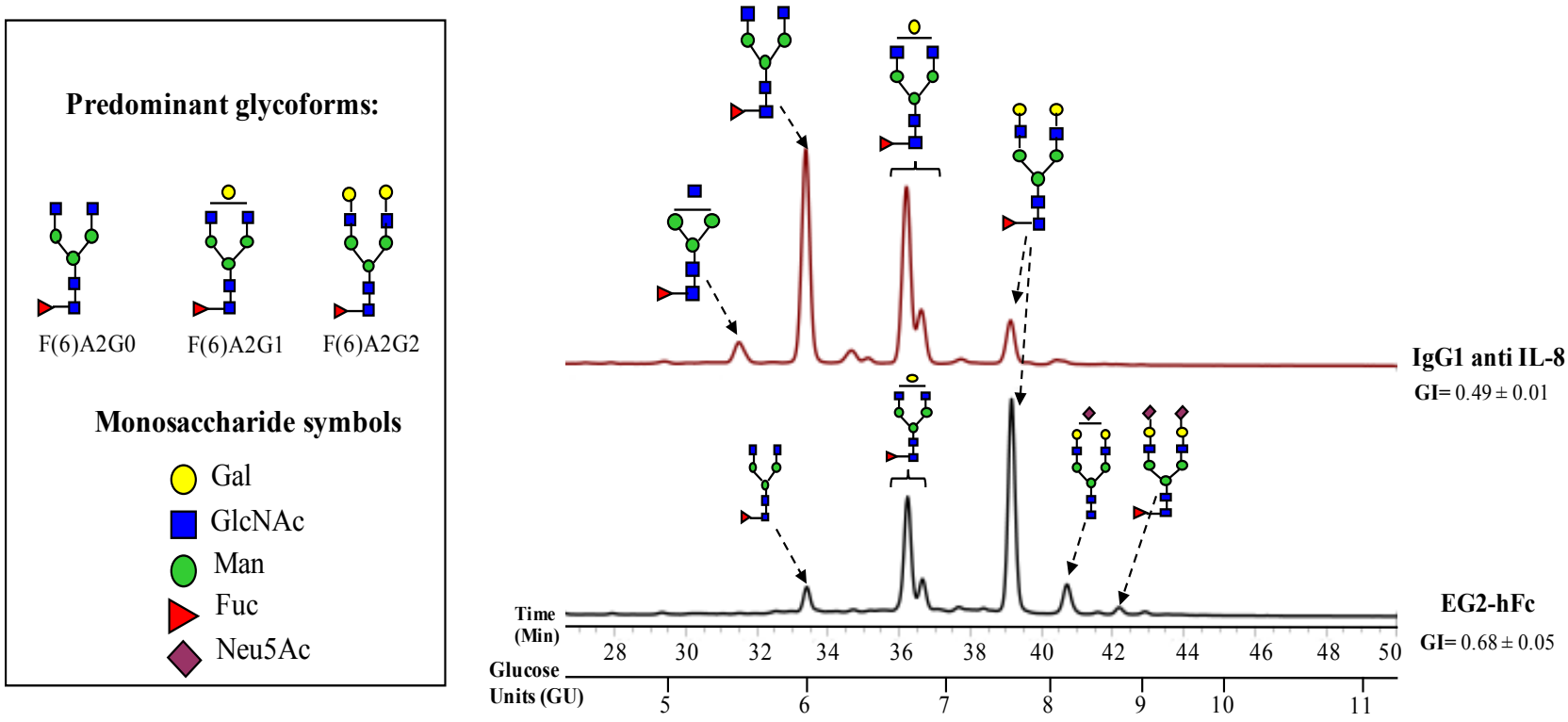


Figure 3. 13 Distribution of glycoforms (%) of DP12-IgG1 Mab produced in media with increasing glucose concentrations.

Relative abundance of glycans containing zero, one and/or two Gal residues (G0, G1 and G2) and/or Sias from DP12 anti-IL8 Mabs cultured in media containing 3 mM glucose and increasing glutamine concentrations (0 mM- 4 mM). Control culture contained 25 mM glucose and 4 mM glutamine. Values correspond to the mean ± SD of triplicates of one independent experiments (n = 3).

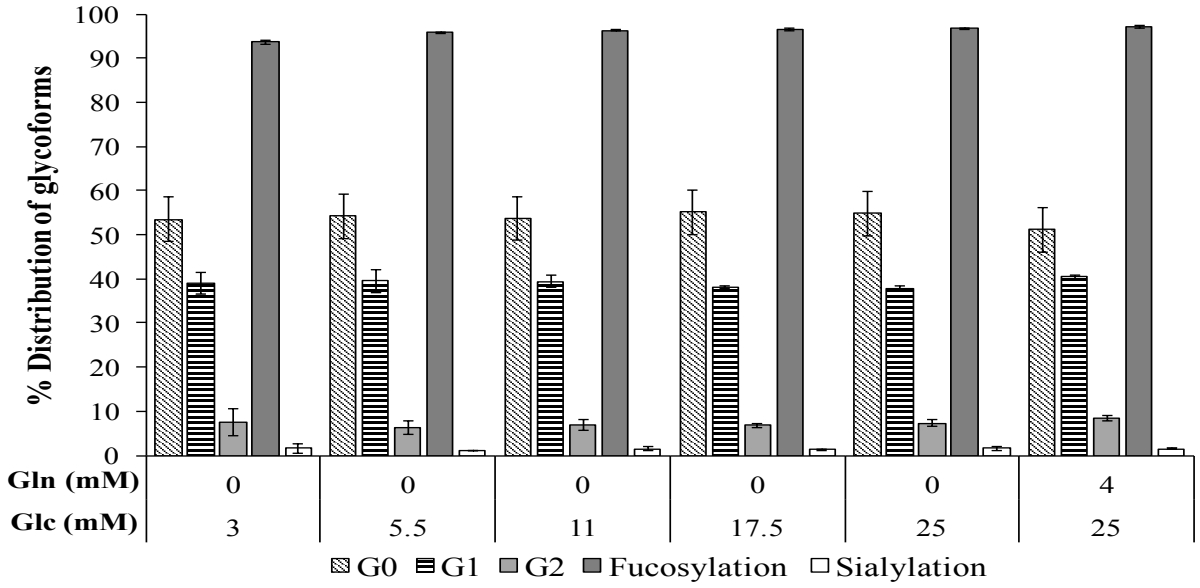


Figure 3. 14 Nucleotide and nucleotide sugar analysis of CHO EG2 cells.
HPAEC separations are shown of nucleotide and nucleotide sugars extracted from CHO EG2 cells cultured in **A.** 25 mM and **B**. 3 mM glucose- containing media and harvested on day four. **C.** Elution profile of standard mixture used for the identification of metabolites. The elution gradient allowed the separation of 19 out of 20 standard nucleotide and nucleotide sugars using 2 mM sodium hydroxide and 1 M sodium acetate in 1 mM sodium hydroxide as eluents. Metabolites were detected by absorbance at 260 nm.**1.** CMP, **2.** CMP-NeuAc, **3.** AMP, **4.** CDP, **5**. UMP, **6**. UDP-GalNAc, **7.** UDP-GlcNAc + CTP, **8.** ADP, **9.** UDP-Gal, **10.** UDP-Glc, **11**. GMP, 1**2.** UDP, **13**. ATP, **14**. GDP-Fuc, **15.** GDP-Man, **16.** UTP, **17**. GDP-Glc, **18.** GDP, **19.** GTP. GDP-Glc was used as an internal standard (IS). The peak shown with an asterisk is unknown.

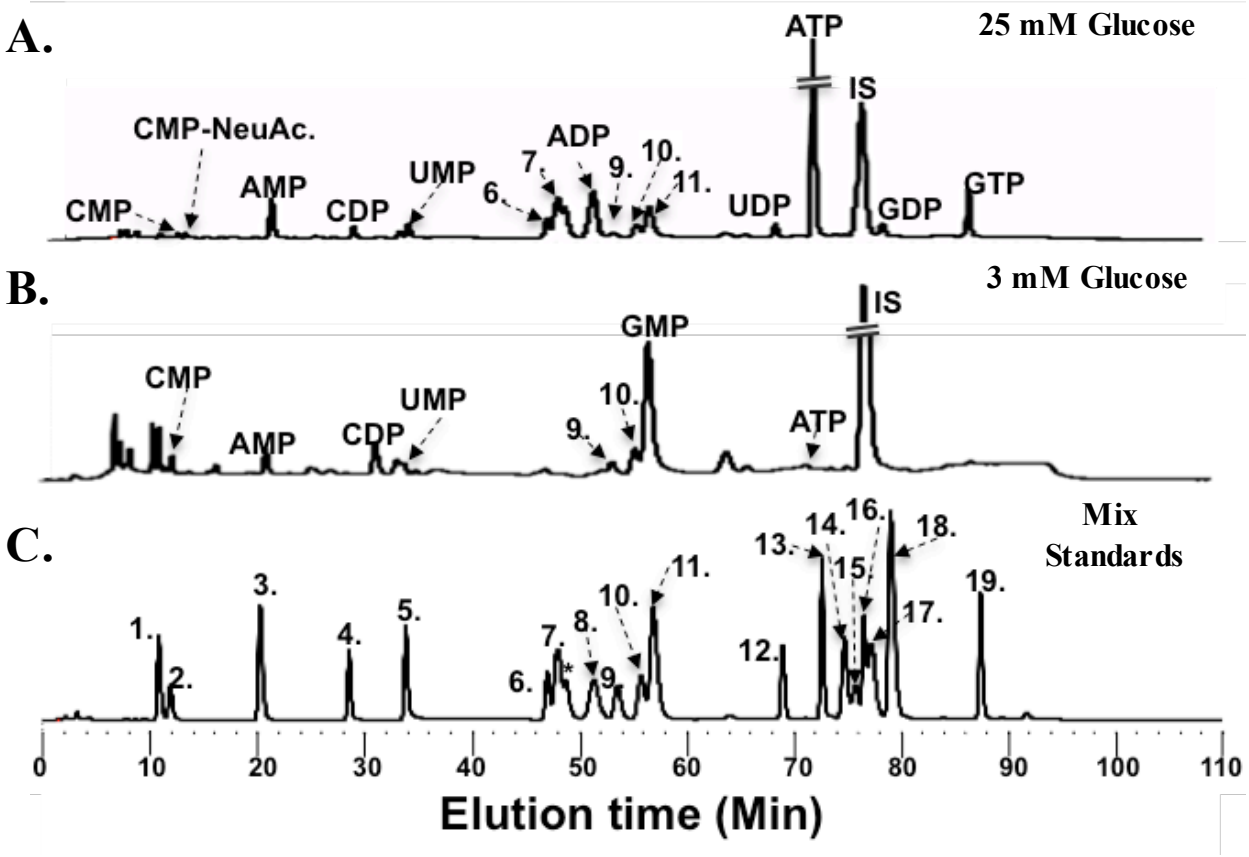


Table 3. 3 Intracellular nucleotide and nucleotide sugar concentrations (fmoles/cell) of EG2 cultures seeded at 2.5 x 10^5 cells/mL in increasing concentrations of glucose without glutamine.

Cell Density Inoculum		**x10^5 cells/ml**																			
Glucose (mM)		**3.00**			**5.50**			**11.00**			**17.50**			**25.00**			**25.00†**				
Glutamine (mM)		**0.00**			**0.00**			**0.00**			**0.00**			**0.00**			**4.00**				
Nucleotide and Nucleotide Sugar (fmole/cell)	**(UMP+UDP+UTP)**	0.04	±	0.01*	0.03	±	0.02*	0.10	±	0.02*	0.58	±	0.07	0.78	±	0.09	0.66	±	0.25		
	(GMP+GDP+GTP)	0.05	±	0.06*	0.07	±	0.01*	1.18	±	0.26	2.45	±	0.44	3.36	±	0.04	2.98	±	0.29		
	(AMP+ADP+ATP)	0.13	±	0.04*	0.23	±	0.05*	1.37	±	0.44	8.12	±	0.13	11.1	±	0.29	9.05	±	1.13		
	(CMP+CDP+CTP)	0.06	±	0.04*	0.23	±	0.04*	0.08	±	0.04*	0.98	±	0.18	0.89	±	0.21	0.71	±	0.32		
	(UDP-Glc+UDP+Gal)	0.47	±	0.23	0.49	±	0.03	0.08	±	0.03	0.60	±	0.07	0.99	±	0.15	0.66	±	0.19		
	(UDP-GalNAc+UDP-GlcNAc)	0.03	±	0.03*	0.10	±	0.05*	0.49	±	0.04*	3.13	±	0.32	3.29	±	0.74	3.27	±	0.29		
	(GDP-Fuc+GDP-Man)	0.03	±	0.01	0.04	±	0.01	0.03	±	0.00	0.11	±	0.03	0.13	±	0.02	0.13	±	0.09		
	CMP-NeuAc	0.05	±	0.07	0.25	±	0.02	0.13	±	0.06	0.08	±	0.05	0.17	±	0.06	0.15	±	0.01		
	AEC	0.01	±	0.02	0.03	±	0.01	0.57	±	0.04	0.78	±	0.01	0.81	±	0.07	0.81	±	0.05		

The media varied in glucose concentrations (3 to 25 mM) in the presence or absence of glutamine. Nucleotide concentrations are represented as the sum of mono, di, and triphosphate nucleotides.

† Control culture

* Pairs of means ± SD (n = 6) that are significantly different (P=0.01) with respect to the corresponding nucleotide/nucleotide sugar control culture.

Table 3. 4. Intracellular nucleotide and nucleotide sugar concentrations (fmoles/cell) of EG2 cultures seeded at 2.5 x 10^5 cells/mL in increasing glutamine with 3 mM glucose.

Cell Density Inoculum		**x10^5 cells/ml**																		
Glucose (mM)		**3.00**			**3.00**			**3.00**			**3.00**			**3.00**			**25.00†**			
Glutamine (mM)		**0.00**			**1.00**			**2.00**			**3.00**			**4.00**			**4.00**			
	(UMP+UDP+UTP)	0.01	±	0.01*	0.06	±	0.06*	0.02	±	0.01*	0.01	±	0.00*	0.02	±	0.01*	0.66	±	0.25
	(GMP+GDP+GTP)	0.03	±	0.04*	0.08	±	0.05*	0.07	±	0.07*	0.10	±	0.07*	0.01	±	0.02*	2.98	±	0.29
Nucleotide	**(AMP+ADP+ATP)**	0.13	±	0.04*	0.22	±	0.06*	0.21	±	0.13*	0.31	±	0.10*	0.12	±	0.04*	9.05	±	1.13
and	**(CMP+CDP+CTP)**	0.06	±	0.04*	0.01	±	0.01*	0.03	±	0.01*	0.05	±	0.06*	0.02	±	0.00*	0.71	±	0.32
Nucleotide	**(UDP-Glc+UDP+Gal)**	0.54	±	0.22	0.54	±	0.00	0.28	±	0.00	0.76	±	0.25	0.36	±	0.17	0.66	±	0.19
Sugar	**(UDP-GalNAc+UDP-GlcNAc)**	0.04	±	0.03*	0.03	±	0.01*	0.01	±	0.00*	0.04	±	0.02*	0.01	±	0.01*	3.27	±	0.29
(fmole/cell)	**(GDP-Fuc+GDP-Man)**	0.03	±	0.02*	0.04	±	0.00*	0.02	±	0.01*	0.04	±	0.01*	0.04	±	0.03*	0.13	±	0.09
	CMP-NeuAc	0.05	±	0.07	0.22	±	0.05	0.22	±	0.11	0.09	±	0.18	0.21	±	0.06	0.15	±	0.01
	AEC	0.00	±	0.00	0.00	±	0.00	0.01	±	0.01	0.03	±	0.05	0.01	±	0.00	0.78	±	0.03

†Control culture

* Pairs of means ± SD (n=6) that are significantly different (p-Value ≤0.01) with respect to the corresponding nucleotide/nucleotide sugar control culture.

Table 3. 5. Intracellular nucleotide and nucleotide sugar concentrations (fmoles/cell) of DP12 cultures seeded at 2.5 x 10^5 cells/mL in increasing glucose concentrations.

†Control culture

Cell Density		**x10^5 cells/ml**									
Inoculum											
Glucose (mM)		**3.00**		**5.50**		**11.00**		**17.50**		**25.00†**	
Nucleotide and Nucleotide Sugar (fmole/cell)	**(UMP+UDP+UTP)**	0.11 ± 0.02*		0.36 ± 0.21*		0.83 ± 0.16		1.00 ± 0.13		0.86 ± 0.25	
	(GMP+GDP+GTP)	0.27 ± 0.12*		0.47 ± 0.01*		1.59 ± 0.06*		1.99 ± 0.26*		3.68 ± 0.68	
	(AMP+ADP+ATP)	0.53 ± 0.22*		1.24 ± 0.20*		4.76 ± 0.18*		6.56 ± 0.56*		10.45 ± 1.65	
	(CMP+CDP+CTP)	0.09 ± 0.04*		0.36 ± 0.24*		0.79 ± 0.15		0.87 ± 0.19		0.87 ± 0.23	
	(UDP-Glc+UDP+Gal)	0.46 ± 0.12*		0.61 ± 0.32*		0.91 ± 0.18*		1.09 ± 0.23		1.65 ± 0.36	
	(UDP-GalNAc+UDP-GlcNAc)	0.07 ± 0.04*		0.27 ± 0.16		0.53 ± 0.20		0.61 ± 0.29		0.77 ± 0.45	
	(GDP-Fuc+GDP-Man)	0.04 ± 0.01*		0.07 ± 0.03*		0.10 ± 0.02*		0.13 ± 0.01		0.14 ± 0.02	
	CMP-NeuAc	0.07 ± 0.04		0.12 ± 0.05		0.20 ± 0.09		0.20 ± 0.08		0.16 ± 0.09	
	AEC	0.40 ± 0.05*		0.68 ± 0.04*		0.76 ± 0.02*		0.72 ± 0.04*		0.85 ± 0.02	

* Pairs of means ± SD (n=3) that are significantly different (p-Value ≤0.01) with respect to the corresponding nucleotide/nucleotide sugar control culture.

3.5. Discussion.

Mammalian glycoproteins are synthesized as a heterogeneous population. The extent of this variability is dependent upon the activities and localization of an array of glycosylating enzymes acting in the endoplasmic reticulum and Golgi as well as the availability of substrates to serve this metabolic network (Sefton 1977). In this study, we have focused on substrate availability in the culture media and their effect on the intracellular levels of nucleotides and nucleotide sugars, which are the immediate precursors for glycosyltransferase enzymes. Cell culture media components have shown to be major parameters responsible for the modulation of recombinant protein glycosylation profiles. Cellular nutrients provided at low concentrations have been implemented in fed-batch cultures to shift cells towards a more efficient metabolism, reducing lactate and ammonia accumulation (Kaluza 1975, Sefton 1977, Liu, Spearman *et al.* 2014). In this study, it was showed that IgG1 and EG2-hFc Mabs glycosylation was sensitive to glucose concentration in the media. When cells had residual glucose in the media, glycosylation proceeded normally; conversely when glucose deprivation occurred, cells produced both non-glycosylated and abnormally glycosylated forms. This led us to investigate the availability of precursors of glycosylation such as nucleotides and nucleotide sugars. We observed a decrease in the intracellular concentrations of nucleotide and nucleotide sugars in glucose-depleted cultures that could be responsible for the changes observed in glycosylation.

Glucose became the growth-rate-limiting nutrient at an early stage of growth in cultures of cells that had been exposed to media containing $\leq$ 5.5 mM glucose. Glucose constitutes the major substrate for energy metabolism and is responsible for the generation of ATP. At low

glucose or glutamine concentrations, a decrease in ATP levels occurs which activates AMP-activated protein kinase (AMPK) (Kaluza 1975). AMPK once activated enhances energy producing catabolic processes while suppresses energy consuming biosynthetic pathways usually functioning in actively growing cells. A decrease of 30% and 65% in intracellular ATP was previously observed in CHO and tumor cells respectively after glucose starvation (Van Venrooij, Henshaw *et al.* 1972, Turco 1980). Thus, enzymes involved in biosynthetic pathways will exhibit little activity at low levels of AEC and cells will lack the energy supply required for initiation of protein synthesis and survival. Burgener *et al. (Burgener, Coombs et al. 2006)* showed that lowering glucose and glutamine concentrations can affect the intracellular adenylate concentration in Vero cells; which in turn affects virus production. In our study, a decrease in q_{Mab} along with a decrease in AEC values was also observed in glucose-starved cells. Actively growing cells have shown to maintain AEC values ≥0.8, while cells depleted of an energy substrate reached values as low as 0.06 when in stationary phase (Chapman, Fall *et al.* 1971). Our data shows that in 3 mM initial glucose cultures, q_{Mab} was 68% of that of the control culture and AEC were as low as 0.01. Negligible amounts of GTP, UTP and CTP were also found in cultures with < 11mM initial glucose. As ATP, these nucleotides serve as substrates, products, effectors or energy donors in many metabolic reactions (Wagner 1997). Thus, a reduction in their pool sizes could be responsible for the changes seen in protein production and cell growth in our glucose-depleted cultures.

In industrial cell cultures, final lactate concentrations depend on the initial glucose concentration present in the media (≥ 35 mM) (Miller, Wilke *et al.* 1988). As a result, lactate accumulates in the media to a concentration (≥ 50mM) that can be detrimental to cell growth, productivity and viability (Glacken, Fleischaker *et al.* 1986); (Ahn and Antoniewicz 2012); and

suggests inefficient use of glucose (Cruz, Moreira *et al.* 1999). CHO EG2 cells showed an increase in q_{Glc} and q_{Lac} with increasing glucose concentrations during exponential phase. Lactate consumption was observed in glucose-depleted cultures (3-5.5 mM). Lactate consumption was previously found when glucose concentration was low or high at late stages of culture and when lactate concentrations reached inhibitory or moderate levels (20mM) (Mulukutla, Gramer *et al.* 2012). This switch in metabolism was attributed to signaling pathways (i.e. P53 and AKT1) causing down-regulation of glycolytic activity in response to the absence of cell growth. In addition, increasing the activity of malate-aspartate shuttle and glycerol 3-phosphate shuttle can decrease lactate production or increase its consumption (Mulukutla, Khan *et al.* 2010). Reduction in q_{Lac} could also be achieved by either replacing glucose with another carbon source like Gal, or by lowering glucose concentrations in the media (Glacken, Fleischaker *et al.* 1986),(Hayter, Curling *et al.* 1992), (Hayter, Curling *et al.* 1993).

Glucose depletion has shown to affect cells' ability to produce the preferred precursor for protein glycosylation ($Glc_3Man_9GlcNAc_2$-PP-Dol), instead truncated precursors $Man_{(2-5)}GlcNac_2$ accumulated (Rearick, Chapman *et al.* 1981); (Turco 1980); (Stark and Heath 1979). These incomplete lipid-linked precursors led to the production of incomplete and non-glycosylated viral proteins (e.g. Sindbis virus membrane proteins, Semliki forest virus envelope proteins, G protein virus membrane proteins) (Sefton 1977); (Kaluza 1975); (Turco 1980), myeloma produced K-46 light chain (Stark and Heath 1979). These aberrant glycosylated proteins showed an increased electrophoretic mobility in SDS-PAGE gels, similarly to our non-glycosylated EG2hFc and IgG1 Mab produced in cultures with ≤5.5 mM. The effect of glucose concentration on the glycosylation site-occupancy of EG2-hFc was previously shown in cultures seeded at $2.5x10^8$ cells/ml over a

period of 24 hours showing an increase in the proportion of non-glycosylated Mab of up to 52% in cultures with no glucose (Liu, Spearman *et al.* 2014). In our study, the relative abundance of non-glycosylated Mab was <39% in Mab cultured in <5.5 mM glucose seeded at $2.5x10^5$ cells/ml. Addition of glucose and/or mannose, has been shown to increase the availability of nucleotide sugar donors (e.g. UDP-GlcNac and GDP-Man) and lipid donors (e.g. Man-P-Dol and Glc-P-Dol) to maintain the cells' ability to produce the complete $Glc_3Man_9GlcNAc_2$-PP-Dol precursor (Stark and Heath 1979); (Gershman and Robbins 1981); (Jones, Tomiya *et al.* 2010). Indeed, a decrease GDP-Man pool has shown to affect the synthesis of Dol-P-Man and subsequent Dol-P-Man derived oligosaccharides in glucose-starved cells (Chapman and Calhoun 1988). In our study, a decrease of up to 41% and 99% in the pool size of GDP-Man and UDP-GlcNAc respectively were observed in 3mM initial glucose cultures, which could have been responsible for the reduction in site-occupancy. UDP-Glc pool is also important for the synthesis of Dol-P-Glc used in the early stages of the glycosylation pathway (Freeze and Elbein 2009). Furthermore, a decrease in UDP-Glc pool can affect the activity of UDP-Glc:glycoprotein glucosyltransferase responsible for the re-glucosylation of incompletely folded proteins before they can be further processed in the glycosylation pathway (Spiro 2000). Ullrey *et al.* found a decrease in the UDP-Hex pool in the hamster cell line NIL maintained in glucose-free media with a decrease in the UDP-Glc/UDP-Gal ratio of 2 in comparison to a ratio of 4 in cells cultured in 25 mM glucose-containing media (Ullrey and Kalckar 1979). In our study, UDP-Hex decreased up to 26% in glucose starved cultures. The decrease in Urd pool in our glucose-starved cultures could also be responsible for this reduction of UDP-Hex pool.

Cells under low glucose concentrations were still able to secrete glycosylated Mab but with an increase in the proportion of pauci-mannose ($Man_{2,3}$-GlcNAc) and non-galactosylated

glycoforms along with a reduction in sialylation. A decrease in galactosylation has been previously reported as a result of low oxygenation in continuous cultures (Kunkel, Jan *et al.* 1998) possibly due to limited availability and reduced transport of UDP-Gal from the cytosol to the Golgi. Another possible reason for a reduction in galactosylation could be steric hindrance between the heavy chains of IgGs, reducing galactosyltransferases accessibility to CH_2 domains (Cabrera, Cremata *et al.* 2005). Reduced galactosylation has shown to affect IgG stability and Fc-mediated effector functions including antibody-dependent cell cytotoxicity (ADCC) and complement activation (Cabrera, Cremata *et al.* 2005); (Abès and Teillaud 2010). Furthermore, a higher content of G0 glycoforms has been related to chronic inflammatory and infectious diseases (Abès and Teillaud 2010, Schroeder and Cavacini 2010). No significant differences were found in GDP-Fuc and CMP-NeuAc levels in the range of glucose concentrations tested. Nakajima *et al.* also observed that the intracellular concentrations of GDP-Fuc and CMP-NeuAc were not affected when CHO cells and MCF7 breast cancer cells were grown in glucose-free medium (Nakajima, Kitazume *et al.* 2010). In our study, the decrease in sialylation could be attributed to the absence of Gal residues observed in glucose-deprived cultures, which are necessary for sialylation to occur. Furthermore, cultures containing low glucose also contained higher levels of CMP in comparison to the control culture where CTP represented the higher proportion in Cyt pool. Previously it has been shown that CMP can act as an inhibitor of CMP-NeuAc transport, reducing sialylation (Cacan, Cecchelli *et al.* 1987). Glutamine has been previously described as an important source of energy at low glucose concentrations (Gambhir, Europa *et al.* 1999). Glutamine oxidation may contribute up to 40% of the energy requirements of the cell and can be more efficient in ATP production than anaerobic glycolysis, contributing to growth-related biosynthetic pathways (e.g. nucleotide biosynthesis) (Zielke, Ozand *et al.* 1978). However, nucleotide levels did not seem to increase when the

glutamine concentration was elevated to 4mM in the media, indicating that glucose depletion was enough to exhaust the cell's energy supplies.

Fed-batch cultivation allows decreased metabolite accumulation (e.g. lactate, ammonia) while increasing cell yield and product concentration by adjusting nutrient concentrations to low levels (Butler 2007). Under these conditions, cellular metabolism shifts from a mainly glycolytic state to a more oxidative state. However, nutrient availability should be monitored closely during biopharmaceutical production so that glucose does not fall to a critical level that can affect product quality (glycosylation) and consistency between batches. In this study, glucose depletion caused a reduced site-occupancy of EG2-hFc Mab and IgG1 Mab as well as changes in the microheterogeneity of EG2-hFc Mab that involved reduced galactosylation and sialylation. This effect could be explained by the reduction of the intracellular nucleotide triphosphate and nucleotide sugar pools which act as immediate precursors of protein glycosylation.

3.6. Conclusions

- Glucose concentrations < 5.5 mM induced a significant decrease in EG2 cells' yield and viability of up to 84% and 25.5% respectively in comparison to the control.
- EG2 cell yields observed in low glucose (3 mM) containing cultures could not be improved by the addition of up to 4 mM of glutamine.
- Low glucose concentrations (< 11 mM) also affected DP12 cells' growth and viability with a decrease of up to 57% and 23% in MCDs and viability when compared with control culture with 25 mM glucose.
- Both cell lines experienced glucose deprivation as early as day two when cultured in media containing < 5.5 mM.

- A significant reduction in glucose consumption rates of up to 88% and 45% were observed in EG2 and DP12 respectively in cultures containing < 5.5 mM with respect to the control culture with 25 mM glucose.

- As a result of glucose deprivation, a swift towards lactate consumption was observed in cultures with < 11 mM glucose.

- Specific antibody production was not affected while glucose was still available in both cell lines.

- Glucose deprived cultures showed two bands in SDS-PAGE gels that differed by 3.3 kDa and corresponded to glycosylated and non-glycosylated Mabs. Presence of non-glycosylated Mab was confirmed by MS analysis.

- A higher proportion of non-galactosylated antibodies (108%) along with a significant decrease in G2-glycans (39%) were observed in EG2 cultures containing < 11 mM in comparison to control cultures with 25 mM glucose. The latter resulted in a significant decrease in GI of up to 29.4% in < 11 mM-containing cultures.

- A correlation between galactosylation and sialylation with the initial glucose concentration was found in EG2-hFc Mabs.

- The glycan distribution observed in IgG1 Mab from control cultures (G0>G1>G2) remained the same regardless of the initial glucose concentration.

- Glucose deprived cultures showed significantly lower levels of nucleotide and nucleotide sugars in comparison to cultures with 25 mM glucose. The latter impacted not only cell metabolism of EG2 and DP12 cells, but also the macroheterogeneity and microheterogeneity of EG2-hFc and IgG1 Mabs respectively.

- High energy EG2 cultures (17.5 mM-25 mM glucose) showed an AEC of 0.78-0.81 respectively in comparison to glucose deprived cultures were AEC were as low as 0.01, which represents a 98.7% decrease with respect to the control.

- A significant decrease (up to 53%) in AEC was also observed in DP12 cultures with <11 mM glucose.

- The decline in nucleotide triphosphates and AEC correlates with the decrease in cell growth and energy metabolism observed in glucose starved cells.

Chapter 4

Effect of initial cell density on CHO EG2 cell growth, metabolism, EG2-hFc Mab production and glycosylation[3]

4.1.Introduction

Batch cultures have been considered as one of the simplest modes of cultivation of high producing cell lines because of their ease of operation and scalability, lower risk of contamination and more efficient process development and validation (Byrne 2014). However, cells have shown higher consumption rates than the ones required to support growth and metabolism. This highly dysregulated metabolism results in nutrient limitation and high accumulation of lactate and ammonia (Restelli and Butler 2002). As a result, cells are exposed to constant changes in the environment which in turn can be detrimental for product's quality. In fact, it has been shown that the extent of glycosylation tends to decrease over time in batch culture (Cruz, Moreira *et al.* 1999).

The effect of cell inoculum has been previously examined during cell culture (Norrby 1974, Ozturk and Palsson 1990, Ng, Berry *et al.* 1996, Castilho 2008). Inoculum density has shown to impact the duration of lag phase which takes place right after inoculation. The lower the cell density the longer the duration of the lag phase, which is detrimental for processing times and productivity (Castilho 2008). In addition, initial cell inoculum has shown to be important for

[3] Partial content of this chapter was included in the following paper:
Villacres, C., et al. "Low Glucose Depletes Glycan Precursors, Reduces Site Occupancy and Galactosylation of a Monoclonal Antibody in Cho Cell Culture." *Biotechnol J* 10.7 (2015): 1051-66.

optimal cell-to-bead distribution in microcarriers (Ng, Berry *et al.* 1996). On the other hand, Ozturk *et al.* determined that initial growth rates as well as the maximum cell densities reached by anchorage-dependent cells are influenced by the initial cell inoculum (Ozturk and Palsson 1990). Similarly, human cells' growth rates were significantly affected by the inoculum size, with the multiplication rate being negatively correlated to the number of cells in the inoculum (Norrby 1974). However, there is no information on the effect of cell inoculum on Mab glycosylation. Previously in Chapter 3, a continuous decrease in glucose consumption, specific cell productivity and Mab glycosylation was observed in glucose-starved CHO cultures. The latter was a response of a reduced energy state as well as a depletion of sugar precursors required for glycosyltransferases. In this chapter, glucose availability was further explored along with initial cell inoculum on their effect on cell growth, metabolism, productivity and particularly glycosylation of CHO EG2-hFc Mabs.

4.2. Objectives

- Investigate the effect of cell inoculum on *N*-linked glycosylation of EG2-hFc Mabs produced in low- and high-glucose media.

-Evaluate the effect of initial cell density on CHO EG2 cells' metabolism

4.3.Experimental set up of cell density and glucose deprivation experiments

CHO EG2 cells were seeded at three initial cell densities: 2.5 x 10^5 cells/mL, 2.5 x 10^6 cells/mL and 2.5 x 10^7 cells/mL in Biogro™ media containing 3 mM/25 mM glucose and 4 mM

glutamine. Control culture consisted of culture media containing 4 mM glutamine and 25 mM glucose, seeded at 2.5 x 10^5 cells/mL. Each experimental condition was run in duplicates and results obtained were confirmed by repeating experiment three times, giving a total of 6 replicates. Daily culture supernatants were taken to check on cell growth, viability and for further metabolite analysis (e.g. glucose consumption, lactate production and antibody production). On day 4, cell culture supernatants were collected and concentrated 10-fold prior to protein A purification. Purified CHO EG2-hFc Mabs were treated with PNGase-F to the release of *N*-glycans prior to 2AB-labelling and HPLC analysis (**See Chapter 2, Section 2.11**). Additional supernatant sampling (containing 1 x 10^7 cells) was used for nucleotide and nucleotide sugar analysis. For this purpose, cell metabolism was stopped on day four and metabolites were extracted prior to RP-HPLC (**See Chapter 2, Section 2.11.8**).

4.4.Results

4.4.1 Effect of cell inoculum on CHO EG2 cell growth and viability.

The influence of initial cell density on cell growth and viability was evaluated for CHO EG2 cells cultured in media containing 3 mM and 25 mM glucose (**Figure 4.1A and 4.1B, Table 4.1**). For this purpose, CHO EG2 cells were inoculated at three different cell densities: Low (L) 2.5x10^5 cells/mL, Medium (M) 2.5x10^6 cells/mL and High (H) 2.5x10^7 cells/mL. For cultures seeded at low cell density, there is an evident exponential growth during the first three days (μ= 0.03 h^{-1}) where maximum cell densities (MCDs) were reached with 1.08x10^6 cells/mL and 3.14x10^6 cells/mL in 3 mM and 25 mM glucose respectively (**Figure 4.1A**). In the case of medium inoculum, a slight cell growth was observed during the first day (μ= 0.02 h^{-1}), after which cultures

reached a plateau for both glucose concentrations. The MCDs observed were $3.55x10^6$ cells/mL and $5.53x10^6$ cells/mL reached on day one (3 mM glucose) and day three (25mM glucose) respectively. Finally, cultures seeded at high cell density, did not show any cell growth (μ= 0.00 h^{-1}) and instead a constant cell density was kept until day two where a decline phase took place. With respect to cell viability, cultures seeded at low cell densities showed the highest viability up until day three with >95%. On day four, viability decreased to 71.7% in 3 mM glucose-containing cultures in comparison to 92.2% in cultures with 25 mM glucose. In contrast, cultures seeded at medium cell density showed higher viabilities during the first two days with up to 96% and then declined to 54% on day four. Finally, high-inoculum cultures with 3 mM and 25 mM glucose cell viabilities start to decline on day two, reaching 66.6% on day four.

These results show that initial cell inoculum had an impact on CHO EG2 cells' growth and viability during a four-day batch culture (**Figure 4.1B**). Further, the initial glucose concentration in the media was also important, with 3 mM glucose cultures showing lower growth rates and viabilities in comparison to cultures with 25 mM glucose regardless of the cell inoculum. Furthermore, MCDs were 65%, 35% and 6% lower in 3 mM glucose cultures seeded at low, medium and high cell density respectively with respect to 25 mM glucose cultures. In contrast to low glucose cultures, the initial cell inoculum did influence cell growth rates and viability in 25 mM glucose cultures. For instance, cultures seeded at low cell density showed a better growth rate and viability all along the culture period when compared to medium and high cell inoculums. The latter showed a slight increase in cell density and a constant decrease in cell viability since day two (**Figure 4.1B**).

4.4.2 Effect of initial cell densities and glucose concentration on CHO EG2 cell's metabolism and productivity

To further explain the effect of the inoculum density and glucose availability on cell growth and viability, glucose and lactate concentrations were measured simultaneously using a biochemical analyzer. Figures **4.2A, 4.2B and 4.2C** show the glucose consumption of EG2 batch cultures seeded at three different cell concentrations designated low (2.5 x 10^5 cells/mL), medium (2.5 x 10^6 cells/mL) and high (2.5 x 10^7 cells/mL) respectively in media containing 3 mM and 25 mM glucose. The data show that all cultures containing an initial concentration of 3 mM glucose resulted in nutrient depletion regardless of the cell inoculum before day two. Cultures seeded at the low cell density containing 25 mM initial glucose did not result in glucose depletion until day four. However, increasing cell inoculum to 2.5 x 10^6 and 2.5 x 10^7 cells/mL caused glucose depletion on day four and day two respectively. This glucose depletion can be related to the lack of cell growth and low viability (≤75%) observed in cultures seeded at the medium and high cell densities in both 3 mM and 25 mM glucose (**Table 4.1**). A switch from lactate production towards lactate consumption was observed in glucose-depleted cultures. Lactate consumption was also observed in cultures with 25 mM glucose inoculated at medium and high cell densities (**Figure 4.2B** and 4.2**C**). This occurred when lactate reached 23 mM and when glucose was still available (medium cell density) or under glucose depletion (high cell density). The ratio ($Y_{Lac/Glc}$) is shown in **Table 4.1** 1 in cultures with 3 mM and 25 mM glucose at different cell inocula. $Y_{Lac/Glc}$* decreased significantly from 1.59 ±0.06 to 0.04 ±0.00 (3 mM glucose; $P < 0.0001$) and from 1.38 ±0.10 to 0.51 ±0.16 (25 mM glucose; $P < 0.001$) when cell inoculum increased from 2.5x10^5 to 2.5x10^7 cells/mL on day one.

The cell-specific productivity of antibody (q_{Mab}) of CHO EG2 cells was determined at varying glucose concentrations (**Table 4.1**). The q_{Mab} was determined from the slope of the plot of EG2-hFc Mab concentration and the IVCD values. In 3 mM initial glucose q_{Mab} decreased from 1.20 ±0.22 μg/10^6 cells/day (low inoculum, 2.5x10^5 cells/mL) to 0.44 ±0.01 μg/10^6 cells/day (medium inoculum, 2.5x10^6 cells/mL) and to 0.41 ±0.07 μg/10^6 cells/day (high inoculum, 2.5x10^7 cells/mL). Similarly, in 25 mM cultures a decrease in q_{Mab} release was also observed from 1.53 ±0.51 μg/10^6 cells/day (low inoculum) to 1.05 ±0.02 μg/10^6 cells/day (medium inoculum) and to 0.51±0.10 μg/10^6 cells/day (high inoculum). These q_{Mab} values differ according to the exposure of cells to glucose depletion. The lowest q_{Mab} (0.41 μg/10^6 cells/day) corresponded to cultures where glucose depletion occurred before day two. Lower antibody production (2.76 ±0.30 to 7.51 ±0.01 μg/mL) was observed in 3 mM initial glucose in comparison to 25 mM initial glucose (9.44 ±0.16 to 43.07 ±2.04 μg/mL) when cell inoculum increased from 2.5 x 10^5 to 2.5 x 10^7 cells/mL. Cultures seeded at the highest cell density (2.5 x10^7 cells/mL) showed no production after day two, so q_{Mab} values were calculated up to this point (**Table 4.1**).

These results show that the glucose limitation observed at higher cell densities caused a decrease in the amount of glucose consumed per cell, which in turn affected the amount of lactate produced per cell. This is reflected in the decrease observed in $Y_{Lac/Glc,}$ which indicates a metabolic shift from a high to low lactate producing metabolism. In addition, the q_{Mab} decreased significantly with increasing cell inoculum at low and high glucose, even though volumetric productivity was higher at higher cell densities (**Table 4.1**).

Figure 4. 1 Effect of cell inoculum and glucose concentration on A. cell growth and B. viability of CHO EG2 cells during a four-day batch culture.

Cells were seeded at low ($2.5x10^5$ cells/mL); medium ($2.5x10^6$ cells/mL) and high ($2.5x10^7$ cells/mL) densities into 75 mL of media in 250 mL shake flasks. Culture media contained 3 mM or 25 mM glucose with 4 mM glutamine. Data points represent mean ±SD of duplicates for each condition for one of two independent experiments (n=4).

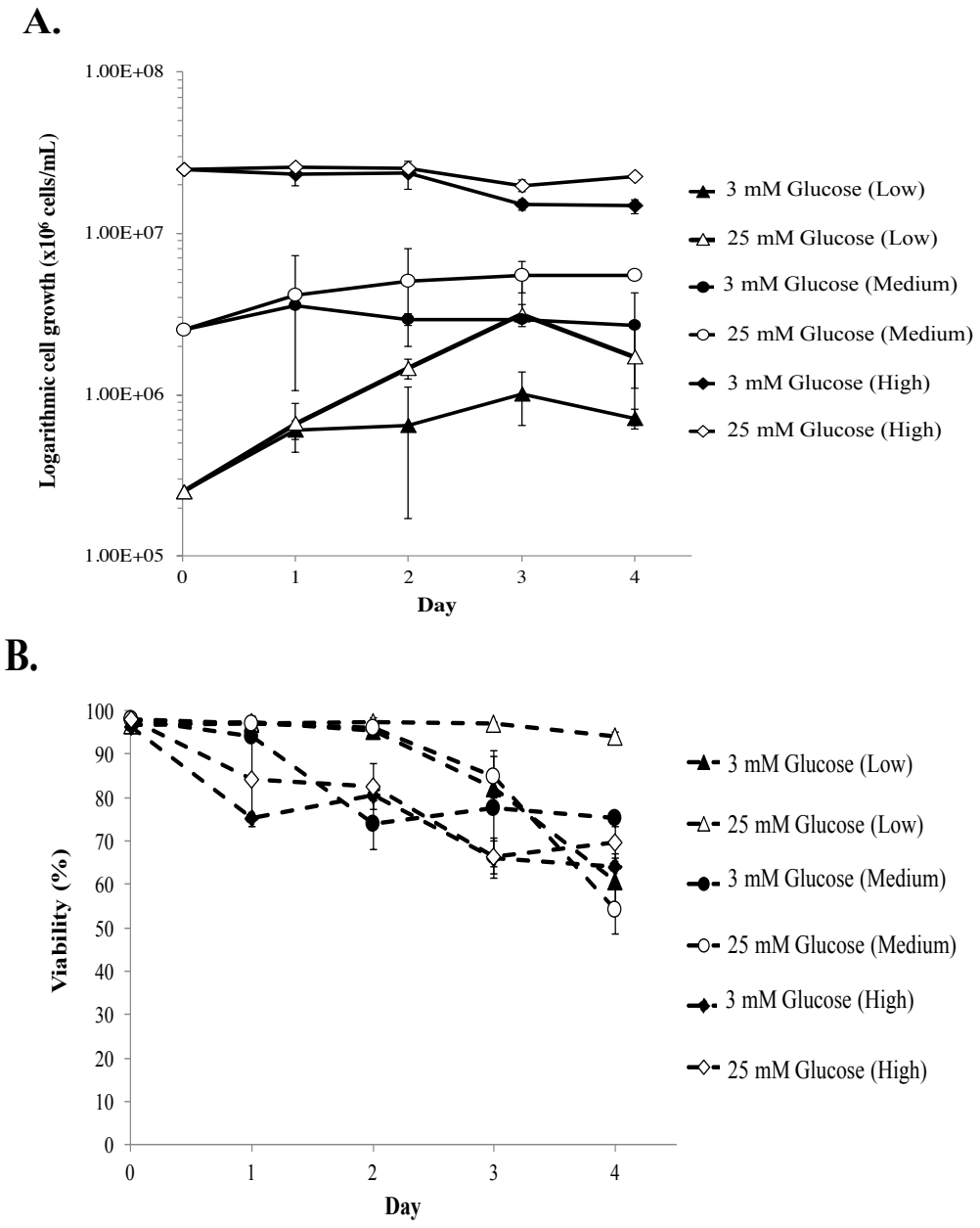


Figure 4. 2. Effect of cell inoculum and glucose on EG2 cells' metabolism.

Dextrose (solid lines) and lactate (dashed lines) concentrations were determined during the batch cultures when inoculated at **A.** $2.5x10^5$ cells/mL, **B.** $2.5x10^6$ cells/mL and **C.** $2.5x\ 10^7$ cells/mL in 75 mL of media containing 3 mM and 25 mM glucose in 250 mL spinner flasks. Data represents mean ±SD of duplicates for each condition for one of three independent experiments (n=6)

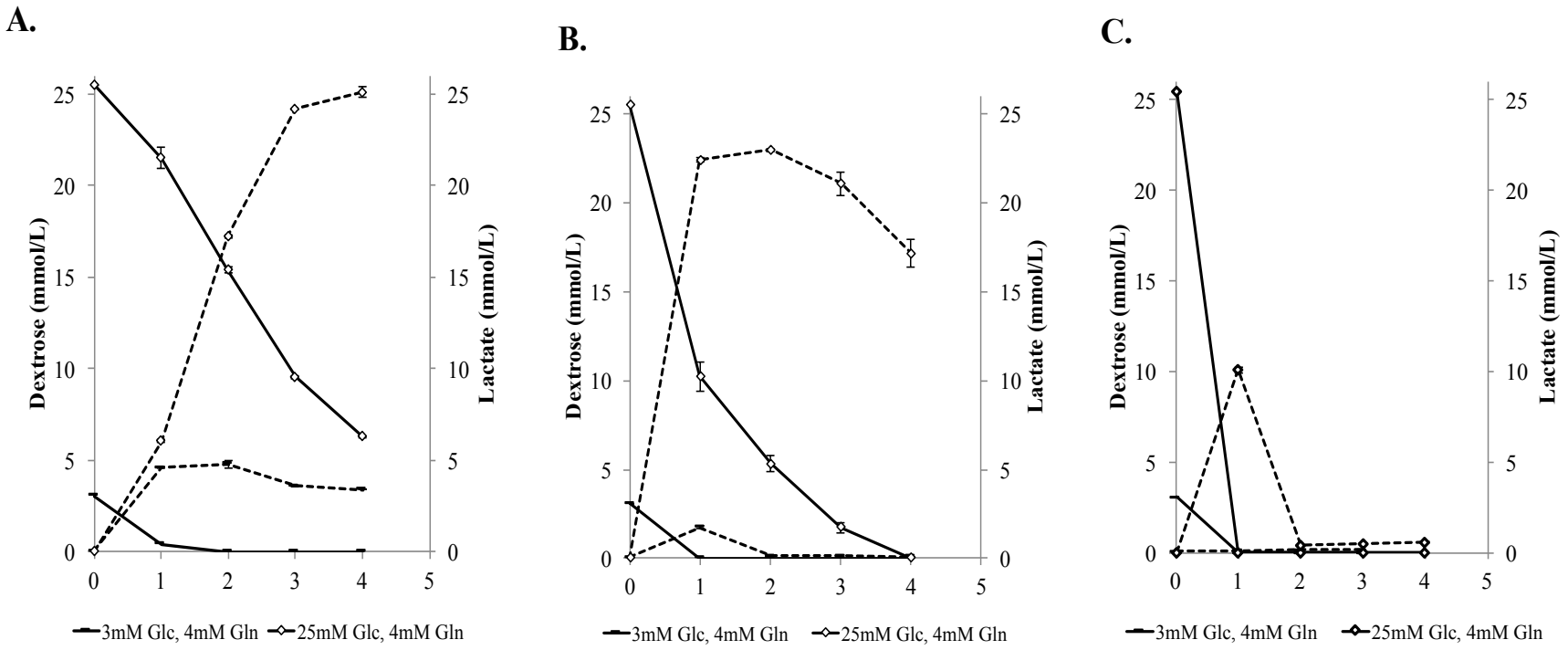


Table 4. 1Culture parameters of CHO-EG2 cells inoculated at low (L=2.5x10^5 cells/ml), medium (M=2.5x10^6 cells/ml) and high (H=2.5x10^7 cells/ml).

Culture Condition		Maximum Viable Cell Density	Viability[a)]	Mab Yield[a)]	q_{Mab}[b)]	Glucose depletion	GI[a)]	$Y_{Lac/Glc}$
Glucose (mM)	Inoculum (cells/ml)	(x10^6 cells/ml)	(%)	(μg/ml)	(μg/10^{6}cell.day)	(day)		(mol/mol)
	L: 2.5x10^5	1.08±0.10	71.7±1.51	2.76±0.30	1.20±0.22	2	0.48±0.03[c)]	1.59±0.06
3	M: 2.5x10^6	3.55±0.09	75.0±1.05	5.27±0.11	0.44±0.01[c)]	<1	0.26±0.01[c)]	0.57±0.01[c)]
	H: 2.5x10^7	22.74±3.20	69.9±3.34	7.51±0.01	0.41±0.07[c)]	<1	0.20±0.01[c)]	0.04±0.00[c)]
	L: 2.5x10^5[d)]	3.14±0.02	92.2±2.55	9.44±0.16	1.53±0.51	>4	0.68±0.05	1.38±0.10
25	M: 2.5x10^6	5.53±1.22	54.0±1.22	19.72±0.42	1.05±0.02	4	0.65±0.04	0.97±0.01[c)]
	H: 2.5x10^7	24.41±2.07	66.6±4.83	43.07±2.04	0.51±0.10[c)]	2	0.43±0.04[c)]	0.51±0.16[c)]

a) Measured on day 4.

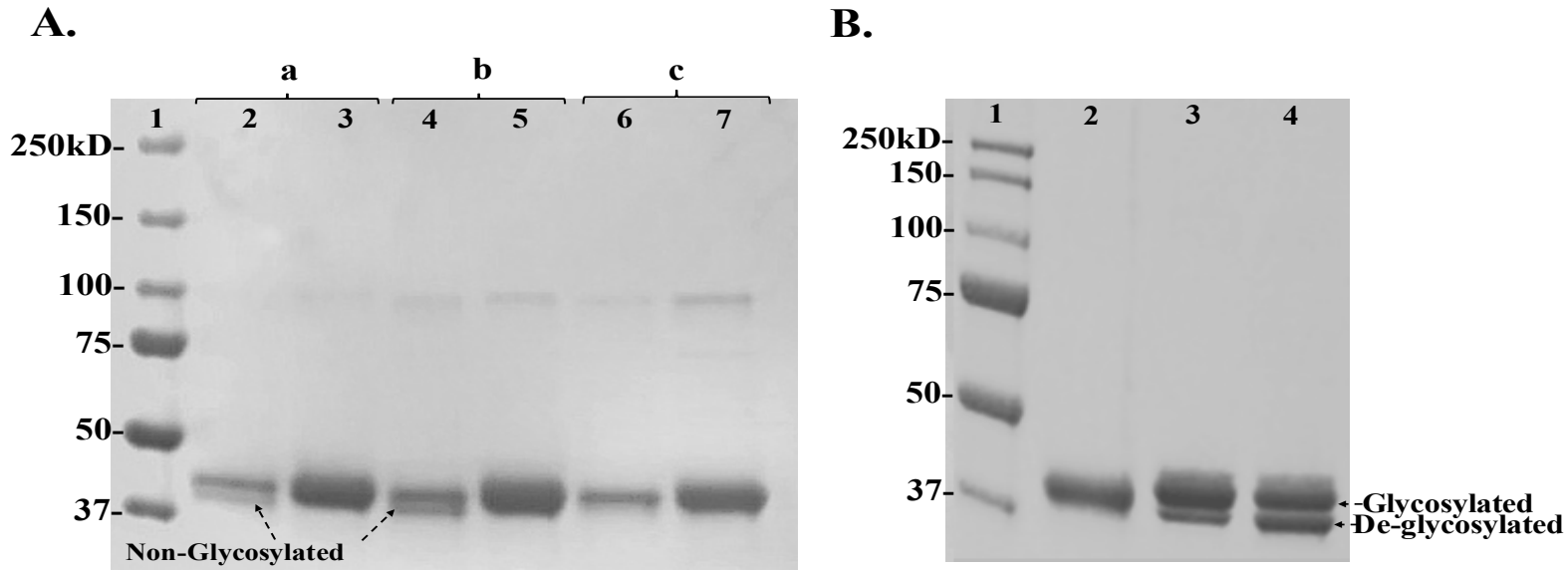
b) Over the period of time where glucose was still available.

c) Pairs of means Å} SD significantly different ($P \leq 0.01$) with respect to control culture. Experiments with low and high cell inoculum experiments correspond to replicates of two independent experiments (n = 4),

medium cell inoculum (n = 2). GI values correspond to three independent experiments (n = 6)

d) Control culture

Figure 4. 3. Effect of cell inoculum and glucose concentration on the macroheterogeneity of EG2-hFc Mab.
A. Mabs were harvested on day four from cultures seeded at a. 2.5 x 10^5, b. 2.5 x 10^6, c. 2.5 x 10^7 cells/mL in media containing 3 mM glucose (lanes 2, 4 and 6) and 25 mM glucose (lanes 3, 5 and 7). **B.** Protein-A purified antibody was produced in control culture (same as Fig. 4.3A, lane 3) after PNGase F digestion over a period of 0, 30 and 60 min (lanes 2, 3 and 4 respectively). Protein samples loaded into gel where 16-fold the original supernatant concentration. Proteins were separated under reducing conditions through 8 to 16% by SDS-PAGE gel and stained with Coomasie blue. Lane 1 in (A) and (B) shows marker proteins. Non-glycosylated, deglycosylated and glycosylated forms of Mab are indicated by arrows.



4.4.3 Initial low glucose and high cell densities influence site-occupancy in EG2-hFc

Differences in site-occupancy were analyzed in the EG2-hFc Mab produced in media containing increasing glucose concentrations and harvested on day four. **Figure 4.3A** shows SDS-PAGE separation of protein A purified Mabs produced from cultures with three different inoculum densities and at two glucose concentrations. Single bands were seen in all cultures supplemented with high glucose but at low glucose and at the inoculum densities of 2.5 x 10^5 and 2.5 x 10^6 cells/mL there was evidence of a second lower molecular weight band. As previously mentioned, this lower band was identified as non-glycosylated Mab. This was consistent with the loss of a glycan when EG2-Mab was digested with PNGase F **(See section 3.2.4).** Cultures with low initial glucose (3 mM glucose seeded at 10^5 cells/mL) resulted in 38.5 ±2.56% non-glycosylated Mabs determined by densitometry analysis. A decrease in site-occupancy was also observed in low glucose cultures seeded at 10^6 cells/mL, with a 55 ±5.10% non-glycosylated Mab (**Fig. 4.3A**). Although glucose depletion occurred in cultures containing 25 mM glucose seeded at medium or high inoculum densities, there was no evidence of non-glycosylated Mabs. A difference of about 3.3 kDa was calculated for bands corresponding to glycosylated vs. non-glycosylated Mabs in SDS-PAGE gels (determined by AlphaView Software). **Figure 4.3B** shows the time-dependent de-glycosylation of Mab produced in 25 mM glucose after incubation with PNGase F. This shows the gradual appearance of a second band at lower molecular weight as previously observed in low glucose cultures **(See Section 3.2.4).** These results indicate that glucose starved cells produced significant amounts of non-glycosylated antibody in cultures with low or medium inocula. The data represent the accumulated antibody over the culture period and extracted on day four. A different pattern was observed at a high inoculum. Surprisingly, only a single band of glycosylated

antibody was observed at low glucose (**Figure 4.3A**). The likely explanation for this apparent anomaly is that the antibody was only produced on the first day before the complete depletion of glucose, after which antibody synthesis ceased. Other deficiencies (e.g. amino acids) could also have affected antibody production.

4.4.4 Glucose deprivation observed at higher cell densities changes the glycosylation profile of EG2-hFc Mabs

The effect of glucose deprivation on *N*-glycosylation was determined in cultures seeded at high cell density. The percentage of pauci-mannose glycans (e.g. M2, M3, F6M2, F6M3) in 3 mM glucose cultures was 12% of the total glycan profile regardless of cell density in inoculum compared to the control culture (25 mM, seeded at $x10^5$ cells/mL) where these glycans were absent. Pauci-mannose glycans were also observed in cultures containing 25 mM glucose and seeded at $x10^6$ and $x10^7$ cells/mL (2.25% and 6.18% respectively). An increase in the proportion of non-galactosylated glycans was observed when the cell inoculum increased from low to high inocula (**Figure 4.4**). At 3 mM glucose, the GI value decreased from 0.48 to 0.20 as the inoculum increased along with an 85% decrease in sialylation. At 25 mM glucose, a slight shift towards G0 was observed by a change in GI values from 0.68 to 0.43 at high inoculation along with a decrease in fucosylation and sialylation by 6.90% and 68.3% respectively. Previously, at a low cell inoculum a correlation of GI and sialylation was found with the initial glucose concentration (R_2 = 0.94) (**Chapter 3, Figure 3.10**). Similarly, a correlation between initial cell inoculum and GI values, sialylation and fucosylation was also found in 25 mM glucose ($R_2 > 0.96$) but not at low glucose cultures probably because the GI values were already low **(Figure 4.5A and 4.5B).** The

data show that the galactosylation decreased at high inoculum density, probably corresponding to the consequent lower intracellular glucose levels. The effect of altered microheterogeneity with inoculum density was observed as a reduction of GI, sialylation and fucosylation at high inoculum as well as at low initial glucose concentration.

Figure 4. 4 Overlay of six profiles of glycans from EG2-hFc Mab produced by cultures seeded at different cell inoculum.

Previously protein A purified Mabs from culture media containing 3 and 25 mM glucose and inoculated at low (L = 2.5 x 10^5 cells/mL), medium (M = 2.5 x 10^6 cells/mL) and high (H = 2.5 x 10^7 cells/mL) cell densities. Peak separation was based on HILIC chromatography. Glycan structures were assigned based on GU values previously reported in the NIBRT Glycodatabase 3.2. Galactosylation index (GI) was defined as [(G2+0.5*G1)/(G0+G1+G2)]. GI values correspond to the mean ±SD of duplicates of three independent experiments (n = 6).

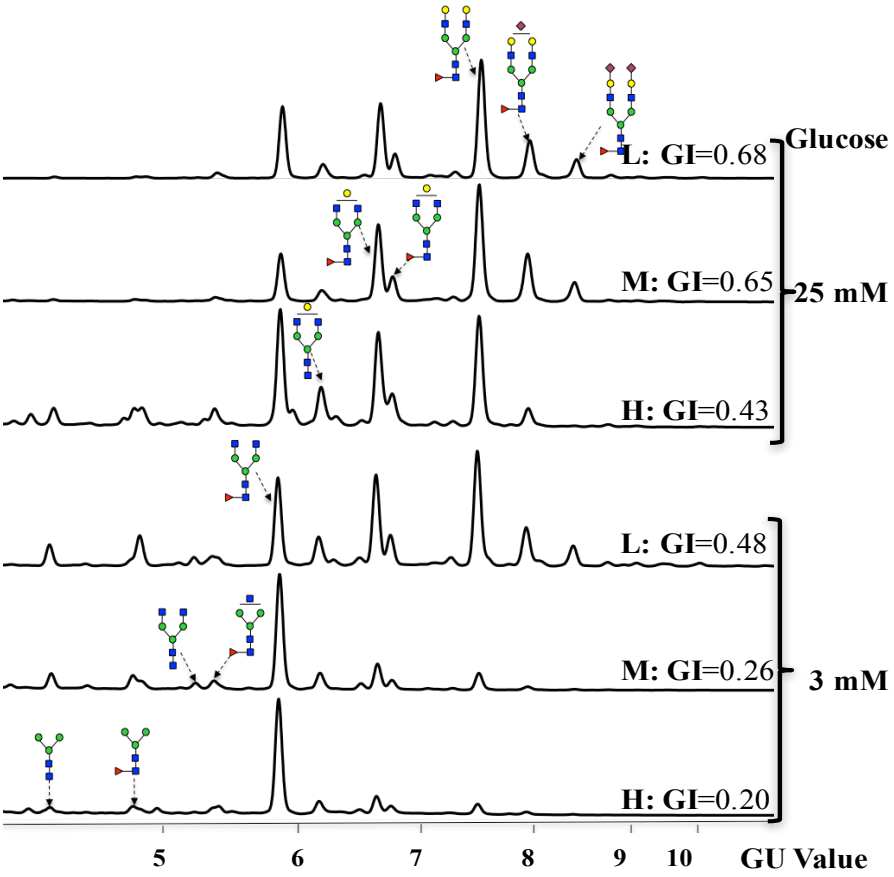


Figure 4. 5 Galactosylation index and sialylation content correlates with initial glucose concentration in the media calculated from EG2-hFc Mab harvested at day four.

Galactosylation index, sialylation and fucosylation percentage decrease correlates with increasing cell density inoculums ($2.5x10^5$, $2.5x10^6$ and $2.5x10^7$ cells/ml) in media containing **A.** 25 mM glucose but not in **B.** 3 mM glucose.

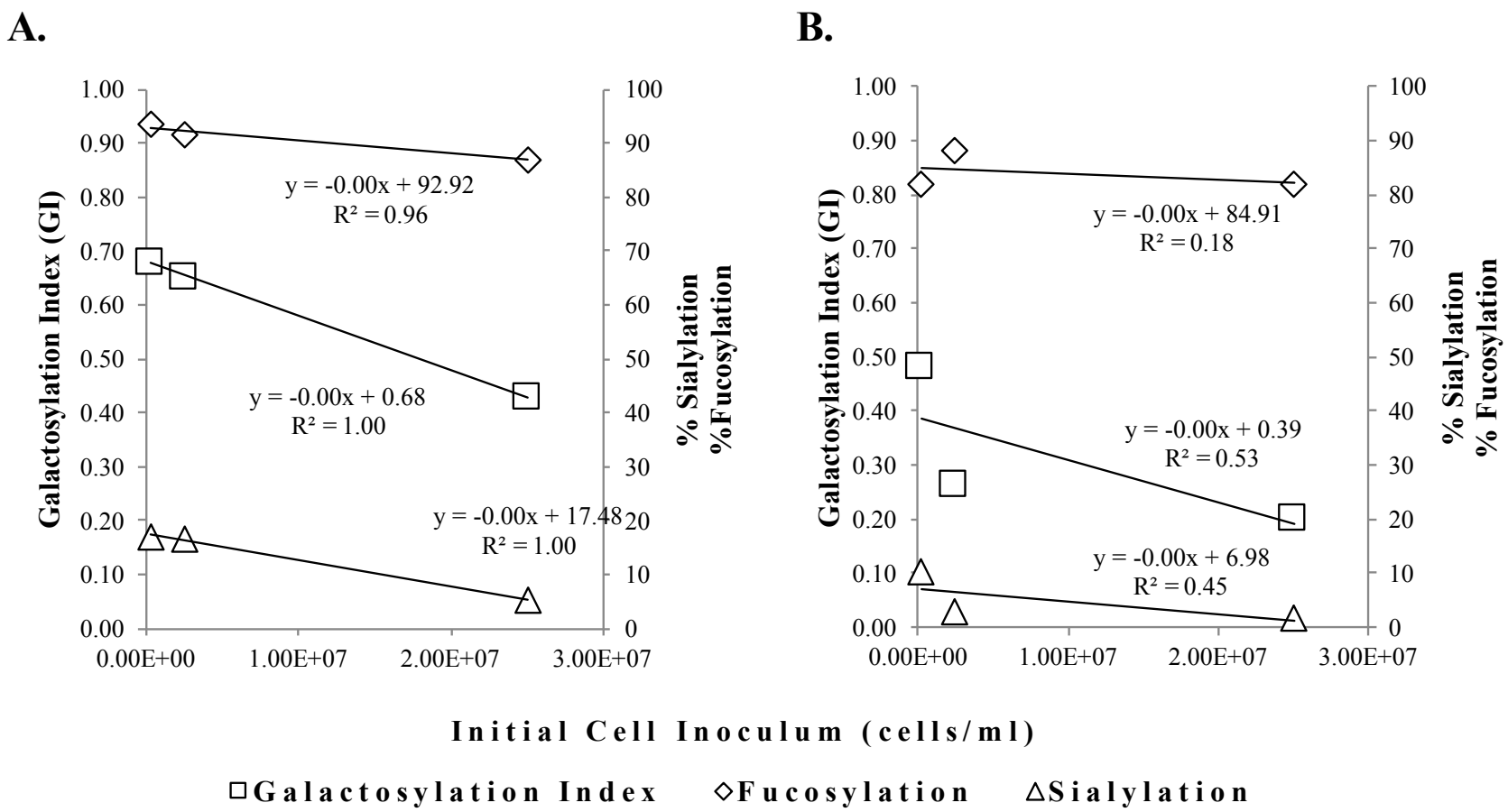


Table 4. 2 Intracellular nucleotide and nucleotide sugar concentrations (fmoles/cell) of EG2 cultures seeded at 2.5x10^5 cells/mL and 2.5 x 10^7 cells/mL.

The media contained 3 mM or 25 mM glucose and 4 mM glutamine. Nucleotide concentrations are represented as the sum of mono, di and triphosphate nucleotides. Pair of means ± SD (n=6) that are significantly different (p-Value ≤0.01) with respect to the corresponding nucleotide/nucleotide sugar control culture †.

Cell Density Inoculum		**x10^5 cells/ml**		**x10^7 cells/ml**	
Glucose (mM)		**3.00**	**25.0†**	**3.00**	**25.0**
Glutamine (mM)		**4.00**	**4.00**	**4.00**	**4.00**
Nucleotide and Nucleotide Sugar (fmole/cell)	**(UMP+UDP+UTP)**	0.02 ± 0.01*	0.66 ± 0.25	0.52 ± 0.22	0.63 ± 0.08
	(GMP+GDP+GTP)	0.01 ± 0.02*	2.98 ± 0.29	0.02 ± 0.02*	0.06 ± 0.03*
	(AMP+ADP+ATP)	0.12 ± 0.04*	9.05 ± 1.13	0.63 ± 0.07*	0.90 ± 0.22*
	(CMP+CDP+CTP)	0.02 ± 0.00*	0.71 ± 0.32	0.31 ± 0.25*	0.27 ± 0.14*
	(UDP-Glc+UDP+Gal)	0.36 ± 0.17	0.66 ± 0.19	0.29 ± 0.18*	0.17 ± 0.07*
	(UDP-GalNAc+UDP-GlcNAc)	0.01 ± 0.01*	3.27 ± 0.29	0.02 ± 0.02	0.21 ± 0.24
	(GDP-Fuc+GDP-Man)	0.04 ± 0.03*	0.13 ± 0.09	0.04 ± 0.01	0.03 ± 0.01
	CMP-NeuAc	0.05 ± 0.07	0.15 ± 0.01	0.00 ± 0.00	0.01 ± 0.02
	AEC	0.01 ± 0.00	0.78 ± 0.03	0.00 ± 0.00	0.04 ± 0.04

4.4.5 Low intracellular concentrations of nucleotide and nucleotide sugars were observed in glucose-depleted cells that were inoculated at high cell densities

A reduction of the intracellular pool of nucleotide and nucleotide sugars could be responsible for the changes observed in both, metabolism and *N*-glycosylation in glucose-depleted cells. To explore this hypothesis, intracellular metabolites of cell extracts cultured in media with 3 mM and 25 mM glucose and inoculated at low and high densities were analyzed and compared with control cultures seeded at $2.5x10^5$ cells/mL in 25 mM glucose. The structural assignment of the peaks was based upon an equivalent chromatographic run of 20 standard molecules. Intracellular concentrations of each of these metabolites was obtained by quantitative analysis of the peak areas in relation to GDP-Glc used as internal standard (IS). There were significant differences in the content of nucleotide and nucleotide sugars from cell extracts analyzed from cultures with differing glucose concentrations. The concentrations of Ade, Cyt, Gua and Urd nucleotides in cell extracts from cultures at 3 mM glucose and a low inoculum decreased by 98.7%, 97.5%, 99.5% and 96.9% respectively compared to the control **(Table 4.2).** Further decrease in Ade, Cyt, Gua and Urd pools was observed in cell extracts from the 3 mM glucose culture at high inoculum with a reduction of up to 93.0%, 56.1%, 99.2% and 21.4% with respect to the control. In addition, decrease of these nucleotide pools was also observed at 25 mM glucose seeded at high inoculum by 90.0%, 61.9%, 98.1% and 3.39% respectively. The adenylate energy charge (AEC) was calculated as the ratio of adenylate nucleotides (AMP, ADP and ATP) as previously mentioned in **Section 2.11.8.3, Eq. 8. Table 4.2** shows the AEC was below 0.04 at high inoculum in comparison to the control value of 0.81 ±0.05. With respect to nucleotide sugars, the intracellular concentrations of GDP-sugars and CMP-NeuAc decreased by up to 78.9% and 91.9%

at higher cells densities. Similarly, a decrease in UDP-hexoses and UDP-hexosamines was observed in both, 3 mM glucose cultures (by 55.7 and 99.4%) and 25 mM glucose cultures (by 74.8 and 93.5%) seeded at $x10^7$ cell/mL compared to the control.

These results show that high cell inoculum cultures resulted in the intracellular depletion of nucleotide and nucleotide sugar pools, which could be responsible for the changes observed in Mab glycosylation. This could indicate that the decreased pool of nucleotides and nucleotide sugars observed under glucose deprivation was instrumental in causing both a low energy status of the cells as well as a limited supply of precursors for the glycosylation process.

4.5.Discussion

During batch culture, nutrients are supplied at high concentrations to supply cell's needs for the full duration of the culture. However, these high nutrient concentrations result in high lactate and ammonia production which have been associated with decreased cell growth, protein production and product quality (Ahn and Antoniewicz 2012) (Cruz, Moreira *et al.* 1999, Butler 2005). The latter is especially important when high cell density cultures are used to produce recombinant proteins. As a result, glucose and glutamine have been supplemented at low concentrations to shift cells towards a more efficient metabolism, reducing the accumulation of cellular waste products (Zielke, Ozand *et al.* 1976, Cruz, Moreira *et al.* 1999). However, a decrease in nutrient availability can impact glycosyltransferases' enzymatic activity, gene expression and the availability of precursor molecules. Thus, cellular waste accumulation along with nutrient depletion need to be avoided so that cells express high volumes of well-defined product that is

biologically active. In this chapter, it was shown that glucose availability affected the glycosylation of EG2-hFc Mab, particularly at high cell inoculum where glucose depletion happened as early as day one. When cells had residual glucose in the media, glycosylation proceeded normally; conversely when glucose deprivation occurred, cells produced both non-glycosylated and abnormally glycosylated forms. This led us to investigate the availability of precursors of glycosylation such as nucleotides and nucleotide sugars. A decrease in the intracellular concentrations of nucleotide and nucleotide sugars was observed in glucose-depleted cultures that could be responsible for the changes observed in cell metabolism and glycosylation. Previous reports have described the effect of initial cell density on mammalian cells' growth. For instance, the initial cell inoculum has shown to be critical for ensuring growth and an even distribution of cells in microcarriers (Ng, Berry *et al.* 1996). In addition, human cells' growth rates were significantly affected by the inoculum size, with the multiplication rate being negatively correlated to the number of cells in the inoculum (Norrby 1974). Similarly, hybridoma cultures showed a lower growth rate as the initial cell density increased from $x10^3$ to $x10^5$ cells/mL (Ozturk and Palsson 1990).

In the experiments outlined in this chapter, EG2 cells' growth rates also varied directly with the initial cell concentration. Low cell inoculum ($x10^5$ cells/mL) showed a higher growth rate of 0.03 h^{-1} in comparison to medium ($x10^6$ cells/mL) cell inoculum where the growth rate was as low as 0.02 h^{-1}. In addition, there was little or no net growth (0.00 h^{-1}) in cultures seeded at much higher cell densities ($x10^7$ cells/mL). The cessation of growth and decrease in viability in all cultures coincided with the time at which glucose became exhausted from culture media. Thus, cultures containing a higher inoculum experienced a faster glucose depletion than cultures seeded

at x10^5 cells/mL. With respect to lactate production, concentrations as high as ≥50 mM can be reached during industrial bioprocesses which can be detrimental to cell growth, productivity and viability (Glacken, Fleischaker *et al.* 1986) (Ahn and Antoniewicz 2012). However, lactate concentrations were not higher than 25 mM in EG2 cultures and instead lactate consumption took place at higher inoculum. Even though lactate consumption has been observed in cultures with low initial glucose concentrations(Barnabe and Butler 2000), lactate consumption was observed in EG2 cultures with both, 3mM and 25 mM initial glucose concentrations. The latter suggests that cell inoculum affected lactate consumption even at high initial glucose concentrations. In EG2 cultures containing 3 mM glucose, lactate concentration increased until glucose was depleted and decreased thereafter regardless of the cell inoculum. In culture media containing 25 mM glucose, lactate consumption happened regardless of whether glucose was depleted or not (at x10^6 cells/mL and x10^7 cells/mL respectively).

As was previously seen in Chapter 3, the relationship between Ade nucleotides (AMP, ADP and ATP) was used to determine the adenylate energy charge (AEC) which has been used to distinguish between active growing cells (AEC= ≥0.8) from cells that have been depleted of an energy substrate (AEC= 0.06) (Chapman, Fall *et al.* 1971). EG2 cells seeded at low inoculum in media with 3 mM or 25 mM glucose showed an AEC of 0.01 and 0.78 respectively. These AEC values were further reduced by up to 83.4% and 94.4% when cell inoculum increased to x 10^7 cells/mL. Thus, AEC was dependent on glucose availability, with depleted cultures lacking the major energy and carbon source required for the generation of ATP. In addition to ATP depletion, accumulation of reactive oxygen species (ROS) may have occurred as previously seen in glucose deprived cells due to inadequate production of NADPH via the penthose phosphate pathway (PPP)

(Liu, Song *et al.* 2003). The pools of UTP, GTP and CTP also decreased but to a lesser extent than ATP pools. As ATP, these nucleotides serve as substrates, products, effectors or energy donors in many metabolic reactions (Wagner 2011). Thus, a reduction in their pool sizes could be also responsible for the changes observed in cell growth and viability in EG2 glucose-depleted cultures.

With respect to specific cell productivity, EG2 cells' q_{Mab} values differed according to the exposure of cells to glucose depletion, with a significant decrease by up to 65.8% and 66.6% when cell inoculum increased from 10^5 cells/mL to 10^7 cells/mL in both 3 mM and 25 mM-containing cultures respectively. Thus, glucose-depleted cultures did not count with the energy supplied required for initiation of protein synthesis. In contrast, Okturk and Palsson found no significant effects of the initial inoculum size on the Mab formation rate during a hybridoma batch culture(Ozturk and Palsson 1990). The lack of effect on q_{Mab} could be because there was still residual glucose present at the end of the culture, opposite to EG2 cultures were glucose was depleted at low initial glucose concentrations and at high cell inoculum. Thus, in the absence of cellular nucleotides, cells will lack the energy supply required for protein synthesis.

Glucose depletion was also responsible for reduced site-occupancy and increase of non-galactosylated glycans in EG2 cells seeded at low inoculum in media containing ≤ 5.5 mM glucose (see Chapter 3). The latter has been associated with a decrease in the production of the lipid linked oligosaccharide $Glc_3Man_9GlcNAc_2$-PP-Dol(Stark and Heath 1979, Turco 1980, Rearick, Chapman *et al.* 1981, Jenkins, Castro *et al.* 1994, Liu, Spearman *et al.* 2014). Similarly, EG2 cells cultured in media containing 3 mM glucose showed a mixture of glycosylated and non-glycosylated proteins regardless the cell inoculum. In contrast, EG2 cells cultured in media with

25 mM glucose showed only glycosylated EG2-hFc Mabs, including glucose-starved cultures (x10^7 cells/mL). The latter suggests that these cultures produced glycoproteins during the first 24 hours after which protein production stopped because there was not an energy source available to support cell growth and protein production. Similarly, Liu *et al.* showed no evidence on non-glycosylated Mab in EG2 cultures seeded high inoculum (x10^8 cells/mL) in media containing >15 mM-25 mM initial glucose over a period of 24 hours (Liu, Spearman *et al.* 2014). However, these cultures had ~8 mM glucose remaining in the culture media at the moment of harvest opposite to EG2 glucose starved cells cultured in media with 25 mM glucose seeded at x10^7 cells/mL for four days.

The changes observed in macro- and micro-heterogeneity in EG2 glucose-starved cultures were suspected to be a result of a decrease in the intracellular pool of nucleotide sugars. In fact, nucleotide sugar activation is dependent upon the availability of tri-phosphorylated nucleotides (Jenkins, Castro *et al.* 1994). Indeed, EG2 glucose-starved cultures lacked sufficient Urd nucleotides for the activation of UDP-Glc and UDP-Gal required at earlier and final steps respectively during glycosylation. A significant decrease of up to 60.0% and 18.3% in UDP-Glc and UDP-Gal respectively was observed in cultures with 3 mM glucose cultures/high inoculum when compared to cultures with 25 mM glucose/low inoculum (control). Similarly, a significant reduction of up to 89.29% and 10.58% in UDP-Glc and UDP-Gal respectively was observed in 25 mM glucose cultures/high inoculum when compared to control cultures. In the absence of UDP-Glc, the synthesis of Dol-P-Glc is compromised at early stages of the glycosylation pathway (Freeze and Elbein 2009). Furthermore, a decrease in UDP-Glc pool can affect the processing of incompletely folded proteins, preventing the transport of proteins out of the ER (Craig Hammond

1995, Spiro 2000). Similar results were found in the glucose-depleted cell line NIL with a decrease in the UDP-Glc/UDP-Gal ratio of two in comparison to a ratio of four in cells cultured in 25 mM glucose-containing media (Ullrey and Kalckar 1979). Finally, a decrease in GDP-Man of up to 6.96% and 41.30% was also observed in 3 mM and 25 mM glucose seeded at high inoculum. Thus, the reduced site-occupancy observed in glucose-starved cells are also the result of a decrease in GDP-Man which may have affected the synthesis of Dol-P-Man and subsequent Dol-P-Man derived oligosaccharides in glucose-starved cells (Chapman and Calhoun 1988). The results showed that inoculum size had a significant effect on EG2 cells' growth, metabolism, specific cell productivity and EG2-hFc Mab's *N*-glycosylation. The effects were more pronounced as the initial concentration of glucose was reduced from 25 mM to 3 mM resulting in lower growth rates, productivity and non-glycosylated Mabs. Equally important is the significant reduction in nucleotide pools and nucleotide sugars that were responsible for the cease of growth and reduction in glycosylation. Thus, it is suggested that the inoculum size should be considered to avoid early depletion of key nutrients that can affect cell growth and product quality.

4.6.Conclusions

- Initial cell inoculum impacted the final MCDs reached on day four. A significant decrease of up to 65.6%, 35.8% and 6.8% were observed in 3 mM glucose containing cultures seeded at 2.5 x 10^5 cells/mL, 2.5 x 10^6 cells/mL and 2.5 x 10^7 cells/mL respectively with respect to 25 mM glucose cultures.
- Lack of cell growth and viability can be related to glucose depletion experienced before day two in cultures containing 3 mM glucose regardless of the cell inoculum

- Increasing the cell inoculum from 2.5 x 10^6 cells/mL to 2.5 x 10^7 cells/mL caused glucose depletion on day four and two respectively in cultures containing 25 mM glucose.

- EG2 cells cultured in media containing 3 mM glucose switched to lactate consumption once glucose was depleted from culture media.

- Lactate consumption was also observed in 25 mM glucose-containing cultures when glucose was still available (medium cell inoculum) or when glucose was depleted (high cell inoculum)

- 3 mM glucose containing cultures showed a significantly lower specific cell productivity by up to 21.6%, 58.1% and 19.6% with respect to cultures containing 25 mM glucose seeded at 2.5 x 10^5 cells/mL, 2.5 x 10^6 cells/mL and 2.5 x 10^7 cells/mL respectively.

- A decrease in site-occupancy was observed in glucose depleted cultures containing 3 mM glucose regardless of cell inoculum.

- There was no evidence of non-glycosylated Mabs in cultures containing 25 mM glucose seeded at medium or high inoculum densities, even though glucose depletion occurred in these cultures.

- Pauci-mannose glycans constituted 12%, 2.25% and 6.18% in 3 mM glucose-containing cultures regardless the cell inoculum and 25 mM glucose-containing cultures seeded at x10^6 and x10^7 cells/mL respectively.

- A significant decrease in GI of up to 58.3% and 85% was observed in cultures containing 3 mM glucose as the inoculum increased from x 10^5 and x 10^7cells/mL.

- At 25 mM glucose, a significant decrease of up to 36.7%, 6.90% and 68.3% was observed in GI values, fucosylation and sialylation respectively as the inoculum increased from x10^5 and x10^7cells/mL.

- Low glucose cultures seeded at low and high cell inoculum showed a significant reduction in the Ade, Cyt, Gua and Urd intracellular pools of up to 98.7%, 97.5%, 99.5% and 96.9% respectively.

- Cultures containing 25 mM glucose seeded at high inoculum also showed a significant decrease of up to 90.0%, 61.9%, 98.1% and 3.39% Ade, Cyt, Gua and Urd intracellular pools respectively.
- An AEC below 0.04 was observed in both cultures containing 3 mM (low and high inoculum) and 25 mM glucose (high inoculum) in comparison to control cultures with an AEC of 0.78.
- A significant decrease of up to 55.7% and 74.8% in UDP-hexoses was observed in 3 mM and 25 mM glucose containing respectively seeded at $x10^7$ cells/mL in comparison to control cultures with 25 mM glucose seeded at $x10^5$cells/mL.
- A significant decrease of up to 99.4% and 93.5% in UDP-hexosamines was observed in 3 mM and 25 mM glucose containing respectively seeded at $x10^7$ cells/mL compared to control cultures.

The data shows that changes in site-occupancy and macroheterogeneity observed in EG2 cultures were a result of nucleotide and nucleotide sugars' low intracellular concentrations caused by glucose depletion, which was more significant at higher cell densities and at low initial glucose concentrations. Nevertheless, 25 mM glucose-containing cultures seeded at high cell densities also experienced glucose depletion.

Section B*

Modulation of Antibody Glycosylation

Control of recombinant protein glycosylation has become one of the key components for biopharmaceutical industry because of its establish role in stability, serum clearance, immunogenicity, proper function and drugs potency (Arnold, Wormald *et al.* 2007, Berger M. 2011). In particular, antibody-Fc glycosylation is important for modulation of immune responses through antibody dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (Jefferis 2002). For example, antibodies lacking core fucose or having bisecting *N*-acetylglucosamine (GlcNAc) have shown an enhanced ADCC while Gal residues have been related to CDC activity (Hodoniczky, Zheng *et al.* 2005, Higel, Seidl *et al.* 2016). Immunoglobulins' glycosylation is highly heterogeneous, consisting on bi-antennary core-fucosylated complex structures with variable levels of galactosylation and minimal sialylation (Raju 2003).

Glycosylation of glycoproteins varies significantly among cell lines and animal species. The most widely used expression system are mammalian cell lines which glycosylate proteins in human-like manner. Many of the FDA-approved monoclonal antibodies in the market are produced by Chinese hamster ovary (CHO) cells or mouse-derived cell lines such as NS0 cells. Even though CHO and NS0 cells are both mammalian cells, nearly every cell type has distinct requirements for cell growth, survival, differentiation and glycosylation machinery, producing glycoproteins with different glycoforms. For instance, CHO cells lack the enzyme GnT-III

- * Content of Section B, including Chapter 5 and Chapter 6 has not been published.

responsible for the transfer of bisecting GlcNAc residues from UDP-GlcNAc whereas mouse myeloma cells express the active GnT-IIII enzyme (Raju, Briggs *et al.* 2000). In addition, glycosylation of recombinant proteins are also strongly influenced by culture conditions.

Different strategies have been used to control recombinant protein glycosylation such us **1.** overexpression or knockout of glycosyltransferase or nucleotide sugar transporters genes (Hodoniczky, Zheng *et al.* 2005); **2.** incorporation of glycosidase inhibitors; **3.** control of culture parameters (e.g. temperature, pH, osmolality, NH^4) (Majid, Butler *et al.* 2007); **4.** *in vitro* manipulation of purified recombinant protein in the presence of specific glycosyltranferases and **5.** incorporation of metabolic precursors of *N*-linked glycosylation pathway (Hills, Patel *et al.* 2001, Wong, Wati *et al.* 2010). The latter have shown to be successful on improving the final content of terminal Gal and sialylation without compromising cell proliferation and productivity (Crowell, Grampp *et al.* 2007, Gramer, Eckblad *et al.* 2011). Thus, in this section, experiments tested nucleotide sugar precursors particularly involved in the galactosylation (e.g. Gal, Urd and M) and sialylation (e.g. GlcN and ManNAc) of NS0-IgG1 and CHO-EG2-hFc.

B.1 Hypothesis of Research

Knowing that there is a relationship between the intracellular levels of nucleotide-sugar pools and protein glycosylation, the galactosylation and sialylation of monoclonal antibodies can be improved by providing cells with precursors (e.g. galactose, uridine, cytidine, glucosamine, mannosamine) and co-factors (manganese chloride, iron).

Chapter 5

Galactosylation and sialylation enhancement through feeding of manganese chloride, uridine and galactose.

5.1. Introduction

Culture media components (e.g. glucose, trace metals and amino acids) have shown to influence the final product's glycosylation profile which in turn is known to influence glycoproteins properties required for proper effector functions (Jefferis 2009). For instance, removal of Gal residues may or not affect C1q binding activity with no effect on ADCC (Boyd, Lines *et al.* 1995, Wright and Morrison 1998, Hodoniczky, Zheng *et al.* 2005). In contrast, lower sialylation has been related to the absence of terminal Gal, which constitutes the appropriate acceptor substrate for sialyltransferases (Crowell, Grampp *et al.* 2007). Exposed Gal residues are prompted to be removed from circulation through uptake by hepatic Gal-specific receptors (Walsh and Jefferis 2006, Gramer, Eckblad *et al.* 2011). Reduction in galactosylation can arise due to multiple factors such as: **1.** glucose starvation (See **Chapter 3**) and amino acids depletion (e.g. cysteine, asparagine, glutamic acid, aspartic acid and tyrosine) (Hayter, Curling *et al.* 1992, Hayter, Curling *et al.* 1993, Crowell, Grampp *et al.* 2007, Gramer, Eckblad *et al.* 2011, Liu, Spearman *et al.* 2014, Villacres, Tayi *et al.* 2015); **2.** low adenylate energy charge (AEC) (Villacres, Tayi *et al.* 2015); **3.** reduced intracellular concentration of nucleotide sugars (UDP-Gal) (Nyberg 1998, Villacres, Tayi *et al.* 2015); **4.** reduced transport; **5.** reduced galactosyltransferase activity or

expression; **6.** sequestration of Gal into *N*-acetylgalactosamine or Urd into UDP-GlcNAc (Gawlitzek, Valley *et al.* 1998, Clark, Griffiths *et al.* 2005).

Knowing that culture media components (e.g. glucose, trace metals and amino acids) have shown to influence the final product's glycosylation profile, the following experiments investigated the effect of precursor feeding on the glycosylation of two cell lines: NS0 producing an IgG1 and CHO cells producing the chimeric EG2-hFc Mab. The experiments showed in this chapter were based on previous reports which improved recombinant proteins galactosylation by increasing the availability of metabolic precursors of UDP-Gal synthesis (Urd; Gal; and M) (Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001, Crowell, Grampp *et al.* 2007, Gramer, Eckblad *et al.* 2011, Grainger and James 2013). For example, supplementation with Urd alone showed to increase the intracellular levels of UDP-Gal, resulting in an increase in galactosylation (Ryll 2006). Hills *et al.* also succeed in increasing the intracellular levels of the nucleotide sugar UDP-Gal but by incorporating 10 mM Gal into the medium, slightly improving galactosylation in IgG1 produced by GS-NS0 (Hills, Patel *et al.* 2001). In addition, incorporation of M has also lead to an increase in the content of galactosylated glycans (Crowell, Grampp *et al.* 2007).

Many glycosylation enzymes are highly dependent on metal ions such as Zn^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+} which act as co-factors along with nucleotide phosphates (UTP, CTP, GTP) and corresponding nucleotide sugars. For example, β-1,4-galactosyltransferase (β-1,4-GalT) requires Mn^{2+} to transfer Gal from UDP-Gal to the 4-OH of terminal *N*-acetylglucosamine (GlcNAc) residues forming a β-1,4 linkage (Powell and Brew 1976, Crowell, Grampp *et al.* 2007, Ramakrishnan and Qasba 2014). Another example is the α-1,3-galactosyltransferase (α-1,3 GalT)

which also uses UDP-Gal as nucleotide sugar donor and Mn^{2+} as a cofactor to transfer Gal to *N*-acetyllactosamine residues (Galβ1-4GlcNAc), producing the known alpha-Gal epitope (See **Chapter 6**). Another enzyme highly dependent on metal ions for optimal activation is CMP- Sia synthetase. This enzyme is responsible for the formation of CMP-Neu5Ac Sia activation in the presence of Neu5Ac and CTP along with metal cations such as Mn^{2+} or Mg^{2+} for maximum activity (Higa and Paulson 1985) (Cao and Chen 2012). Finally, Gramer *et al.* supplemented UDP-Gal precursors in a form of a cocktail (M+Urd+Gal) to GS-CHO cells and observed an improvement in antibody Fc galactosylation which resulted from a decrease in the amount of non-galactosylated glycans (G0) and an increase in glycans with one Gal (G1) (Gramer, Eckblad *et al.* 2011).

5.2. Objectives

The objectives of the experiments outlined in this chapter are:

- To enhance antibody galactosylation through feeding of M, Urd and Gal.
- To test galactosylation precursors feeding on their effect on antibody sialylation.

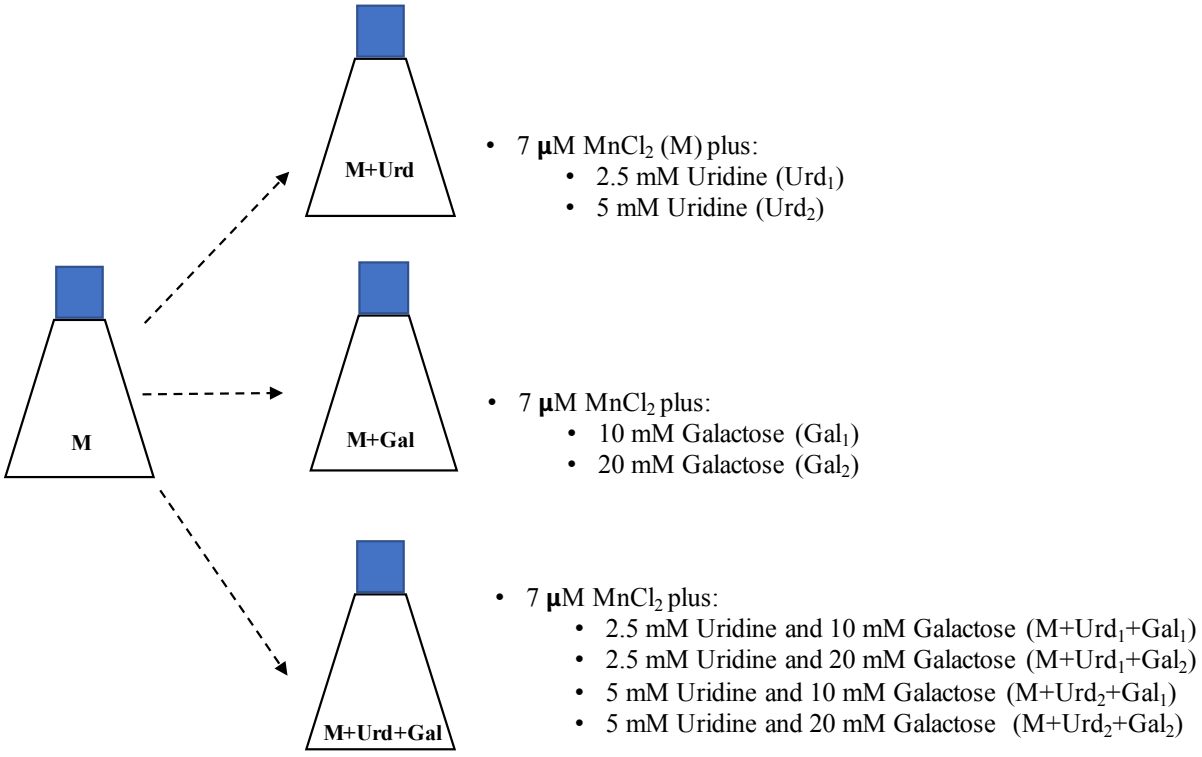
5.3. Experimental set up

Preliminary studies were performed to evaluate the effect of M, Urd and Gal concentrations on cell growth and viability. For this purpose, NS0 cells were seeded at a cell density of 2.5 x 10^5 cells/mL in Biogro™ medium containing M alone or in combination with Urd and Gal as shown in **Figure 5.1.** The M concentration used was based on a previous report by Markely (Markely 2011). With respect to Gal concentrations, the report by Hills *et al.* was used as

a reference (Hills, Patel *et al.* 2001). Finally, Urd concentrations were based on Baker *et al.* and Gramer *et al* (Baker, Rendall *et al.* 2001, Gramer, Eckblad *et al.* 2011). Each condition was performed in duplicates and the results shown are the average of two independent experiments (n=4). To determine whether the impact was cell line-specific, we applied the M+Gal+Urd cocktail to CHO (DUXB) cell line expressing a chimeric human-llama (EG2-hFc) monoclonal antibody. Cells were cultured in 125 mL shake flasks containing 35 mL of Biogro™ media containing 7μM M alone or in combination with: 5 mM Urd ($M+Urd_2$), 20 mM Gal ($M+Gal_2$), 5 mM Urd and 20 mM Gal ($M+Urd_2+Gal_2$). Biogro™ media did not contain M, Urd and Gal in its basal formulation (control cultures). Each condition was done in duplicates and results were averaged (n=2). During the experiments, culture shake flasks were placed in a shake platform at 120 rpm were incubation took place for four days at 37°C with 10% CO_2. Daily sampling (900 μL) was performed to determine cell growth and viability of each condition by trypan blue exclusion method and for metabolite analysis and protein production. On day four, cell culture was terminated and supernatants were collected, filtered and concentrated as previously indicated for further analysis. An extra sample containing $1x10^7$ cells was taken for nucleotide and nucleotide sugar analysis. The purpose of these set of experiments was to study the effect of M, Gal and Urd on the cell growth, viability, productivity and mainly glycosylation of antibodies produced by two different mammalian cell lines.

Figure 5. 1 Experimental set up of galactosylation precursors for NS0 cultures.

Gal, Urd and/or M-containing media were used to evaluate the effect of these supplements on Mabs galactosylation by action of galactosyltransferases.



5.4. Results

5.4.1 NS0 and EG2 cell growth in presence of M, Urd and Gal.

First, the effect of M and Gal was evaluated on NS0 cells' growth and viability (**Figure 5.2**). For this purpose, Biogro™ media was supplemented with 7µM M, 20 mM Gal alone or combined (M+Gal).A lag phase was observed during the first day where cultures had a similar growth rate (0.03 h^{-1}). All cultures reached their MCDs on day four, with the control culture having the highest MCD of 4.01 x 10^6 cells/mL followed by the culture containing Gal with 3.56 x 10^6 cells/mL. Cultures containing M showed a decrease in cell density of 16% with respect to the

control with a cell viability of 92% (p- Value <0.0003). MCD of M+Gal culture consistently showed a significant decrease of about 22.7% and a high cell viability of 91.7% (p-Value <0.0001). A separate set of experiments evaluated the combination of M, Urd and Gal on NS0 cells' growth and viability during a four-day batch culture and the results are shown in **Figure 5.3**. Similar growth rates (0.02 h^{-1}) were observed in each culture over the first 48 hours, after which cell yield and viability depended upon the precursor. On day four, cultures containing M and $M+Urd_1$ showed comparable MCDs of 3.52 x 10^6 cells/mL and 3.12 x 10^6 cells/mL respectively to the control culture with 3.33 x 10^6 cells/mL. Furthermore, cell viability in these cultures were >91.2%. However, cultures containing 5 mM uridine ($M+Urd_2$) showed a significant decrease in MCD and cell viability of up to 28.7% and 5.61% respectively when compared to the control. With respect to M+Urd+Gal cultures showed a significant decrease of up to 50.5% and 6% in cell growth and viability respectively when compared to the control (p-Value <0.0001). Cultures containing M+Gal also showed a decrease in cell growth of about 21% (p-Value <0.001) on day four but not as significant as when supplemented with Urd. This decrease in cell growth cannot be attributed to a decrease in cell viability (>92.1%) and probably the lower cell density observed in M+Gal cultures did not reach their MCD on day four and therefore were still growing.

To determine whether the impact was cell line-specific, the MGU cocktail was applied to CHO (DUXB) cell line expressing a chimeric human-llama (EG2-hFc) monoclonal antibody. Cells were cultured in Biogro™ media containing 7μM M alone or in combination with: 5 mM Urd ($M+Urd_2$), 20 mM Gal ($M+Gal_2$), 5 mM Urd and 20 mM Gal ($M+Urd_2+Gal_2$).

Figure 5. 2 The effect of galactosylation precursor supplementation on cell growth and viability of NS0 cells.

A. Biogro™ media (Control, -●-) containing **B.** 7 μM M, -✕-); **C.** Gal, -▲-); **D.** M+Gal, -❍. Data points represent mean ± SD of triplicates for each condition for two set of independent experiments (n=6). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.

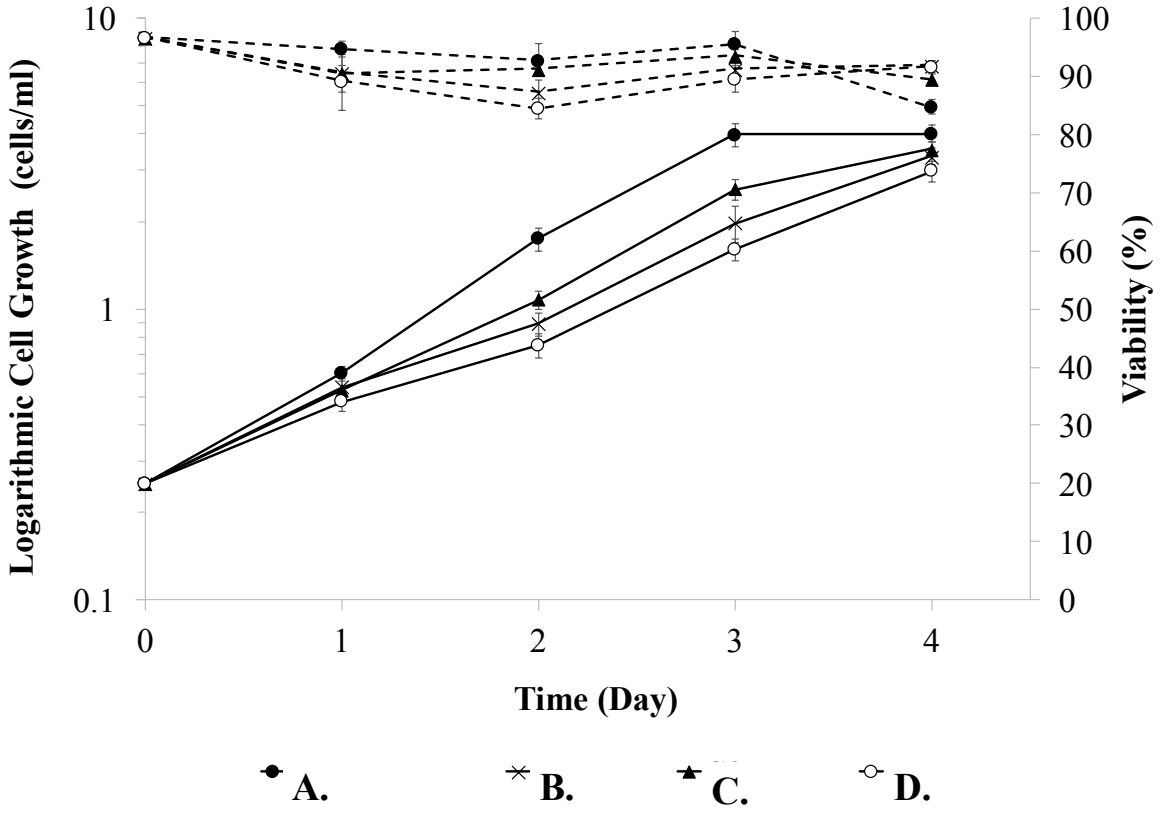


Figure 5.4 shows that CHO EG2 cells reached their MCDs on day four and that cell viability was >95% in all culture conditions. M, Gal_2 and M+Gal cultures showed comparable MCDs (3.35 x 10^6 cells/mL, 3.55 x 10^6 cells/mL and 3.40 x 10^6 cells/mL respectively) on day four with respect to the control culture (3.54 x 10^6 cells/mL). Cultures containing Urd, M+Urd or M+Urd+Gal showed a significant decrease in cell growth of about 11.5%, 18.8% and 20.8%

respectively in comparison to the control culture (p-Value 0.02, p-Value 0.0005, p-Value 0.0002). All cell cultures maintain cell viabilities over 90% on day four.

Figure 5. 3 Effect of M, Urd and Gal on growth and viability of NS0 cells.

A. Biogro™ media (Control,-●-) supplemented with **B.** 7µM M,-✕-; **C.** 7µM M + 2.5 Urd_1,-△-; **D**. 7µM M + 5mM Urd_2, -◆-; **E.** 7µM M+10 mM Gal_1,-□-; **F**. 7µM M + 20 mM Gal_2,-○-; **G.** 7 µM M + 2.5mM Urd_1+ 10mM Gal_1,-■-); **H.** 7µM M +2.5mM Urd_1 +20mM Gal_2,-✳-); **I.** 7µM M + 5mM Urd_2 +10 mM Gal_1,-◇-); **J.** 7µM M + 5mM Urd_2 +20 mM Gal_2, –-. Data points represent mean ± SD of duplicates for each condition for two set of independent experiments (n=4). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay using a computer-based image analyzer.

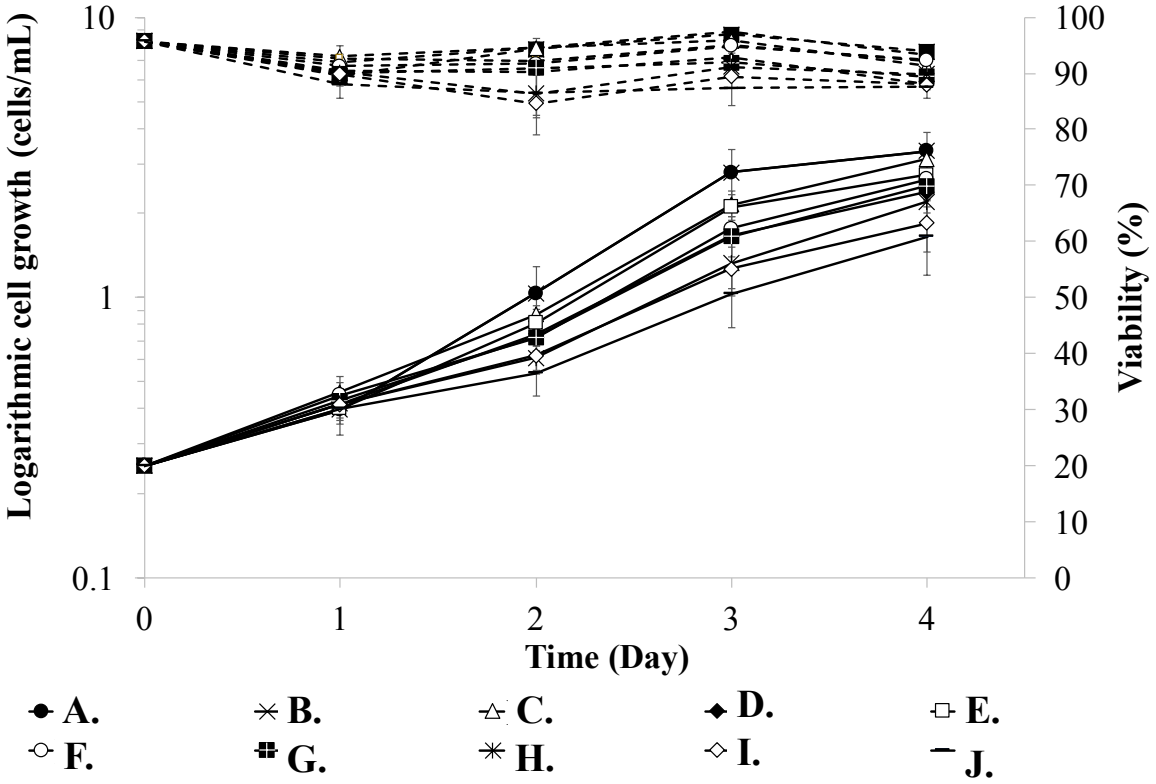
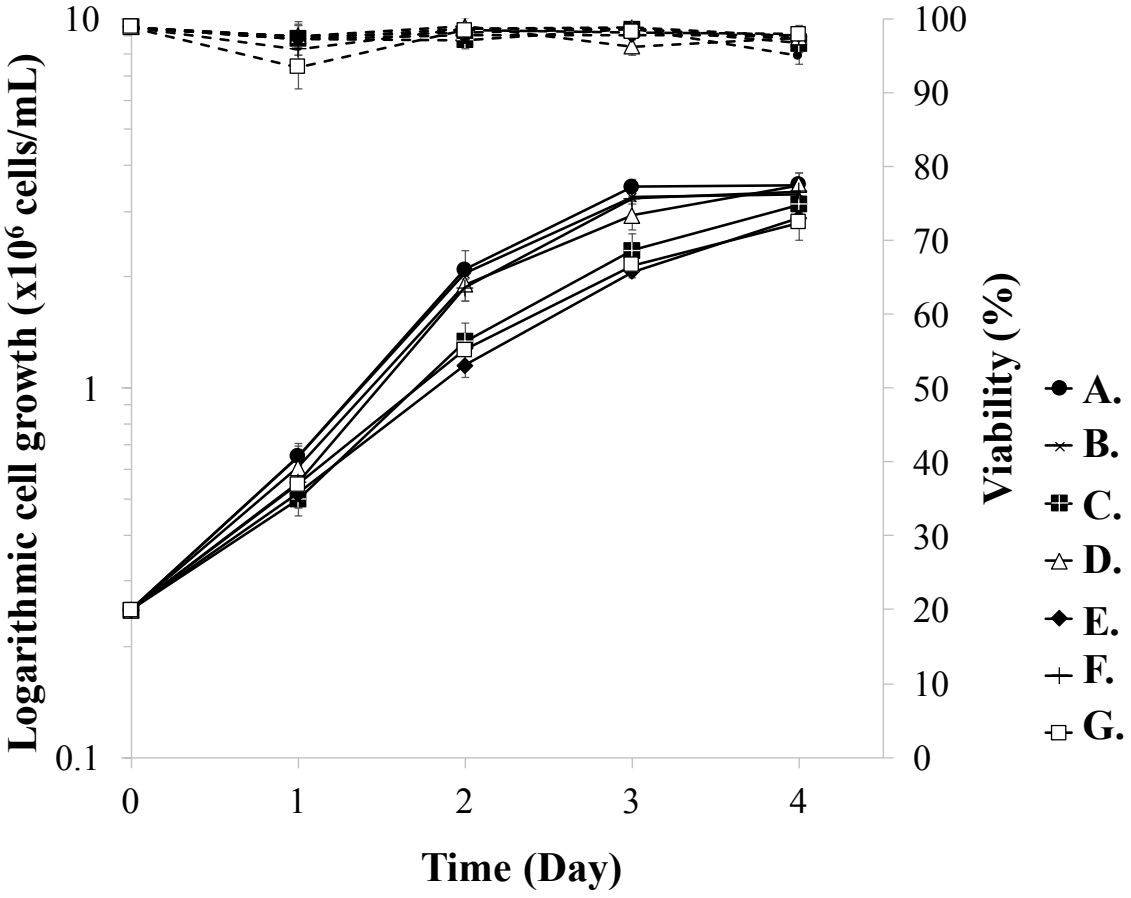


Figure 5. 4 The effect of galactosylation precursors supplementation on cell growth and viability of CHO-EG2 cells.

A. Biogro™ culture medium (Control, -●-) supplemented with **B.** 7 μM M, -×-; **C.** 5 mM Urd, -■-; **D.** 20 mM Gal, -△-; **E.** 7 μM M plus 5 mM Urd, -◆-; **F.** 7 μM M plus 20 mM Gal, -+-; **G.** 7 μM M plus 20 mM Gal and 5 mM Urd, -□- . Data points represent mean ± SD of triplicates for each condition (n=3). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.



In summary, a decrease of up to 16%, 28.7%, 21% and 50.5% in MCDs was observed in NS0 cultures containing M, M+Urd, M+Gal and M+Urd+Gal respectively. The combination of the three supplements showed the highest growth inhibition, with Urd having the most negative impact on cell growth and viability. Higher cell densities were obtained when either Gal or M

were supplemented alone than when they were combined (M+Gal), suspecting that the latter where still in exponential growth phase. Furthermore, since M+Gal cultures showed high viabilities but not as high as the control, it would be necessary to extend the cultivation period further than day four to see if higher MCDs can be reached. With respect to CHO EG2 cells, supplementation of M and Gal allowed MCDs that were comparable to control culture. However, a significant decrease of about 11.5% 18.8% and 20.8% in MCDs were also observed in Urd-, M+Urd- and M+Urd+Gal-containing cultures respectively. All culture conditions showed high viability no matter the supplement added in EG2 cultures.

5.4.2 Glucose uptake and lactate production under different culture conditions

The precursor-supplemented cultures showed significant differences in cell growth and viabilities that could have being a result of changes in cellular metabolism. Thus, the effect of M, Urd and Gal on NS0 cells' metabolism was assessed by measuring the concentrations of dextrose and lactate by using a YSI biochemistry analyzer. **Figure 5.5** shows the profile of dextrose consumption and lactate production for NS0 cultures. Cultures with higher MCDs (M, M+Urd and control) had less amount of glucose at the end of the culture (< 4.33 mmol/L). Notice that glucose and glutamine are the only carbon source in these cultures (initial concentration of 25 mM and 4 mM respectively). In contrast, cultures containing Gal (M+Gal_1; M+Gal_2; M+Urd_1+Gal_1; M+Urd_1+Gal_2; M+Urd_2+Gal_1 and M+Urd_2+Gal_2) showed a higher amount of residual dextrose at the end of the culture (>6.98 mmol/L), possibly because these cultures have two carbon sources: glucose and Gal. **Table 5.1** shows a comparison of the cumulative specific dextrose consumption rates (q_{Dex}) calculated from the slope between the amount of dextrose/glucose (μmol/mL) and

integral viable cell values from the start of cell culture until day four (IVCD). On day four, slightly higher consumption rates (q_{Dex}) were observed in M+Urd cultures (>1.19 ± 0.37 μmol/10^6 cells/day; p-Value <0.05) in comparison to the control with 0.90 μmol/10^6 cells/day). Incorporation of Gal showed a slightly increase in consumption rates but these changes were not significant. However, increasing Gal concentrations from 10 mM to 20 mM (Gal_1 and Gal_2 respectively) showed a significant increase in consumption rates in Gal_2 cultures (**Table 5.1**; p-Value ≤0.0045). A slightly higher but not significant increase in q_{Dex} was observed in cultures containing M+Urd_1+Gal_1, M+Urd_1+Gal_2, M+Urd_2+Gal_1 when compared to control cultures. However, cultures containing M+Urd_2+Gal_2 showed a significant higher q_{Dex} of 244% when compared to the control (p-Value <0.0001). These cultures showed the lowest MCDs in comparison to untreated cultures (**Figure 5.4**).

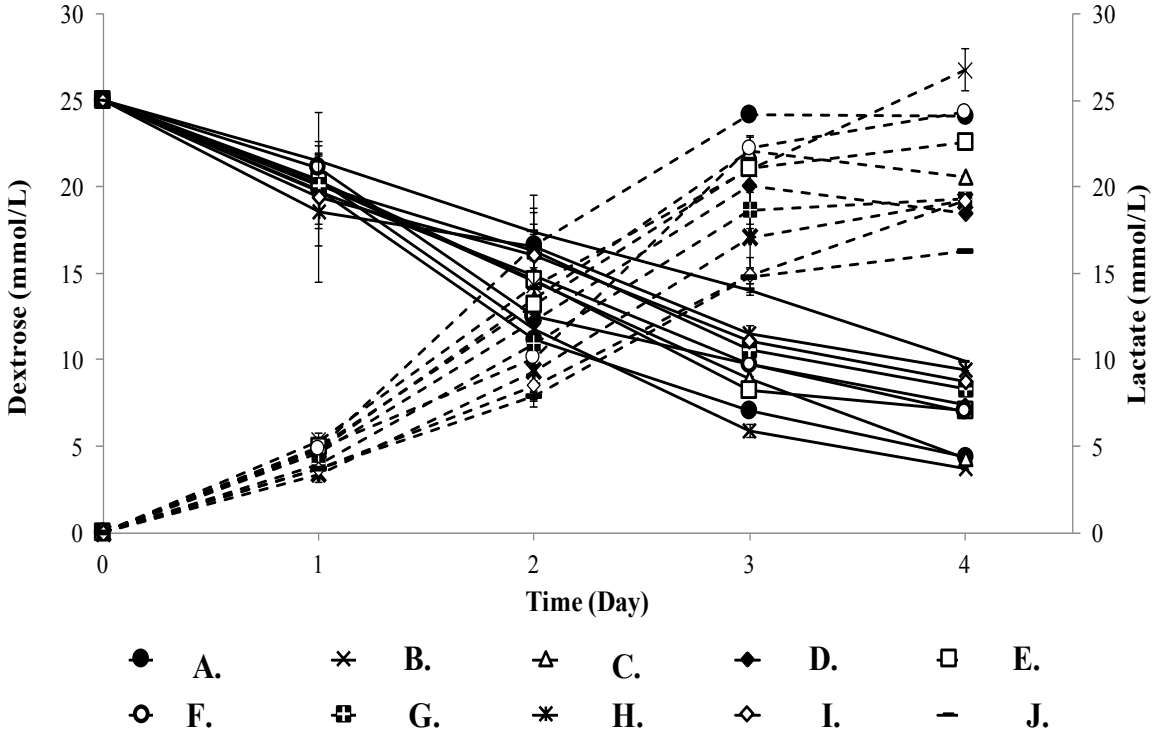
Higher glucose uptake rates have translated into higher lactate productions when high glucose concentrations are provided to cells in culture (Hayter, Curling *et al.* 1991). In these experiments, NS0 cultures contained 25 mM glucose regardless of the supplementation with galactosylation precursors. In control cultures, lactate reached 24.1 mM on day four in a similar manner to cultures containing M, M+Gal_1/Gal_2 with 26.75 mM, 22.6 mM and 24.3 mM respectively. However, a slightly but significantly higher yield of lactate produced per dextrose consumed ($Y_{Lac/Dex}$) was observed in cultures containing M (1.26 ± 0.06 mol/mol), M+Gal_1 and M+Gal_2(1.26 ± 0.00 mol/mol and 1.35 ± 0.00 mol/mol), and M+Urd_1+Gal_2 (1.23 ± 0.00 mol/mol) (p-Value < 0.004) in comparison to the control (1.16 ± 0.01 mol/mol. $Y_{Lac/Dex}$ can give information regarding glucose metabolism. It has previously been seen that lactate is produced as an inhibitory metabolite in a ratio of about 1.1-1.7 mol lactate per mole of glucose (Regine Eibl 2009). Thus, a

more energetically efficient utilization of glucose translates into lower lactate production. Only cultures containing M+Urd_1/Urd_2 (1.00 ± 0.01 mol/mol; 1.05 ± 0.00 mol/mol respectively) and M+Urd_2+Gal_2 (1.08 ± 0.00 mol/mol) had a slight but significant decrease in $Y_{Lac/Glc}$ with respect to the control

These results indicate that M, M+Urd and control cultures, containing only glucose as a carbon source, showed less than 4.33 mmol/L of residual glucose on day four. The latter is consistent with the significant increase (up to 92%) in specific glucose consumption observed in M+Urd cultures in comparison to the control with 0.90 μmol/10^6 cells/day. In addition, cultures containing M+Urd_1/Urd_2 showed the lowest $Y_{Lac/Glc}$ in comparison to the control, indicating a more efficient glucose metabolism. With respect to cultures containing Gal, (M+Gal_1; M+Gal_2; M+Urd_1+Gal_1; M+Urd_1+Gal_2; M+Urd_2+Gal_1 and M+Urd_2+Gal_2) the amount of residual dextrose at the end of the culture was over >6.98 mmol/L because these cultures contained both glucose and Gal. Cultures containing M+Urd+Gal showed comparable consumption rates with respect to the control, except cultures with M+Urd_2+Gal_2 which showed a significant increase in q_{Dex} of about 244% when compared to the control. Cultures containing M, M+$Gal_{1/2}$ and M+Urd_1+Gal_2 showed an increase in $Y_{Lac/Dex}$ of about 8.6%, 16.4%, 6.03% respectively when compared to the control with 1.16 ± 0.01 mol/mol).

Figure 5. 5 Effect of galactosylation precursor supplementation on NS0 cells' metabolism.

Glucose (solid lanes) and lactate (dashed lanes) concentrations were determined during NS0 batch cultures when inoculated at an initial cell density of $2.5x10^5$ cells/mL in 35 mL A. culture medium (control, -●-) containing: **B.** 7μM M, -×-); **C**. 7μM M + 2.5 mM Urd_1, -△-; **D**. 7μM M + 5mM Urd_2, -◆-; **E**. 7μM M+10 mM Gal_1, -□-); **F.** 7μM M + 20 mM Gal_2, -○-); **G.** 7 μM M + 2.5mM Urd_1+ 10 mM Gal_1, -■-; **H.** 7μM M + 2.5mM Urd_1 +20 mM Gal_2, -✳-; **I.** 7μM M + 5mM Urd_2 +10 mMGal_1,-◇-; **J**. 7μM M + 5mM Urd_2 + 20 mM Gal_2, –-. Data points represent mean ± SD of duplicates for each condition from one set of experiments (n=2).



5.4.3 M, Urd and Gal effect on productivity in NS0 cells

IgG1 production was quantified by ELISA to determine the effect if any of M, Urd or Gal on NS0 cells productivity. Volumetric productivity showed a typical increase during exponential phase (day 2 – day 4) and it was significantly higher in cultures containing M+Gal (128 μg/mL) in comparison to the control with 91.9 μg/mL on day four (p-Value <0.0009). In contrast, Urd containing cultures also showed a significantly higher production with up to 123 μg/mL (p-Value 0.02) and when combined with Gal reached up to 104 μg/mL. The cell-specific productivity of antibody (q_{Mab}) was determined from the slope of the plot of NS0-IgG1 Mab concentration and the integral viable cell density (IVCD) values. Cultures containing M showed a higher q_{Mab} of 43.5 $\mu g/10^6$ cell-day than the control with 30.3 $\mu g/10^6$ cell-day (p-Value 0.01). This specific productivity further increased by up to 17% and 39.6% in cultures with M+Gal_1 and M+Gal_2 respectively. A further increase of up to 46.2% and 131% was observed in M+Urd_1- and M+Urd_2-containing cultures respectively when compared to untreated cultures (p-Value <0.001). Incorporation of Gal seems to have had a negative effect on specific cell productivity. This is reflected by a significant decrease in q_{Mab} of up to 32% in M+Urd_1+Gal_2 with respect to M+Urd_1 cultures. Similarly, M+Urd_2+Gal_1 and M+Urd_2+Gal_2 where a decrease of up to 39% and 21.6% was observed in comparison to M+Urd_2 cultures (**Table 5.1**). Nevertheless, supplementation of the three components also significantly increased NS0 cells' specific cell productivity by up to 83.5% (p-Value <0.0001) in comparison to the control.

The results indicate that M, Urd and Gal improved both volumetric and specific cell productivity in NS0 cells. Thus, a significant increase of up to 43.5%, 131%, 39.6%, 83.5% was observed in q_{Mab} in cultures containing M, M+Urd, M+Gal and M+Urd+Gal respectively. The latter showed a synergistic effect when the three supplements were combined with respect to the

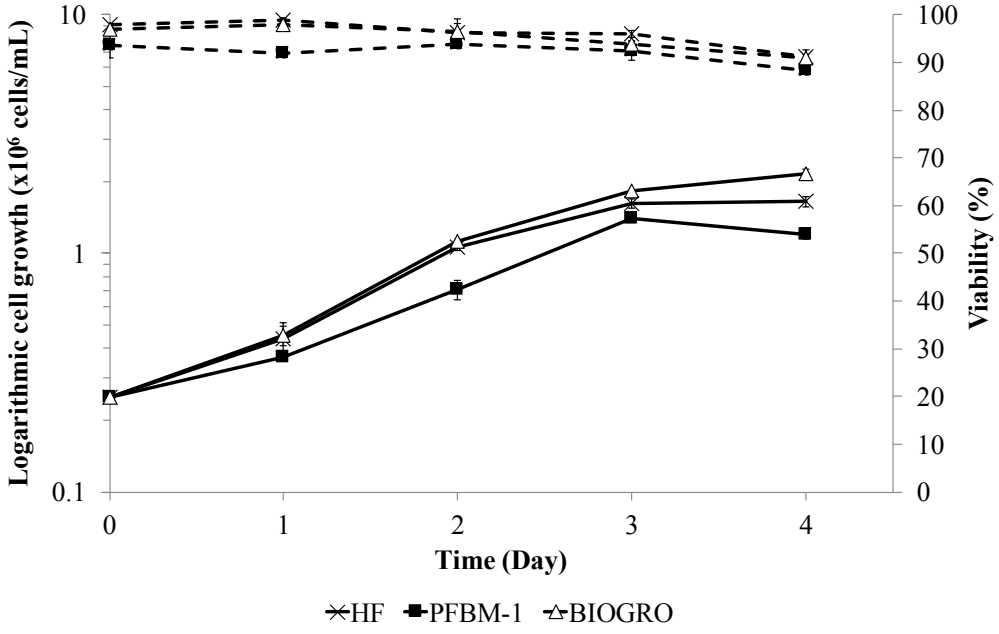
control culture. However, Gal- cultures did not reach specific cell productivities as high as M+Urd-containing cultures suggesting a negative effect in q_{Mab} when Gal was added along M and Urd.

5.4.1 NS0-IgG1 *N*-glycosylation

NS0 cells were initially cultured in a proprietary chemically defined protein free basal media (PFBM-1) from Abbvie (Redwood City, CA, USA). Since the PFBM-1 media's composition was unknown, NS0 cells were gradually adapted to grow in suspension in shake flasks in Biogro™ media with 0.5 g/L yeast extract. **Figure 5.6** shows a comparison of NS0 cells' growth in Biogro™ media with 0.5g/L yeast extract, in Biogro™ without hydrolysate (HF) and PFBM. A higher growth was observed in Biogro™ media with 2.17 x10^6 cells/mL which represents a significant increase of 81% with respect to the MCDs reached in by NS0 in PFBM. Thus, the proprietary media Biogro™ with 0.5 g/L YE was chosen over PFBM and HF formulations for further experiments. **Figure 5.7** shows a comparison of NS0-IgG1 *N*-glycosylation profiles obtained when cells were cultured in PFBM-1 and Biogro™ media. The *N*-glycan population identified seemed to be entirely bi-antennary structures of the complex-type with core fucosylation, lacking bisecting GlcNAc residues and with variable terminal galactosylation and sialylation. IgG1 produced in PFBM-1 shows a higher proportion of glycans containing zero Gal residues (F6A2G0) with 43% ± 0.80, followed by glycans containing one (F6A2G1) and two Gal residues (F6A2G2) with 41.4% ± 0.13 and 15.6% ± 0.93 respectively (top **Figure 5.7**).

Figure 5. 6. Adaptation of NS0 cells to grow in Biogro™ media with 0.5 g/L yeast extract (YE).

Comparison of NS0 cells' growth performance in protein free basal media (PFBM-1), in Biogro™ media without hydrolysate (HF) and Biogro™ media with 0.5g/L YE.



Sialylated glycans including F6A2G1S1, F6A2G2S1, F6A2G2S2, F6A2G2Ga1S1 and F6A2G2G2Ga1S2 constituted 15.0% ± 0.97 of the glycan pool. The relative abundance of alpha-1,3-Gal containing glycans was 3.55% ± 1.21 and included F6A2G1Ga1, F6A2G2Ga1, F6A2G2Ga1S1 and F6A2G1GaS1/S2. The GU values from F6A2G2Ga1 (GU=8.33) and F6A2G2GaS1/S2 (GU=9.28) overlapped with F6A2G2S1 and F6A2G2S2 respectively. Therefore, part of the alpha-Gal percentage showed above includes the area of these overlapped structures. IgG1 produced in Biogro™ media showed the same glycan structures but the distribution of Gal-containing glycans differed with respect to the ones observed in PFBM-1, Mabs showed a shift in the proportion of Gal-containing glycans **(bottom Figure 5.7)**. There was a

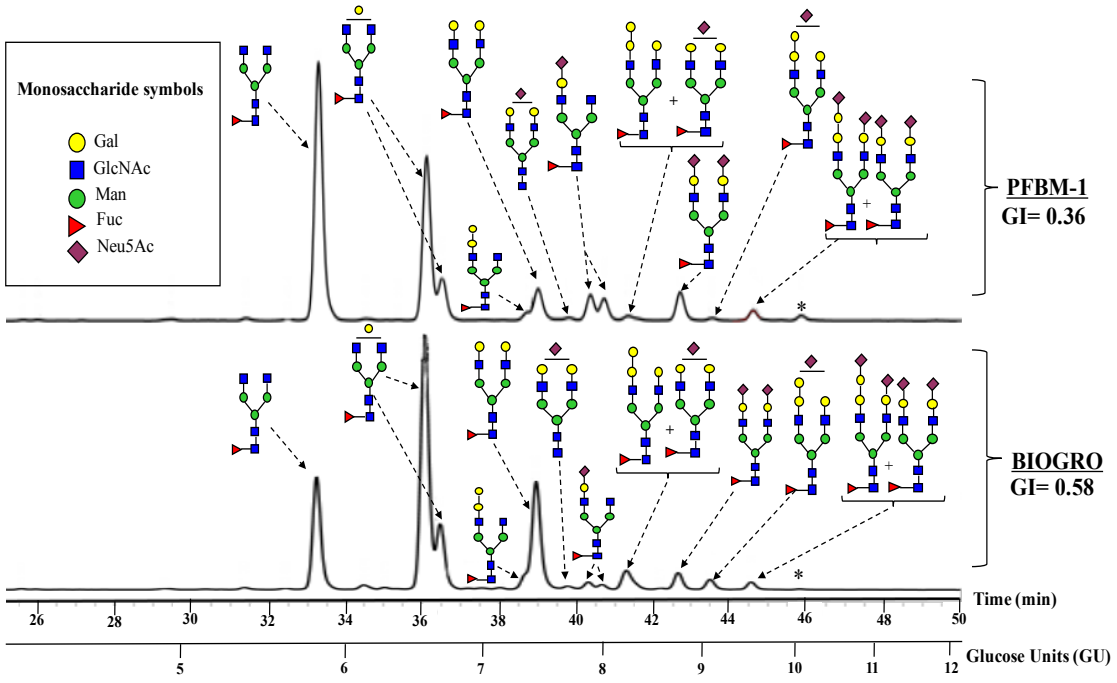
higher proportion of glycans containing one Gal residue (F6A2G1) with 47.2% ± 3.28, followed by glycans containing two (F6A2G2) and zero Gal residues (F6A2G0) with 35% ± 4.37 and 17.8 ± 1.41 respectively. Sia content about 14.4% ± 5.27 and consisted of the same structures previously described in PFBM-1 media. Alpha-1,3 Gal glycans were also observed in Mabs produced in Biogro™ media with a 11.9% ± 4.08. This percentage included F6A2G2Ga1 (GU=8.33) and F6A2G2GaS1/S2 (GU=9.28) which continued to overlap with F6A2G2S1and F6A2G2S2 respectively. Exoglycosidase digestion in combination with NP-HPLC was used to better assess the amount of alpha-Gal residues present in NS0-IgG1. Previously 2-AB-labeled glycans were incubated overnight with α-2-3,6,8 neuraminidase alone or in combination with β-1,4 galactosidase at 37°C. Neuraminidase removes terminal Sias while β-galactosidase removes the terminal Gal residues found in *N*-glycans. Digested glycans were collected and prepared for injection onto the HPLC. **Figure 5.8** shows the enzyme digestion array for NS0-IgG1 glycans produced in Biogro™ media. After digestion with **α**2-3,6,8 neuraminidase, all sialylated structures including A2G2S1, F6A2G1S1, F6A2G2S1/S2 and F6A2G2Ga1S1/S2 shifted mainly to three major peaks: F6A2G1, F6A2G2 and F6A2G2Ga1.

A consecutive digestion with **β**1-4 galactosidase (neuraminidase *plus* galactosidase) caused these peaks to further move to two main peaks: F6A2G0 and F6A2G1Ga1 with an 85.1% and 6.37% respectively **(Figure 5.8**). Thus, the alpha-Gal structures previously identified migrated all to F6A2G1Ga1 allowing the determination of the relative abundance of alpha-Gal residues in NS0-IgG1 produced in Biogro™ media with around 6.37%. The proportion of glycans containing or not Gal residues (G2, G1 and G0) was used to calculate the galactosylation index (GI) (**see section 2.11.6)**. NS0-IgG1 produced in PFBM-1 showed a significantly lower GI of 0.36 ± 0.01

(p-Value 0.002) in comparison to NS0-IgG1 produced in Biogro™ media with 0.58 ± 0.03. The percentage of fucosylation in PFBM-1 was slightly lower but not significantly with 86.3% ± 1.70 in comparison to Biogro™ media with 94.2% ± 6.27.

Figure 5. 7 Overlay of N-glycosylation profiles from NS0-IgG1 Mab produced in a chemically defined protein free basal (PFBM-1) media and/or Biogro™ media supplemented with 0.5g/L yeast extract.

Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Glycan structures were assigned based on GU values previously reported in the NIBRT Glycodatabase. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). * Corresponds to a sialylated and fucosylated glycan.



These results showed that adapting NS0 cells from PFBM-1 media to Biogro™ media increased the GI of the antibody from GI=0.36 to GI=0.58. This was a result of a shift in the

proportion of glycans containing Gal residues from G0>G1>G2 in PFBM-1media to G1>G2>G0 in Biogro™ media. However, an increase in alpha-1,3-Gal residues was also observed in Biogro™ media along with similar sialylation in comparison to PFBM.

Figure 5. 8 Sequential digestion of alpha-1,3 Gal residues present in NS0-IgG1.

Sequential exoglycosidase digestions of the IgG glycan pool with **α**2-3,6,8 neuraminidase and **β**1-4 galactosidase were performed to determine the percentage of **α**2-3 Gal residues in NS0-IgG1 produced in Biogro™ media.

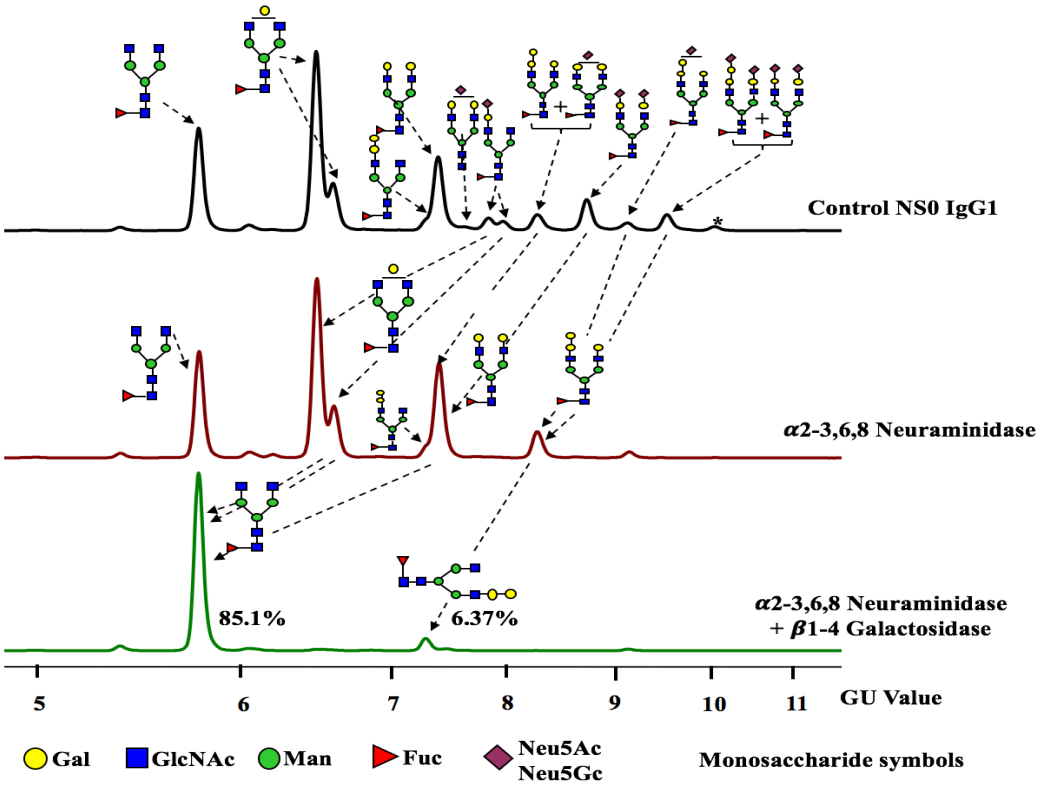


Table 5. 1 Specific glucose uptake (q_{Glc}), by-product formation (q_{Lac}) and productivity (q_{Mab}) determined during a four-day batch culture.

The $Y_{Lac/Glc}$ corresponds to the amount of lactate produce per mole of glucose. NS0 cells were cultured in media containing M, Urd and Gal. Daily samples were taken to measure glucose consumption, lactate production and cell productivity. The control sample did not include any supplement. The data shows the average and standard deviation corresponding to two samples from one independent set of experiments (n=2).

	Control	**$MnCl_2$ (M)**	**M+U1**	**M+U2**	**M+G1**	**M+G2**	**M+U1+G1**	**M+U1+G2**	**M+U2+G1**	**M+U2+G2**
q_{Dex}	0.90 ± 0.12	0.72 ± 0.14	1.73 ± 0.29*	1.19 ± 0.37	0.45 ± 0.06	1.24 ± 0.10	1.09 ± 0.24	1.20 ± 0.24	1.49 ± 0.14*	3.10 ± 0.26*
q_{Lac}	0.03 ± 0.09	1.86 ± 1.03*	0.55 ± 0.30	0.79 ± 1.12	0.62 ± 0.08	0.95 ± 0.06	0.31 ± 0.51	1.23 ± 1.01	2.77 ± 0.64*	1.12 ± 0.32
$Y_{Lac/Glc}$	1.16 ± 0.01	1.26 ± 0.06*	1.00 ± 0.00*	1.05 ± 0.00*	1.26 ± 0.00*	1.35 ± 0.00*	1.15 ± 0.00	1.23 ± 0.00*	1.18 ± 0.00	1.08 ± 0.00*
q_{Mab}	30.3 ± 1.02	43.5 ± 4.36	44.3 ± 1.54	70.0 ± 0.63	35.7 ± 9.21	42.3 ± 7.70	46.4 ± 2.69	29.2 ± 1.90	42.6 ± 1.95	55.6 ± 3.00

* Pair of means significantly different to the control

5.4.2 Comparison of the effect of MGU on NS0-IgG1 and CHO EG2-hFc Mabs' galactosylation.

The following is a comparison of NS0-IgG1 and CHO-EG2-hFc's *N*-glycosylation analysis when cultured in the presence of galactosylation precursors. For this purpose, NS0 and EG2 cells were inoculated at $2.5x10^5$ cells/mL in media containing M, Urd and Gal (**Figure 5.1**). Both protein A purified Mabs were incubated with PNGase F digestion for *N*-glycans release and then glycans were 2-AB labeled prior to analysis by HILIC chromatography. **Figure 5.9** shows a comparison of *N*-glycans found in NS0-IgG1 and CHO EG2-hFc Mabs which consisted on entirely complex bi-antennary structures, core fucosylated but with different Gal and terminal sialylation content. As previously mentioned, IgG1 Mab consisted of a glycan distribution of G1>G2>G0 in comparison to EG2-hFc Mabs with G0<G1<G2 in Biogro™ media without additives. Thus, a difference in GI was observed between antibodies, with a of GI=0.58 ± 0.03 and a GI= 0.68 ± 0.05 in IgG1 Mab and EG2-hFc Mab respectively. Incorporation of 7μM M induced a significant increase of up to 8.5% in GI (GI=0.63 ± 0.01) in comparison to NS0 cells control sample (GI=0.58 ± 0.03) (p-Value 0.0029). In contrast, M- supplemented CHO EG2 cultures did not show have a significant difference in EG2-hFc Mab's GI (0.65 ± 0.03) when compared to the control (**Table 5.2**). With respect to M+Urd_1/Urd_2– containing NS0 cultures, a significant increase of up to 22.4% (p-Value < 0.0001) was observed in the GI in comparison to the control. Increasing Urd concentration from 2.5 mM (Urd_1) to 5 mM (Urd_2) did not cause a significant effect on galactosylation with a GI= 0.70 ± 0.02 and GI=0.72 ± 0.01 respectively. Thus, the effect of only 5 mM Urd in combination with M was tested on EG2 cells which was also responsible for a

significant increase of about 27.7% in GI in comparison to EG2-control cultures (p-Value 0.001; **Table 5.2**).

The highest GI value was observed in NS0 cultures containing M+$Gal_{1/2}$ (GI= 0.78 ± 0.0) with a significant increase of about 33% with respect to the control (p-Value < 0.0001) **(Table 5.2)**. In addition, M+$Gal_{1/2}$ showed a significantly higher GI with respect to all NS0-cultures except M+Urd1+Gal1 (p-Value < 0.05). In contrast, EG2-cultures also showed the highest increase in GI = 0.81 ± 0.01 in M+Gal_2, which represents a 31% increase when compared to untreated cultures (p-Value 0.0009). These results suggested that M and Gal could be interacting with each other in a manner that a further increase in GI is observed when combined. To test if there is in fact a synergistic effect, cells were cultured in media containing: 7 μM M; 20 mM galactose (Gal_2)or 7 μM M plus 20 mM Gal (M+Gal_2). In NS0 cells, an increase of 17.2% (GI = 0.69±0.02; p-Value 0.0004) and of 29% (GI= 0.75 ± 0.00; p-Value < 0.0001) was observed in M- and Gal- cultures respectively. NS0 cultures supplemented with both additives (M+Gal) showed an even higher increase in the GI than when the supplements were alone, to up to 34% in comparison to the control (GI= 0.78 ± 0.01; p-Value < 0.0001; **Table 5.2)**. Accordingly, Gal- EG2 containing also showed a significant increase of 17% in GI in comparison to the control. Furthermore, EG2 cells cultured in the presence of both supplements, M+Gal_2, showed an even higher increase in GI (up to 31%) when compared to the control (p-Value 0.0429). Thus, a significant synergistic effect was observed in the increase of GI in both Mabs in cultures supplemented with M+Gal_2- than M and control cultures (**Table 5.2**). Finally, addition of Urd along with M and Gal (M+Urd_1/Urd_2+Gal_1/Gal_2) also increased the GI to up to 27% (GI=0.74 ± 0.01; p-Value < 0.0001) in NS0 cells. In contrast, EG2-hFc Mabs showed a significant increase of up to 29% in cultures containing M+Urd_2+Gal_2

respectively when compared to the control. Both Mabs showed that M+Urd+Gal cultures could not further increase the GI observed in M+Gal containing cultures.

The significant increase in GIs observed in all supplements came from a decrease in the amount of glycans containing zero and one Gal residue (G0, G1) along with an increase in glycans containing two Gal residues (G2) as shown in **Table 5.3**. The highest increment in G2 was observed in cultures containing M+Gal_1 and M+Gal_2 with 76.1% and 76.3% respectively followed by cultures containing M+Urd+Gal with an increase of up to 59% in comparison to the control. When examining the synergistic effect of M and Gal (**Table 5.3**), cultures containing M alone showed a decrease of G0-glycans of about 45.5% along with an increase of 27% in G2 glycans in comparison to the control (p-Value < 0.0001), while in the presence of Gal, a significant decrease of 62.9% in G0 (p-Value < 0.0001) and 16.2% in G1 (p-Value 0.0002) containing glycans was observed along with a significant increase of 52% of G2 glycans (p-Value <0.0001). Finally, cultures with M and Gal showed the most significant decrease of 68% and 26% in G0 and G1 containing glycans respectively (p-Value <0.0001; p-Value <0.0001) along with a significant increase of 64% in G2 glycans (p-Value <0.0001). With respect to EG2-hFc Mabs, the similar effect was observed in a reduction in G0 and G1- glycans along with an increase in G2- glycans (**Table 5.3**). For instance, M+Gal_2-containing EG2 cultures seemed to influence G0 and G1-glycans represented by the significant decrease of up to 62.0% and 30.9% in G0 and G1 (p-Value 0.001) respectively along with a significant increase of up to 49.6% in G2 glycans (p-Value 0.008) when compared to untreated cultures. With respect to Urd containing cultures, a biggest influence was observed over G0- than G1- glycans, with a reduction of up to 67.1% and 11.2% (p-Value

<0.05). However, G2-glycans increased to up to 42.2% when compared to the control (p-Value 0.001).

These results show that M, Urd and Gal could increase the GI of both NS0-IgG1 and EG2-hFc Mabs. The increment observed in GIs was a result of a significant increase in glycans containing two Gal residues along with a decrease in glycans containing zero or one Gal residues. Thus, an increment of up to 33%, 22.4% and 27% where observed in NS0-cultures containing M+Gal_2, M+Urd_2 and M+Urd_2+Gal_2 respectively in comparison to the control. In contrast, EG2-hFc Mabs showed also a significant increase in the GI of up to 31%, 27.7% and 29% in cultures containing M+Gal_2, M+Urd_2 and M+Urd_2+Gal_2 respectively in comparison to untreated cultures. Finally, a synergistic effect was observed in the increase of GI in both Mabs in cultures supplemented with M+Gal_2- than M and control cultures.

5.4.3 Comparison of the effect of M, Urd and Gal on NS0-IgG1 and CHO EG2-hFc Mabs' sialylation.

N-glycans terminating in Gal are required for sialylation to occur. NS0-IgG1 Mabs sialylation was determined after culturing NS0 cells in Biogro™ media containing M, Urd and Gal (**Table 5.2)**. NS0-IgG1 showed 14.4% ± 5.27 of sialylation in the control culture, constituted by the following glycans: F6A2G2S1, F6A2G1S1, A2G2S1, F6A2G2S2, F6A2G2Ga1S1 and F6A2G2Ga1S2. In contrast, CHO-EG2 cells cultured in Biogro™ media without any supplement showed a sialylation of 17.9% ± 0.22 which corresponded to mainly two glycans: F6A2G2S1and F6A2G2S2 **(Figure 5.9)**. A significant increase of up to 40% and 71% was observed in NS0

cultures supplemented with M- and M+Urd_2 respectively (p-Value 0.026; <0.001) in comparison to untreated cultures. In contrast, EG2-hFc Mabs' sialylation was not influenced by M-supplementation. However, a significant decrease of up to 23.5% was observed in media containing M+Urd_2 when compared to the EG2-control cultures. The highest Sia increase (up to 142.4% and 62%) in sialylation was observed in M+Gal_2 NS0 and EG2 cultures respectively (p-Value <0.0001). Finally, M+Urd+Gal showed a significant increase of up to 86.8% in NS0-IgG1. In contrast, EG2-hFc Mabs showed a significant reduction of up to 15.6% in Sia content in M+Urd_2+Gal_2 cultures (p-Value<0.0001). The latter suggests that Urd had a negative effect over sialylation in EG2-hFc Mabs. Finally, the previous synergistic effect of M and Gal observed on GI of NS0 IgG1 Mabs was also observed in sialylation, with a significant increase of up to 40%, 120.1% and 142.4% in NS0 cultures supplemented with M-, Gal- and M+-Gal respectively. In EG2 cells, the synergistic effect of M+Gal was also observed in EG2-hFc sialylation, represented by an increase of 3%, 27.4% or 62% in cultures supplemented with M-, Gal-, or M+Gal respectively.

In summary, the increase in GI observed in NS0-IgG1and EG2-hFc Mabs provided with available sites for sialyltransferase to add Sia. Thus, the highest percent of sialylation was observed in M+Gal cultures, with an increase of up to 142.4% and 62% in IgG1 and EG2-hFc Mab respectively. Feeding M, Gal and Urd together did not further increase the sialylation previously observed in M+Gal cultures in both Mabs. Urd affected IgG1 differently than EG2-hFc Mabs. For instance, a significant increase of up to 70.8% and 86.8% was observed in M+Urd and M+Urd+Gal- containing NS0-cultures respectively. In contrast, a significant decrease of up to

23.5% and 15.6% was observed in sialylation in M+Urd and M+Urd+Gal- containing EG2 cultures respectively.

5.4.4 Improvement in galactosylation and sialylation results from an increase in nucleotide sugar pools.

Changes in galactosylation and terminal sialylation were observed in both NS0-IgG1 and EG2-hFc Mabs. After observing that NS0 cells showed the best improvement in galactosylation and sialylation, in this section the intracellular levels of nucleotides and nucleotide sugars were analyzed in NS0 cells cultured in media containing M, Gal, Urd alone or a combination of these supplements. For this purpose, $1x10^7$ cells were harvested on day four and rapidly quenched using 60% methanol plus AMBIC to stop cells' metabolism. Then, the metabolites were extracted using solid-phase extraction cartridges, recovered and separated by ion-pair reversed phase high-performance liquid chromatography (RP HPLC). Nucleotide sugar and nucleotides were identified by comparison with the retention time of a standards mixture and quantified using GDP-Glc as an internal standard. Nucleotides were evaluated individually and as a pool (i.e Urd pool = UMP + UDP +UTP; Ade pool = AMP + ADP + ATP; Cyt pool = CMP+CDP+CTP and Gua pool = GMP + GDP + GTP). Nucleotide sugars were defined as follows: UDP-Hex = UDP-Gal *plus* UDP-Glc; UDP-GNAc = UDP-GlcNAc *plus* UDP-GalNAc. The gradient and eluent composition used yielded the co-elution of UDP-GalNAc+CTP.

Figure 5.10 shows the concentrations of nucleotides from NS0 cells cultured in the presence of M, Urd and Gal. Nucleotide monophosphates were observed at higher concentrations

than nucleotide di-phosphates and tri-phosphates in all cell cultures, including the control. **Figure 5.10A** shows a significant increase of 2-fold in the Cyt pool in Urd, M+Gal and M+Gal+Urd cultures in comparison to the control (1.17 ± 0.41 fmole/cell; p-Value ≤ 0.04). When evaluated individually, CMP content was higher by about 2-fold in Urd, M+Gal and M+Gal+Urd cultures in comparison to the control (0.92 ± 0.32 fmole/cell; p-Value ≤0.01). CDP levels were also higher in Urd, M+Gal and M+Gal+Urd cultures, but just the latter was significantly higher with a ~5-fold increase in comparison to the control (0.25 ± 0.09 fmole/cell; p-Value 0.01). CTP levels were not possible to define as this nucleotide's peak eluted with UDP-GalNAc. CMP-Neu5Ac content was not affected by any of the supplements, remaining close to the content found in the control sample (0.2 ± 0.37 fmole/cell).

The Urd-pool content found on untreated control cultures was 4.5 ± 0.79 fmol/cell. Incorporation of Urd or in combination with M and Gal (M+Gal+Urd) increased significantly the Urd-pool value previously found by ~2.5 fold (p-Value ≤0.0087) (**Figure 5.10B**). The Urd- pool was unaffected in cultures containing Gal, remaining comparable to the control with 3.40 ± 0.71 fmole/cell. However, incorporation of Gal along with M showed the opposite effect, increasing the Urd-pool by ~1.5 fold. UTP content was almost negligible in all cell cultures and without any significant difference. Urd, M+Gal and M+Gal+Urd cultures showed an increase in the content of UMP, with Urd and M+Gal+Urd cultures being 2-fold higher than the control sample with 3.49 ± 0.69 fmole/cell (p-Value ≤0.02). Gal -fed cultures showed comparable amounts of UMP and UDP with respect to the control. The amount of UDP in M+Gal+Urd cultures was significantly higher by 3 times when compared to untreated cultures.

Figure 5. 9 Comparison of N-glycosylation profiles from NS0-IgG1 Mab and EG2-hFc Mab produced in Biogro™ media supplemented with 0.5g/L yeast extract.

Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Glycan structures were assigned based on GU values previously reported in the NIBRT Glycodatabase. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). GI values correspond to the mean ± SD of duplicates (n = 2).

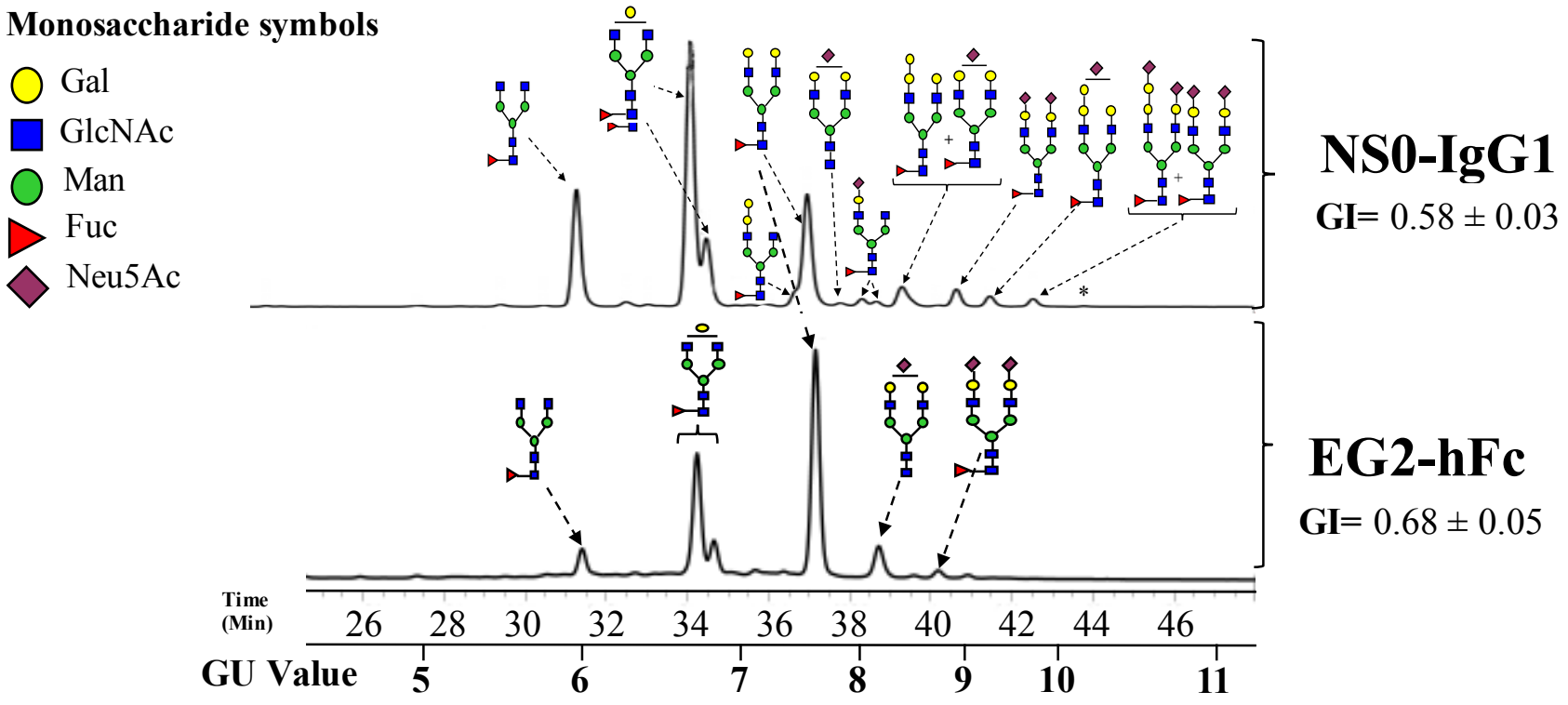


Table 5. 2 GI and percentage sialylation from NS0-IgG1 and CHO EG2-hFc samples cultured in Biogro™ media supplemented with galactosylation precursors

7 μM M; 2.5 Urd_1 or 5 mM Urd_2); 10 mM Gal_1or 20 mM Gal_2) or a combination of these supplements (M+Urd; M+Gal; M+Urd+Gal). Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). GI values correspond to the mean ± SD of duplicates of two independent experiments (n = 4). *Pairs of means ± SD significantly different (p-Value ≤0.05*; <0.001**; <0.0001***) with respect to the control culture. Upward diagonal filled cells were not determined for EG2 cells.

Culture conditions	NS0-IgG1	CHO EG2-hFc	NS0-IgG1	CHO EG2-hFc
	Galactosylation Index (GI)		Sialylation (%)	
Control	0.58 ± 0.03	0.62 ± 0.07	14.4 ± 5.27	17.9 ± 0.22
M	0.63 ± 0.01*	0.65 ± 0.03	20.2 ± 0.73**	18.5 ± 2.72
M+Urd_1	0.70 ± 0.02***		23.4 ± 0.41**	
M+Urd_2	0.72 ± 0.01***	0.79 ± 0.01*	24.6 ± 0.63**	13.7 ± 2.85*
Gal_2	0.75 ± 0.00***	0.73 ± 0.03	31.7 ± 0.63***	22.8 ± 1.43*
M+Gal_1	0.78 ± 0.01***		32.8 ± 3.92***	
M+Gal_2	0.78 ± 0.01***	0.81 ± 0.01*	34.9 ± 1.03***	29.0 ± 0.53**
M+Urd_1+Gal_1	0.74 ± 0.01***		26.9 ± 0.80***	
M+Urd_1+Gal_2	0.73 ± 0.02***		25.2 ± 1.85***	
M+Urd_2+Gal_1	0.71 ± 0.04***		19.6 ± 6.01*	
M+Urd_2+Gal_2	0.74 ± 0.02***	0.80 ± 0.00*	24.4 ± 1.82**	15.1 ± 0.45

Table 5. 3 Comparison of relative abundance of G0- , G1- and G2-containing glycans from NS0-IgG1 and CHO EG2-hFc produced in media containing galactosylation precursors.

Biogro™ media supplemented with: 7 μM M; 2.5 Urd_1 or 5 mM Urd_2; 10 mM Gal_1 or 20 mM Gal_2 or a combination of these supplements (M+Urd; M+Gal; M+Urd+Gal). Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). GI values correspond to the mean ± SD of duplicates of two independent experiments (n = 4). *Pairs of means ± SD significantly different (p-Value ≤0.05*; <0.001**; <0.0001***) with respect to the control culture. Upward diagonal filled cells were not determined for EG2 cells.

Culture conditions	**NS0-IgG1 Mab**			**CHO EG2-hFc Mab**		
	G0	**G1**	**G2**	**G0**	**G1**	**G2**
Control	17.8 ± 1.41	47.2 ± 3.29	35.0 ± 4.38	23.5 ± 6.91	29.1 ± 0.31	47.4 ± 7.11
M	15.1 ± 0.62*	43.3 ± 1.66*	41.6 ± 1.25*	22.1 ± 2.11	26.0 ± 0.86	51.9 ± 2.97
M+Urd_1	9.98 ± 1.23***	40.5 ± 1.25**	49.6 ± 2.46***			
M+Urd_2	8.64 ± 0.18***	39.5 ± 0.42**	51.9 ± 0.59***	7.73 ± 0.93***	25.8 ± 1.04*	66.4 ± 1.97***
Gal_2	8.03 ± 0.32***	33.2 ± 0.33***	58.8 ± 0.61***	16.1 ± 2.51*	23.0 ± 1.18***	61.0 ± 3.69*
M+Gal_1	6.49 ± 0.74***	32.0 ± 0.67***	61.6 ± 1.18***			
M+Gal_2	6.76 ± 0.45***	31.6 ± 0.12***	61.6 ± 0.57***	8.93 ± 0.80***	20.1 ± 0.21***	71.0 ± 1.01***
M+Urd_1+Gal_1	7.67 ± 0.88***	36.8 ± 0.61***	55.5 ± 1.46***			
M+Urd_1+Gal_2	8.12 ± 0.74***	37.2 ± 1.63***	54.7 ± 2.29***			
M+Urd_2+Gal_1	8.98 ± 1.15***	40.8 ± 5.16**	50.2 ± 5.70***			
M+Urd_2+Gal_2	8.11 ± 1.75***	36.6 ± 1.16***	55.3 ± 1.95***	8.01 ± 0.39***	24.5 ± 0.41**	67.5 ± 0.80***

The Ade-pool content in control culture (8.55 ± 2.30 fmole/cell) was comparable to Urd, Gal and M+Gal+Urd cultures (**Figure 5.11A)**. However, the Ade-pool content increased 1.5-fold in M+G cultures in comparison to the control. When looking at AEC, growing cells are expected to have values over 0.8. NS0 cultures showed AEC values <0.2, which indicates that cell metabolism was not properly stopped at the moment of extraction. As a result, quenching of NS0 cells did not prevent metabolite interconversions. The latter indicates that the extraction protocol failed on preventing metabolite interconversions and as a result higher concentrations of monophosphate nucleotides are observed in Figure 5.10 and 5.11. AMP content was the lowest in Urd -fed cultures (p-Value 0.01) with a decrease of 52% in comparison to the control. No significant difference was observed in AMP content in Gal and M+Gal+Urd cultures. With respect to ADP and ATP, comparable amounts were found in Urd, Gal, and M+Gal cultures, and slightly but significantly higher in M+Gal+Urd cultures (p-Value 0.02).

With respect to the Gua-pool, Urd, M+Gal and M+Gal+Urd cultures had comparable amount with the control culture content (~3.63 ± 0.57 fmole/cell) (**Figure 5.11B**). In contrast, Gal-fed cultures showed the lowest Gua-pool with a decrease of 30% with respect to the control. This drop resulted from a reduction in GMP levels (p-Value 0.086). No significant differences were detected with GMP in Urd, M+Gal and M+Gal+Urd cultures. However, a significant increase of 1.4-fold and 3-fold in GDP and GTP content respectively was observed in M+Gal+Urd cultures in comparison to untreated cultures with 0.70 ± 0.1 and 0.12 ± 0.03 fmole/cell respectively (p-Value ≤0.04).

As shown in **Figure 5.12A**, addition of 5 mM Urd caused a ~3-fold increase in the total UDP-Hex content of NS0 cells from untreated cultures (1.36 ± 0.50 fmole/cell; p-Value 0.0002). UDP-Gal and UDP-Glc content was evaluated separately and these nucleotides were 2.5 times and 3 times respectively in comparison to the control with 0.40 ± 0.20 fmole/cell (UDP-Gal) and 0.96 ± 0.32 fmole/cell (UDP-Glc) (p-Value ≤0.0025). Cultures containing M+Gal+Urd did not show further increase in the amount of UDP-Gal and UDP-Glc previously found when Urd was supplemented alone. Thus, M+Gal+Urd cultures had a comparable amount of UDP-Hex with Urd cultures. Addition of 20 mM Gal caused a 3-fold increase in the total UDP-Hex content compared with the control. Despite that UDP-Glc content was not affected, there was a 3.5-fold increase in the UDP-Gal content in Gal -fed cultures (p-Value 0.0001).

A synergistic effect was observed when M+Gal were added together, showing a 2.7-fold increase in the UDP-Hex content in comparison to the control and a 1.6-fold increase in comparison to Gal cultures (p-Value 0.0004). Furthermore, UDP-Gal and UDP-Glc content represent a 5- and 3.5 times increase in M+Gal cultures over the control sample (p-Value <0.0001; 0.02). It is noticeable that the proportion of UDP-Gal was higher with respect to UDP-Glc in Gal-supplemented cultures, opposite to what was observed in the control and Urd-containing cultures where UDP-Glc content was higher than UDP-Gal. These results show that there was an increase in the UDP-Gal pool at the expense of UDP-Glc when Gal was added to the culture media. Finally, M+Gal+Urd cultures where comparable to Urd cultures, showing a significantly higher UDP-Hex content with a 2.6-fold increase with respect to the control (p-Value 0.0003). Thus, cellular levels of UDP-Gal and UDP-Glc were elevated 2.7- and 1.75-fold in comparison to the control. UDP-GNAc pool was unaffected by any of the supplements tested (**Figure 5.13B**). Thus, M, Urd and

Gal treated cultures had a comparable amount of UDP-GNAc with the control (10.1 ± 2.69 fmole/cell). Gal containing cultures had a slightly lower content of UDP-GNAc with 8.55 ± 0.23 fmole/cell, but was not significant with respect to the control. The proportion of UDP-GlcNAc was higher in all cultures with respect to UDP-GalNAc, with UDP-GlcNAc constituting up to 78% of the total UDP-GNAc pool.

These results show that the changes observed in glycosylation are a response of a change in the intracellular concentrations of nucleotides and nucleotide sugars, depending on the feeding strategy used. Urd-containing cultures showed an increase in the content of Urd- nucleotides as well as of UDP-sugars required for an increase in GI (UDP-Gal pool). Gal-feeding alone or in combination with M increased even more the content of the UDP-Gal pool, but Urd and Cyt pools remain lower than in Urd-fed cultures

Figure 5. 10. Effect of galactosylation precursors on the intracellular concentrations of Cyt - and Urd -containing nucleotide and nucleotide sugars of NS0 cells .

A. CMP, CDP, CTP and CMP-Neu5Ac and **B.** UMP, UDP and UTP. Nucleotide amounts are also represented as pools of Cyt and Urd, respectively. NS0 cells were seeded in shake flasks containing Biogro™ media supplemented with: 7 μM M, 5 mM Urd, 20 mM Gal; M+Urd; M+Gal; and M+Gal+Urd and harvested on day 4. Nucleotide sugars were extracted and resolved by anion-exchange chromatography. Values correspond to the mean ± SD of triplicates (n = 3). Pairs of means ± SD significantly different (p-Value ≤0.05*) with respect to the control culture. CTP content are not represented as they co-eluted with UDPGalNAc

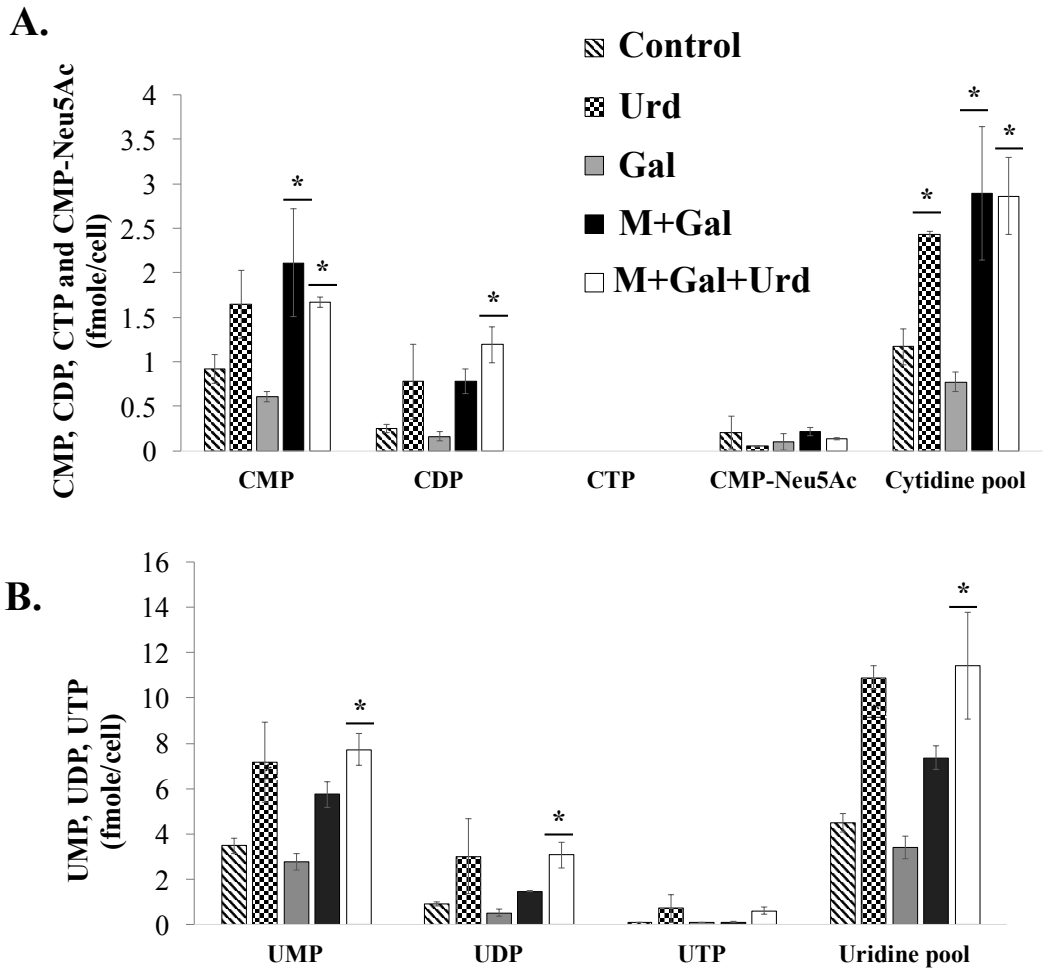


Figure 5. 6. Effect of galactosylation precursors on the intracellular concentrations of Ade- and Gua-containing nucleotide and nucleotide sugars of NS0 cells

A. AMP, ADP and ATP and **B.** GMP, GDP and GTP. Nucleotide amounts are also represented as pools of Ade and Gua nucleotides respectively. NS0 cells were seeded in shake flasks containing Biogro™ media supplemented with: 7 μM M, 5 mM Urd, 20 mM Gal, M+Urd, M+Gal and M+Gal+Urd and harvested on day 4. Nucleotide sugars were extracted and resolved by anion-exchange chromatography. Values correspond to the mean ± SD of triplicates (n = 3). Pairs of means ± SD significantly different (p-Value ≤0.05*) with respect to the control culture.

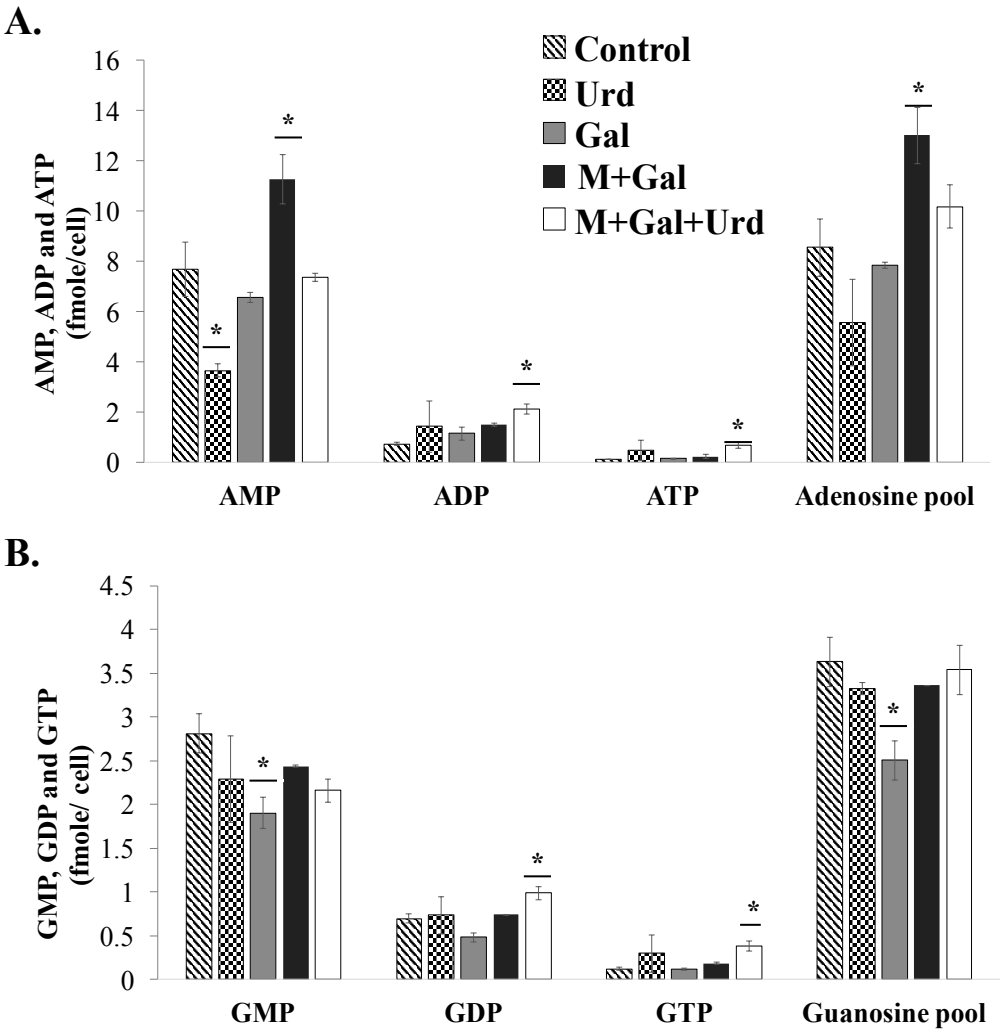
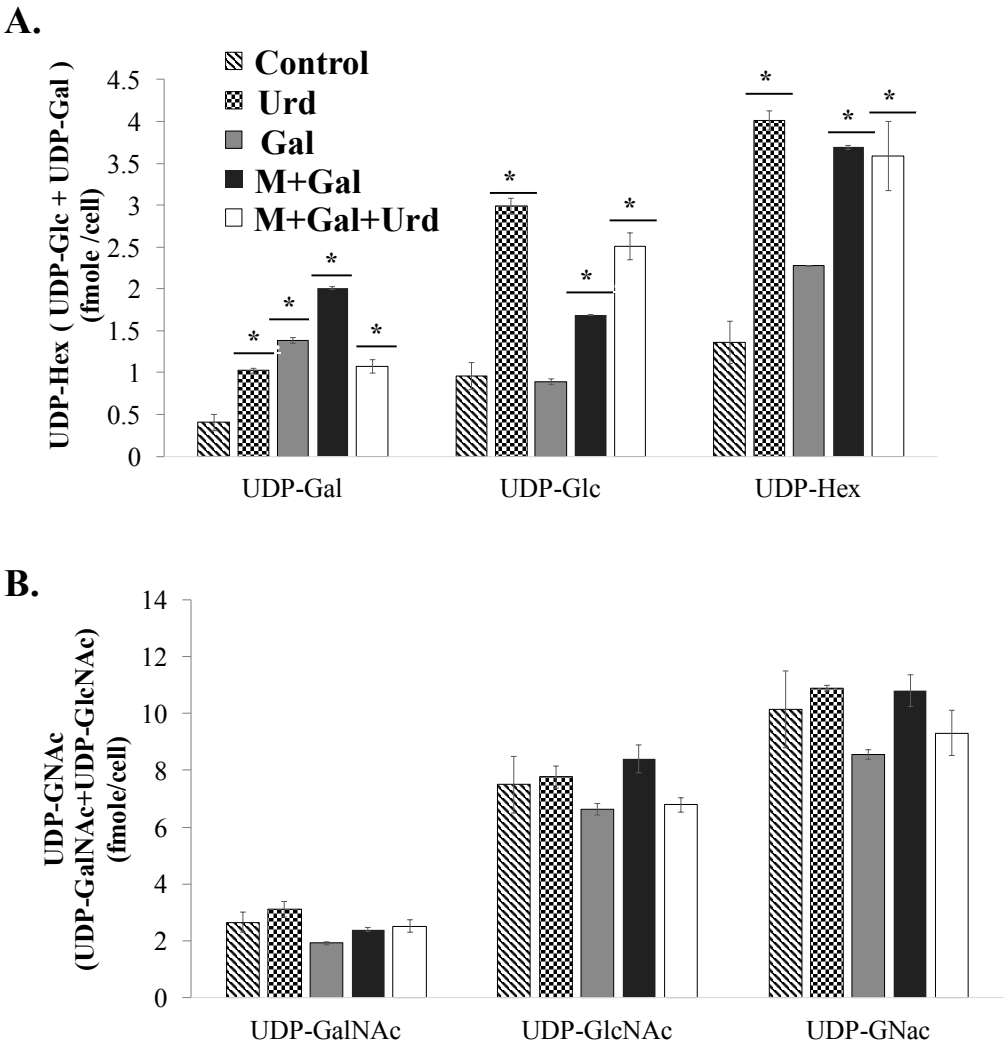


Figure 5. 12. Effect of galactosylation precursors on the intracellular concentrations of UDP-hexosamines and UDP-hexoses of NS0 cells

A. UDP-Hex and **B.** UDP-GNAc pools. NS0 cells were seeded in shake flasks containing Biogro™ media supplemented with: 7 μM M, 5 mM Urd, 20 mM Gal, M+Urd, M+Gal and M+Gal+Urd and harvested on day 4. Nucleotide sugars were extracted and resolved by anion-exchange chromatography. Values correspond to the mean ± SD of triplicates (n = 3). Pairs of means ± SD significantly different (p-Value ≤0.0025*) with respect to the control culture. UDP-Hex corresponds to UDP-Glc plus UDP-Gal and UDP-GNAc to UDP-GalNAc plus UDP-GlcNAc.



5.5. Discussion

Different nutrient supplementation strategies have been focused not only on increasing cell longevity and productivity, but more importantly on assuring comparability between batches with a more consistent glycosylation. Glycan biosynthesis is mediated by glycosyltransferases which are responsible for adding monosaccharides one at a time to specific positions on branched sugar chains. Thus, glycoproteins are first galactosylated and then sialylated. In this chapter, experiments focused on increasing the content of Gal in IgG1 and EG2-hFc Mabs produced by NSO and EG2 cells by culturing them in media containing galactosylation precursors (**Figure 5.14**). The latter was expected to increase the availability of terminal Gal residues required for sialyltransferases to add Sias in the *trans*-portion of the Golgi. Galactosylation precursor- and trace metal- feeding strategies have been previously used to improve glycosylation of different glycoproteins (Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001, Crowell, Grampp *et al.* 2007, Gramer, Eckblad *et al.* 2011). For instance, most of glycosyltransferases are dependent on divalent cations (typically Mg^{2+} and Mn^{2+}) for their activity (Varki A 2009). Thus, manganese was used in the form of $MnCl_2$ to support the activity of β1-4 galactosyltransferase (GalT) responsible for utilizing UDP-Gal as a donor to incorporate Gal to exposed *N*-acetylglucosamine residues. In addition, Urd and Gal were supplemented to NS0 and EG2 cells to increase the abundance of the nucleotide sugar precursor UDP-Gal required by galactosyltransferases.

Different studies have shown that human cells and CHO cells experience a metabolic shift when cultures are exposed to glucose and Gal (Wilkens, Altamirano *et al.* 2011). Under these conditions, Glc becomes the first carbon source to be consumed followed by Gal and lactate

(Altamirano, Paredes *et al.* 2000, Altamirano, Illanes *et al.* 2006, Wilkens, Altamirano *et al.* 2011). The latter is supported by the low uptake rate of Gal in comparison to Glc. In contrast, NS0 and EG2 cell cultures showed only one growth phase in cultures with Glc (control, M, M+$Urd_{1,2}$) and cultures containing Glc and Gal (M+$Gal_{1,2}$, M+$Urd_{1,2}$+$Gal_{1,2}$). The absence of a second growth phase is because NS0 and EG2 cells contained between 35-45 mM of hexose (25 mM Glc + 10 or 20 mM Gal) in comparison to previous studies were Glc was supplemented at low concentrations (2 -6 mM) when combined with Gal (18-14 mM). Thus, the observation of two growth phases in Glc+Gal cultures in previous reports is a response of cells accessing to Gal once Glc was depleted from the media (Altamirano, Illanes *et al.* 2006, Wilkens, Altamirano *et al.* 2011). As a result, culture periods and cell viabilities were longer and higher respectively in Gal-fed cultures (Clark, Griffiths *et al.* 2005, Altamirano, Illanes *et al.* 2006). Based on the results observed for NS0 and EG2 cells, future experiments should be focused on extending the culture period to investigate if Gal -fed cultures can indeed have a longer exponential phase as well as higher cell densities than the ones observed on day 4. Furthermore, it would be interesting to test if NS0 cells are able to consume Gal and lactate once Glc is not available as previously seen in other studies (Altamirano, Paredes *et al.* 2000, Grainger and James 2013). The latter was not possible as our Gal-fed cultures still had residual glucose (~3-9 mM) on day four.

Consumption rates were not affected by the incorporation of M nor by the addition of Gal; however M+Gal cultures accumulated lactate above previously reported inhibitory levels (> 18 mM) (Altamirano, Illanes *et al.* 2006). Combination of M+Urd+Gal caused a more significant drop in cell densities. Indeed, Urd seem to act as a potent inhibitor of NS0 and CHO cells' growth, especially when concentrations increased from 2.5 mM to 5 mM (M+Urd_1 vs. M+Urd_2;

M+Urd_1+Gal_1 vs. M+Urd_2+Gal_1). It has been seen that free Urd reach higher concentrations in tissues (10-fold more) than those present in plasma (1-3 μM) and that concentrations in the millimolar range can produce a concentration-dependent inhibition of cellular proliferation (Sokoloski JA1 1991, Grainger and James 2013). The reduction in cell growth has been related to a reduction in ATP along with an increase of the abundance of UTP (Sokoloski JA1 1991). In NS0 cells, the level of nucleosides triphosphate (NTP) was almost negligible in all conditions because cultures had passed the exponential growth phase. For this reason, the nucleotide pools were considered instead (NMP+ NDP+ NTP). Urd containing cultures showed the lowest Ade-pool (AMP+AMP+ATP) along with the highest content of Urd-pools (UMP+UDP+UTP) in comparison to control cultures and Gal-containing cultures. Finally, Mn^{2+} alone had minimal impact on NS0 cells' growth, but had a significantly higher productivity alone or when combined with Urd or Gal when compared to the control.

Divalent cations were already present in Biogro™ media in the form of $MgCl_2$; however, the concentrations used may not have been the adequate for GalT optimal activity (Crowell, Grampp *et al.* 2007, Grainger and James 2013). In EG2 cells, 7 μM did not have a significant impact when added alone. This result is consistent with Grainger and James, which observed that the trace-metal Mn^{2+} exerted any effect over Mab galactosylation (1μM- 40 μM)(Grainger and James 2013). However, NS0 cells behave differently, as M slightly improved the galactosylation of NS0-IgG1 Mab represented by an increase of 8.6% in GI. However, this increment in GI was more significant in M+Urd (24%) or M+Gal (34.5%); which indicates a synergistic effect between these galactosylation precursors in both cell lines. This is consistent with previous reports where an increase in protein glycosylation resulted from an enhanced intracellular concentration of UDP-

Gal induced by M, Urd and Gal (Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001, Ryll 2006, Wong, Wati *et al.* 2010, Gramer, Eckblad *et al.* 2011). Accordingly, Gal and M+Gal supplementation induced a significant increase of 3.5-fold and 5-fold respectively in the intracellular concentration of UDP-Gal in NS0 cells. However, this increase in UDP-Gal happened at the expense of UDP-Glc where only an increase of 1.75-fold was observed in M+Gal. These results are consistent with Wong *et al.*'s report where a higher increase in UDP-Gal (20- and 25-fold) was observed with respect to UDP-Glc (2- and 8-fold) in Gal- and Gal+Urd cultures respectively. Wong *et al.* did not incorporate M along with Urd and Gal, which may suggest that the main contributors to the increment in UDP-Gal and subsequently in GI is a result of Gal and Urd supplementation.

The data also demonstrate that feeding Gal had no effect over the amount of Urd-pool unlike Urd-fed cultures which showed the highest levels of Urd-nucleotide pool. The latter was consistent with previous studies by Ryll *et al.* where UTP increased by up to 7-fold and 5-fold in cultures with 0.2 mM Urd but not in 11 mM Gal-containing cultures (Ryll 2006). However, having higher concentrations of Urd-pool was not enough to increase galactosylation in Urd-containing cultures as efficiently as Gal-fed cultures. Thus, the concentrations of UDP-Gal in Urd-containing cultures were 73.9% and 50.7% of that of Gal, and M+Gal- supplemented cultures. In contrast, UDP-Glc was the most predominant nucleotide sugar from the UDP-Hex pool in Urd-cultures. Thus, an increase of about 3.3-fold and 1.77- in UDP-Glc concentrations was observed in Urd-cultures with respect to Gal- and M+Gal- containing cultures. Similarly, Wong *et al.* observed a higher increase of UDP-Glc in Urd+Gal cultures than cultures containing only Gal. Thus, the lower GI values Urd-cultures with respect to Gal-containing cultures are a result of lower intracellular

concentrations of UDP-Gal required for galactosylation to occur. Finally, the incorporation of the three galactosylation precursors (M+Urd+Gal) was not as successful in further increasing the galactosylation levels observed in M+Gal cultures. The concentrations of UDP-Gal in these cultures where similar to the ones observed in Urd-cultures. Thus, the lack of higher concentrations of UDP-Gal may be responsible for the lower GI observed in M+Urd+Gal with respect to M+Gal cultures.

With respect to the effect that MGU cocktail has on the sialylation of glycoproteins is contradictory. For instance, Gramer *et al.* observed a minimal impact on sialylation (~1%) in two different cell lines expressing human Mabs cultured in media containing a MGU cocktail (Gramer, Eckblad *et al.* 2011). In contrast, an improvement in sialylation was observed in NS0 and EG2 cultures when M, Urd and Gal were added alone or combined. Thus, the lack of galactosylation sites available for Sia addition was responsible for the lower sialylation observed in NS0 control cultures. Similarly, Schilling *et al.* described galactosylation as a rate-limiting step of product sialylation after improving CHO-produced CTLA4Ig fusion protein's sialylation by Gal-feeding (12.5 g/L)(Schilling, Gangloff *et al.* 2008). Interestingly, Urd supplementation had a different effect on the sialylation of EG2-hFc Mab with respect to NS0-IgG1Mab. Urd seemed to affect negatively the addition of Sia when incorporated alone or in combination with M and Gal, even though an improvement in GI was observed in Urd, M+Urd and M+Urd+Gal cultures. High concentrations of Cyt nucleotides (CMP, CDP and CTP) or/and Urd nucleotides (UMP, UDP and UTP) have shown to inhibit the translocation of CMP-Neu5Ac into Golgi vesicles (Capasso and Hirschberg 1984, Pels Rijcken, Overdijk *et al.* 1995). The latter may suggest that the lower sialylation observed in our Urd cultures could have been a response of an inhibition caused by high

concentrations of Urd and Cyt pools (**Figure 5.13**). In the case of EG2-hFc Mabs produced in media containing M+Gal and M+Gal+Urd, both showed comparable increments in GI. In contrast, Gramer *et al.* observed a synergistic effect in antibody galactosylation when using the three components rather than each component alone (Gramer, Eckblad *et al.* 2011).

Insufficient or inconsistent galactosylation and sialylation of glycoproteins is a big problem during the manufacture of recombinant glycoproteins because non-galactosylated and therefore non-sialylated glycoproteins are rapidly removed from circulation by either the mannose/GlcNAc receptor- or asialoglycoprotein receptor. The latter can compromise the pharmacokinetic properties of a given recombinant glycoprotein. The results of the present study show that the glycosylation machinery of our cell lines may be subject to regulation at the level of nucleotide sugar availability. This was evident when an increment in the intracellular levels and transport of UDP-Gal resulted in an increased in galactosylation in both IgG1 and EG2-hFc Mabs by feeding M, Urd or Gal. The increment observed in sialylation can be attributed to the added number of Gal moieties available for sialylation in NS0 cells, but not for Urd - containing CHO EG2 cultures. The latter is consistent with the fact that different cell lines differ in their requirements for optimal glycosylation and that any media composition optimization needs to be tested individually.

5.6. Conclusions

- Cultures fed with galactosylation precursors reached lower maximal cell densities with respect to untreated cultures.

- The highest specific cell productivities (131%) were observed in Urd containing cultures with respect to the control.

- NS0 cells' adaptation from a PFBM-1 to Biogro™ media induced an improvement in GI from 0.36 to 0.58 respectively.

- M+Gal- containing showed the maximum GI of 0.78 and 0.81 in both IgG1 and EG2 Mabs respectively.

- To increase galactosylation, the combination of 7μM M and 20 mM Gal is preferable than adding each component alone.

- Combination of M+Urd+Gal did not further improve galactosylation observed in M+Gal- containing cultures in either Mab.

- A lower content in UDP-Gal in Urd- cultures resulted in lower GI values with respect to Gal containing cultures.

- In the presence of more Gal-terminal residues, the percentage of sialylation observed in untreated NS0 and EG2 cultures improved significantly in supplemented cultures with some differences between cell lines:

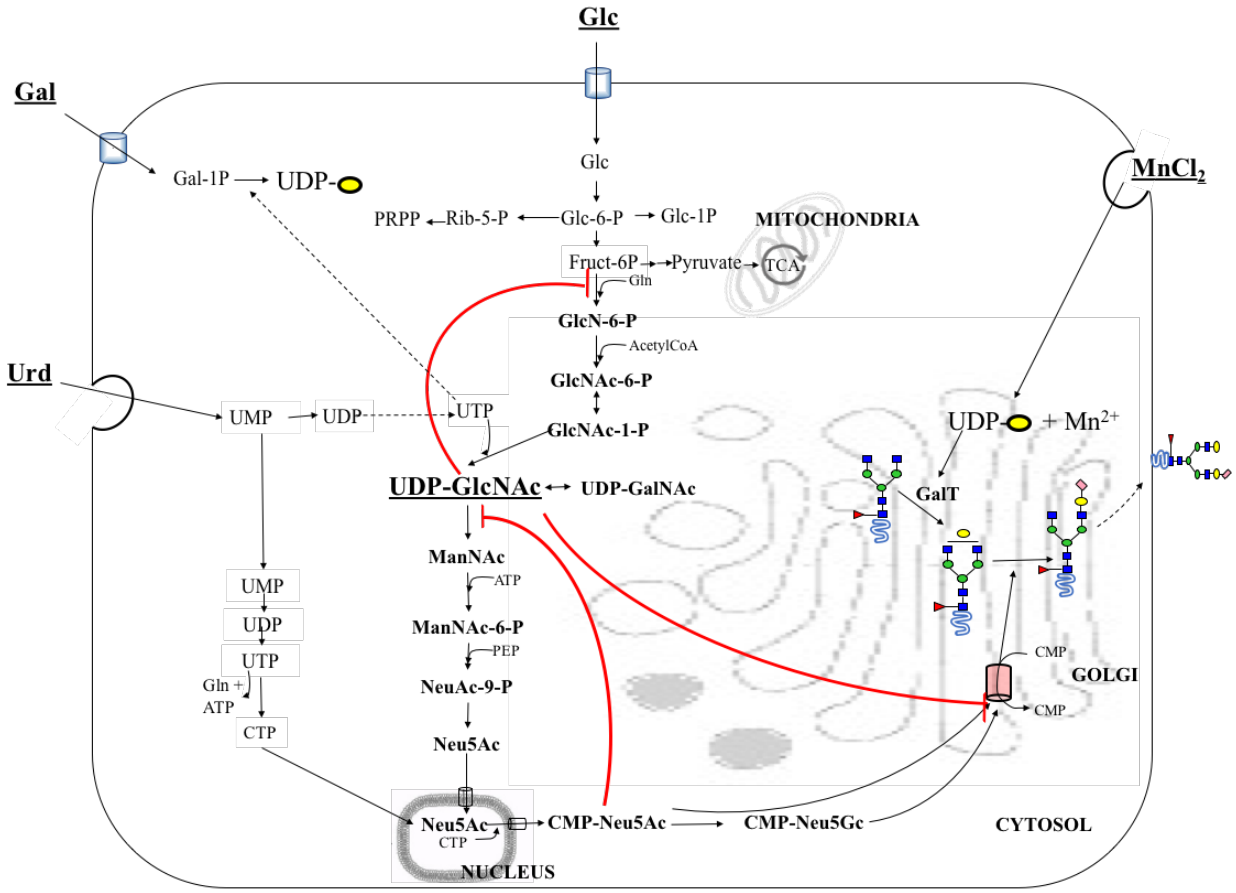
- M+Gal- supplemented cultures showed the highest percentage increase in sialylated glycans (34.9% and 29%) in NS0 and EG2 cells respectively in comparison to untreated cultures.

- Sialylation of IgG1 improved significantly (24-26% of total glycan pool) in Urd-supplemented cultures with respect to NS0 control culture with 14% of sialylated glycans. In contrast, Urd had a negative effect in EG2-hFc Mab with 13% in comparison to control cultures with 17.9% of sialylated glycans.

- Galactosylation precursor supplementation allowed for a greater increase in GI and higher sialylation of NS0 cells compared to CHO cells. However, it is important to consider that NS0

cells incorporate both immunogenic residues Neu5Gc and alpha-Gal. This would limit the use of NS0 in the production of therapeutics with respect to EG2 cells.

Figure 5. 13 Galactosylation precursor feeding strategy used to improve galactosylation and sialylation of IgG1 and EG2-hFc Mabs.
Galactosyltransferases use UDP-Gal as nucleotide sugar donor and Mn^{2+} as a cofactor to transfer Gal to *N*-acetyllactosamine residues (Galβ1-4GlcNAc). Availability of terminal Gal residues allows for the incorporation of Sia by sialyltransferases in the trans-portion of the Golgi.



Chapter 6*

Galactosylation precursor feeding effect on the content of alpha gal and *B*-1,4 galactosylation

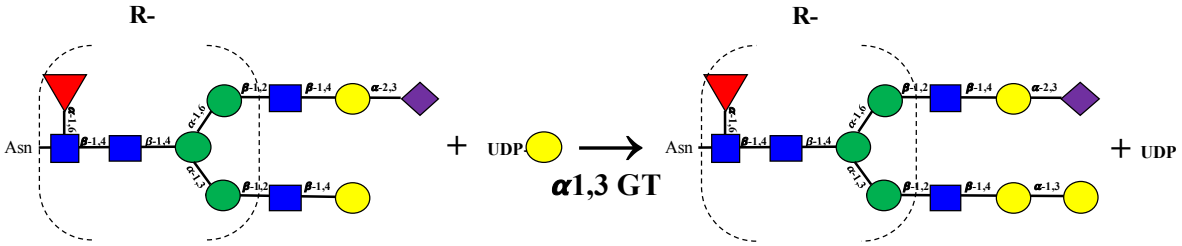
6.1 Introduction

Mammalian cells can produce an array of glycoforms depending on their glycosylation machinery as well as on the expression of specific glycosylation genes. The most commonly used production cell lines to produce monoclonal antibodies are CHO, NS0 and Sp2/0, the same which can introduce sugar residues that are not present in normal serum-derived IgG. The glycan epitopes that can elicit an immunological reaction in humans are the alpha-Gal epitope and the Sia Neu5Gc (Ghaderi, Zhang *et al.* 2012). In this chapter, the focus will be to evaluate the content of the alpha-Gal epitope in NS0-IgG1 Mab. For the content of Neu5Gc in NS0-IgG1 Mab refer to **Section C, Chapters 9-12**.

Despite the common believe that CHO cells lacked the machinery to synthesize the alpha-Gal epitope, the FDA-approved therapeutic glycoprotein produced by CHO cells: Abatacept, Orencia® was shown to contain alpha-Gal residues (Bosques, Collins *et al.* 2010, Ghaderi, Zhang *et al.* 2012). Therefore, CHO cells have a functional ortholog of *N*-acetyllactosaminide-3-α-galactosyltrasferase-1 (α1,3GT) (Bosques, Collins *et al.* 2010, Lammerts van Bueren, Rispens *et al.* 2011). In contrast, for the CHO-derived EG2-hFc Mab, the level of alpha-Gal containing

* Data presented in this chapter has not yet being published.

glycans was below the limit of quantification, which lead us to analyse NS0-IgG1 Mab instead. The α1,3GT enzyme is localized in the *trans*-Golgi network where it uses UDP-Gal as a sugar donor and either glycosphigolipids or glycoproteins (R-) carrying *N*-acetyllactosamine residues ($\mathrm{Gal}\beta 1-4\mathrm{GlcNAc}-\mathrm{R}$) as sugar receptor as shown in the following reaction (Galili 2001, Macher and Galili 2008):



Humans, Old World monkey and apes have lost expression of the gene encoding for α1,3GT and instead humans and higher primates produce antibodies against alpha-Gal residues (Salama, Mosser *et al.* 2017). It is believed that expression of alpha-Gal epitope was suppressed in the ancestral lineages of Old World primates less than 30 million years ago before the divergence of apes and Old World monkeys (Galili, Shohet *et al.* 1988). The titers against the alpha-Gal epitope have been shown to reach as much as 1% of the total human circulating antibodies and its ubiquitous presence throughout life suggests a constant antigenic stimulation which may originate from the intestinal flora bearing alpha-galactosyl groups on their cell wall (e.g. *E. coli* and Shigella) (Galili, Rachmilewitz *et al.* 1984, Ghaderi, Zhang *et al.* 2012). Thus, anti alpha-Gal antibodies have been considered for years as the first barrier to successful transplantation of pig organs into humans (referred to as xenotransplantation) (Macher and Galili 2008). With respect to therapeutic glycoproteins, alpha-Gal containing glycoforms have shown to adversely affect both the safety and half-life of the final product. The best example is the FDA-approved monoclonal antibody

Erbitux (Cetuximab), produced by SP2/0 cells for the use in colorectal cancer and squamous-cell carcinoma of the head and neck (van Bueren, Rispens *et al.* 2011). Cetuximab was associated with IgE-mediated anaphylactic responses within minutes after exposure to terminal alpha-Gal residues (up to 30%) mainly localized in the Fab portion of the heavy chains (Steinke, Platts-Mills *et al.* 2015, Commins, Jerath *et al.* 2016, Zhang, Woen *et al.* 2016). Interestingly, a connexion between the maximum incidence of rocky mountain spotted fever and hypersensitivity reactions to Cetuximab were registered in a group of southern US states (Commins, Jerath *et al.* 2016). Thus, tick bites were considered as the primary source for IgE antibodies against alpha-Gal. In addition, similar but delayed hypersensitivity responses were observed after 3-6 hours of eating meat carrying the alpha-Gal epitope (e.g. beef, pork, lamb), making alpha-Gal a relevant food allergen (Commins and Platts-Mills 2009). These delayed allergic reactions induced by eating read meat were also believed to be enhanced by previous tick bites (Commins and Platts-Mills 2009, Commins and Platts-Mills 2013). In fact, it was found that ecto-parasitic bites increased the level of anti alpha-Gal IgE antibodies by 20-fold (Zhang, Woen *et al.* 2016).

The α1,3GT expression has shown to be higher in mouse cell lines (e.g. C127, Itk-, NS0, Sp2/0 and hybridoma cells) in comparison to hamster cell lines (e.g. CHO, BHK-21). The latter makes mouse cell line products more likely to induce an antigenic response than those from CHO cells (Gadgil 2017). In addition, the extent of alpha-Gal containing glycoforms relies on the competition between α1,3GT and α2,3 /α2,6 ST enzymes to cap the carbohydrate chain with either alpha-Gal or Sia respectively. Furthermore, α1,3GT and α2,3 /α2,6 ST enzymes' activity may change depending on culture conditions (van Bueren, Rispens *et al.* 2011). For these reasons, IgG1

Mabs produced in media containing galactosylation precursors were analyzed on their content of alpha-Gal epitope.

6.2 Objectives

Even though the proportion of alpha-Gal epitope required to induce a hypersensitive reaction is not known, it has become mandatory to minimize the content of alpha-Gal epitopes during biotherapeutics development and production. For this reason, the focus of this chapter was to:

- Determine the content of alpha-Gal residues in IgG1 Mab produced by NS0 cells.
- Evaluate the effect of galactosylation precursors on the relative abundance of alpha-Gal epitope in IgG1 Mab produced by NS0 cells.

6.3 Experimental set up

To evaluate the effect of galactosylation precursors over the content of alpha-Gal residues in IgG1 Mab produced by NSO cells, Biogro™ media was supplemented with M=7 μM, Urd_1 = 2.5 mM; Urd_2 = 5 mM) and Gal_1 = 10 mM; Gal_2 = 20 mM. For more detail regarding cell culture parameters refer to **Chapter 5**. Cell culture was terminated on day four where cells were spun down and supernatant was collected, filter and Mab was protein A purified prior to glycosylation analysis. Alpha-Gal glycans were determined by sequential exoglycosidase digestion, particularly digestion with the coffee bean alpha-galactosidase which has been commonly used to cleave the non-reducing terminal Gal residues linked α1-3,4,6 to glycoproteins. Comparison of retention /

elution times of the exoglycosidase digestions products with an oligosaccharide ladder allowed the calculation of the migration shifts after cleavage. For more detail about the treatment of Mab with exoglycosidases refer to the material and methods section (See **Chapter 2.12.5**). The relative abundance of alpha-Gal residues was determined by addition of the area under the peaks corresponding to F6A2G1**Ga1,** F6A2G2**Ga1,** F6A2G2**Ga1**S1 and F6A2G2**Ga1**S1/S2. However, the peaks corresponding to F6A2G2Ga1 and F6A2G2Ga1S1/S2 overlapped with GU values of F6A2G2S1 and F6A2G2S2 respectively. Thus, consecutive exoglycosidase digestions were performed to determine the real amount of alpha-Gal (**Figure 6.1**) vs. beta-Gal containing glycans **(Figure 6.2).** The consecutive digestions shown in **Figure 6.1** and **6.2** allowed the calculation of glycans containing beta-Gal residues by using the following equation:

$$G0 + (G1 + G2) - (G1 + Ga1) = 100\ \%\ \text{glycan pool}$$

where non-galactosylated glycans (G0) were taken after digestion with neuraminidase plus alpha-galactosidase (**Figure 6.2**). G1 and G2 correspond to glycans containing one and two Gal residues including glycans containing G1+Ga1 after alpha-galactosidase digestion. For this reason the contributing value of alpha-Gal (G1+Ga1) was subtracted to determine the amount of beta-Gal. The 100% glycan pool refers to the total of glycans after neuraminidase digestion, which gathers glycans with zero, one and two Gal residues.

6.4 Results

6.4.1 Effect of MGU cocktail on the content of alpha-Gal

Previously, M, Urd and Gal were evaluated in their ability to improve NS0-IgG1 *N*-linked glycosylation. An improvement in galactosylation of up to 8.5%, 23%, 33% and 27% was observed in cultures containing M, M+Urd, M+Gal and M+Urd+Gal respectively in comparison to the control (**See Chapter 5**). This increment in Gal-containing glycans could also mean an increase in alpha-Gal residues commonly present in NS0 cell lines. Our NS0 cell line was not different and showed the alpha-Gal residues presented in **Table 6.1.**

A GU value was assigned to each peak by entering the corresponding retention times into a 5th polynomial equation previously fit with a standard dextran ladder. Then the GU values were used to assigned a structure to each peak based on comparison to previously published data provided by the Glycobase database. Individual peaks were further confirmed by exoglycosidase digestion. **Figure 6.1** and **6.2** shows the detailed exoglycosidase cleavage sites of previously labeled glycans when incubated overnight with neuraminidase and galactosidase to determine the content of alpha-Gal vs. beta-Gal glycans. Thus, neuraminidase of *Clostridium perfringens* which releases α2-3,6,8–linked non-reducing terminal Sias and galactosidase from *Streptococcus pneumoniae* which hydrolyzed the non-reducing terminal Gal with β1-4 linkages. Digested glycans were collected and prepared for injection onto the HPLC (**See section 2.11.5**). The aforementioned consecutive exoglycosidase digestion gathered alpha-Gal residues in one peak, F6A2G1Ga1 allowing us to determine the relative abundance of glycans containing the alpha-Gal epitope (**Figure 6.1**). In contrast, glycans digestion with neuraminidase from *Clostridium*

perfringens released (α2-3,6,8–linked non-reducing terminal Sia) plus the alpha-galactosidase from Green coffee bean which hydrolyzed the non-reducing terminal Gal with α1-3 linkages was used to determine the amount of beta-Gal residues (See equation **Section 6.2**).

Table 6.2 shows the results of enzyme digestion array for NS0-IgG1 glycans when cultured in Biogro™ media without additives and when supplemented with 20 mM Gal and with 7μM M plus 20 mM Gal respectively. These cultures where chosen to further investigate the contribution of alpha-Gal vs. beta-Gal. First, addition of α-2-3,6,8 neuraminidase (*Clostridium perfringens*) alone caused the shift of all sialylated structures present on the undigested sample (top profile): A2G2S1 (GU=7.85), F6A2G1S1 (GU=7.76), F6A2G2S1 (GU=8.29), F6A2G2S2 (GU=9.41), F6A2G2Ga1S1/S2 (GU=9.41) and the last sialylated and fucosylated unidentified glycan (*) which migrate mainly to A2G2 (GU=7.02), F6A2G1 (GU=6.71) isomers, F6A2G2 (GU=7.48) or F6A2G2Ga1 (GU=8.27). Subsequently glycans were incubated with α-2-3,6,8 neuraminidase (*Clostridium perfringens*) and β-1,4 galactosidase (*Streptococcus pneumoniae*), expecting that sialylated and galactosylated glycans (β-1,4 linkage) to be removed. Glycans such as A2G2, F6A1G1, F6A2G2, F6A2G2Ga1 disappeared and migrated to the peak corresponding to A2 (GU=5.41), F6A1, or F6A2G1Ga1 (GU=7.39). The latter structure also contains a Gal-residue in the *β*-1,4 linkage but cannot be cleaved due to the presence of an alpha-1,3 Gal residue blocking the access of the *β*-1,4 galactosidase enzyme. This gathered alpha-Gal residues in one peak, F6A2G1Ga1 allowing us to determine the relative abundance of glycans containing this immunogenic residue. Control samples showed 6.37% of alpha-Gal residues in comparison to cultures containing 20 mM Gal alone or in combination with 7 μM M with 17.2% and 23.5% respectively (**Table 6.2**). The peak corresponding to F6A2G2Ga1S1 (identified as **) was not

digested completely in samples containing 20 mM Gal and M+Gal. This peak had an area higher than in the control sample, indicating that more enzyme was required for a complete digestion. Thus, the area of alpha-Gal residues corresponds to the area of F6A2G1Ga1 and F6A2G2Ga1S1 (**Table 6.2**).

Table 6. 1 Structures containing alpha-Gal residues identified in IgG1 Mab produced by NS0 cells.

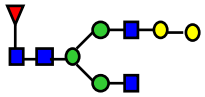
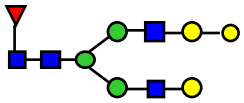
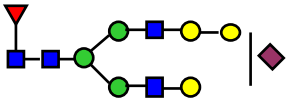
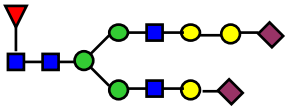
Name	Structure	GU Value
F6A2G1**Ga1**		7.39
F6A2G2**Ga1**		8.27
F6A2G2**Ga1**S1		9.08
F6A2G2**Ga1**S1/S2		9.44

Figure 6. 1 Detailed structural analysis of alpha-Gal residues of IgG1 Mab using exoglycosidase sequencing.

Neuraminidase from *Clostridium perfringens* released α2-3,6,8–linked non-reducing terminal Sias (Red arrows and red HPLC profile). Galactosidase from *Streptococcus pneumoniae* (Green arrow and green HPLC profile) hydrolyzed the non-reducing terminal Gal with β1-4 linkages. The consecutive exoglycosidase digestion gathered alpha-Gal residues in one peak, F6A2G1Ga1 allowing us to determine the relative abundance of glycans containing the alpha-Gal epitope.

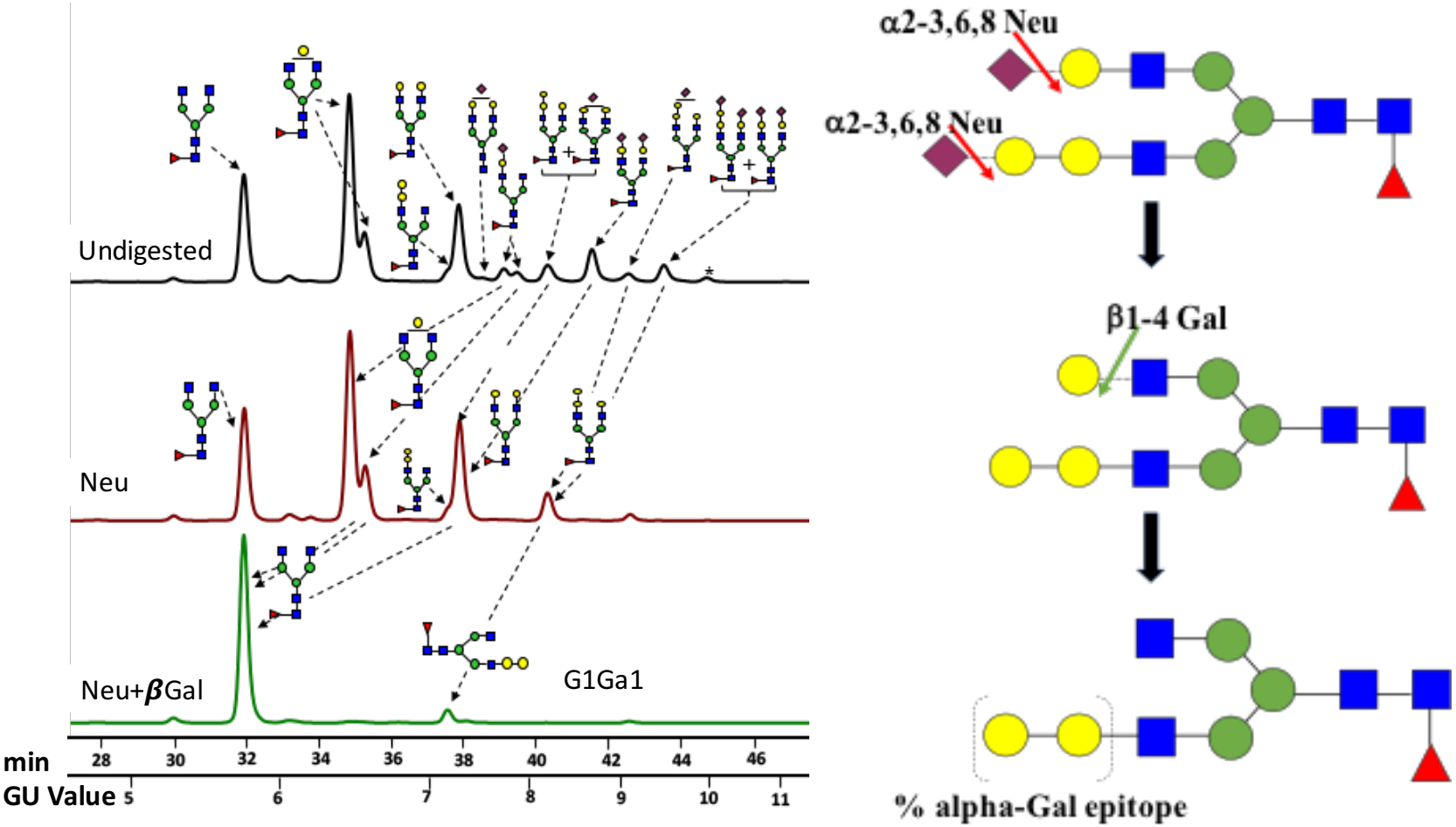


Figure 6. 2 Detailed structural analysis of beta-Gal residues of IgG1 Mab using exoglycosidase sequencing.

Neuraminidase from Clostridium perfringens released α2-3,6,8–linked non-reducing terminal Neu5Ac and Neu5Gc (Red arrows and orange HPLC profile). Alpha-galactosidase from Green coffee bean hydrolyzed the non-reducing terminal Gal with α1-3 linkages (Blue arrow and blue HPLC profile). The sum of R-G1 and R-G2 was used to determine the relative abundance of glycans containing the beta-Gal glycans. The latter includes glycans containing the alpha-Gal epitope after digestion.

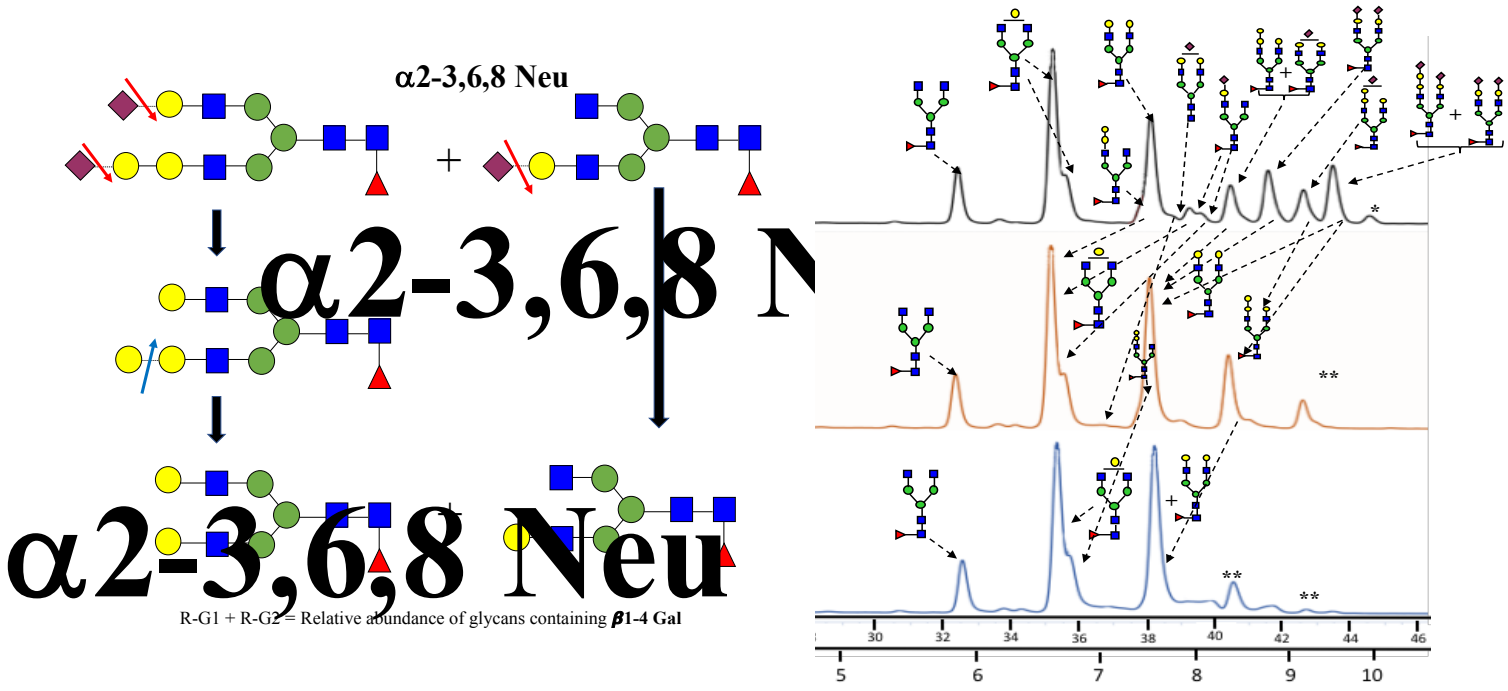


Table 6. 2 Summary of exoglycosidase treatment results.

The relative abundance of glycans containing Gal in the β-1,4 linkage (beta-Gal) or α-1,3 linkage (alpha-Gal). The percentages were determined after digestion with: **A.** α 2-3,6,8 Neuraminidase plus β1-4 galactosidase which resulted in one peak (F6A2G1Ga1) corresponding to the pool of alpha gal containing glycans and another peak with non-galactosylated glycans (F6A2G0). **B.** Digestion with α 2-3,6,8 Neuraminidase plus α1-3 galactosidase resulted in one peak corresponding to non-galactosylated glycans (F6A2G0) and another two peaks (F6A2G1 and F6A2G2) corresponding to glycans containing β-1,4- Gal glycans, including the glycans digested by alpha-gal galactosidase. The relative abundance of beta-Gal containing glycans were obtained by subtracting the relative abundance previously found for alpha gal-containing glycans* from the area obtained from F6A2G1+F6A2G2**.

Culture condition	**EXOGLYCOSYDASE TREATMENT**				**Percentage of Gal containing glycans**	
	A		**B**			
Resulting peaks after digestion	**F6A2G0**	**F6A2G1Ga1**	**F6A2G0**	**F6A2G1+F6A2G2***	**Beta-Gal** (β-1,4)	**Alpha-Gal** (α-1,3 gal)
Control	85.1%	6.37%	26.9%	73.1%	66.7%	6.37%
Gal	**75.0%**	17.2%	10.9%	89.1%	71.9%	17.2%
Mn^{2+}+Gal	**68.4%**	23.5%	93.0%	90.7%	67.2%	23.5%

To determine the relative abundance of glycans containing Gal in the β-1,4 linkage (beta-Gal), NS0-IgG1 Mabs were subjected to exoglycosidase digestion with α2-3,6,8 neuraminidase, α2-3,6,8 neuraminidase *plus* α-1,3 galactosidase (**Figure 6.2.**). Glycans containing Sias such as **A2G2S1** (GU=7.85)**, F6A2G1S1** (GU=7.76)**, F6A2G2S1 (**GU=8.29**), F6A2G2S2 (**GU=9.41)**, F6A2G2Ga1S1/S2** (GU=9.41) were cleaved by neuraminidase and migrated to peaks corresponding to **A2G2** (GU=7.02), **F6A2G1** (GU=6.71) isomers, **F6A2G2** (GU=7.48) or **F6A2G2Ga1** (GU=8.27). The bottom profile shows the digestion after incubation of glycans with both α2-3,6,8 neuraminidase plus α-1,3 galactosidase. Peaks containing alpha-Gal residues such as **F6A2G2Ga1** (GU=8.27) and **F6A2G1Ga1** (GU=7.39) migrated to **F6A2G2** (GU=7.48) and **F6A2G1** (GU=6.71) respectively. Thus, the area of F6A2G1 and F6A2G2 can be assumed to correspond to glycans containing Gal in the β-1,4 linkage. This is considering the glycans containing alpha-Gal epitopes which migrated after cleavage with α-1,3 galactosidase. To determine the relative abundance of glycans containing only beta-Gal, the percentage of alpha-Gal containing glycans (previously determined by digestion with β-1,4 galactosyltransferase) was subtracted from the area of F6A2G1 and F6A2G2 (**See Table 6.2).**

As a result, control samples showed 66.7% and 6.37% of glycans containing beta-Gal and alpha-Gal residues respectively. Cultures containing 20 mM Gal showed an increase in Gal-containing glycans with a distribution of 71.9% and 17.2% glycans with beta-Gal and alpha-Gal residues respectively. Here, an increase of alpha-Gal residues of about 170% was observed with respect to the control. Finally, cultures containing 20 mM Gal and 7μM M showed 67.2% and 23.5 % of beta-Gal and alpha-Gal residues respectively which represents an increase of 269% of alpha-Gal containing glycans in comparison to non-supplemented cultures. These results show that M

and Gal induce an increment in Gal containing glycans, but mainly alpha-Gal containing residues. This is reflected by the relative abundance of beta-Gal glycans which was determined to be constant between 67%-71%.

The effect of M and Urd on the relative abundance of alpha-Gal residues is shown in **Figure 6.3.** Feeding M did not influence the proportion of alpha-Gal residues in comparison to the control (11.9%±4.08). In contrast, M+Urd_1/Urd_2 showed a significant increase of up to 68% in alpha-Gal containing glycans with respect to the control (p-Value 0.02). Increasing Urd's concentration from 2.5mM (Urd_1) to 5 mM (Urd_2) did not affect significantly the proportion of alpha-Gal residues. Cultures containing M+Gal_1/Gal_2 showed the highest increase of up to 174% in alpha-1,3 Gal residues in comparison to the control. However, increasing Gal concentration from 10 mM (Gal_1) to 20 mM (Gal_2) did not cause a significant effect in the proportion of alpha-1,3 Gal residues. Combining the three galactosylation precursors did not impact the relative abundance of alpha-Gal when comparing to M+Urd and M+Gal. However, a significant increase of up to 135% was observed in M+Urd+Gal cultures when compared to the control (p-Value <0.0001). No significant differences were observed if Urd or Gal concentrations were increased in the M+Urd+Gal cocktail.

Knowing that M+Gal cultures showed the highest increase in galactosylation and alpha-Gal residues, the next step was to further evaluate the effect of these galactosylation precursors but individually. This would allow us to identify any synergistic effect happening between these two supplements. The following set of experiments also explored the real content of alpha-Gal residues in NS0-IgG1 Mab produced in media containing $MnCl_2$ and Gal by exoglycosidase digestion.

Figure 6. 3 Relative abundance of glycans containing alpha-1,3 Gal residues from NS0-IgG1 samples cultured in galactosylation precursors-supplemented media

A. Biogro™ media (control); supplemented with: **B**. 7µM M; **C.** 7µM M+2.5 mM Urd_1; **D.** 7µM M + 5 mM Urd_2; **E.** 7µM M +10 mM Gal_1; **F**. 7µM M+ 20 mM Gal_2; **G**. Mn^{2+}+Urd_1+Gal_1; **H.** Mn^{2+}+Urd_2+Gal_1; **I.** Mn^{2+}+Urd_2+Gal_1; **J.** Mn^{2+}+Urd_2+Gal_2. Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Values correspond to the mean ± SD of duplicates of two independent experiments (n = 4). Pairs of means ± SD significantly different (p-Value <0.05*; <0.001**; <0.0001***) with respect to the control culture.

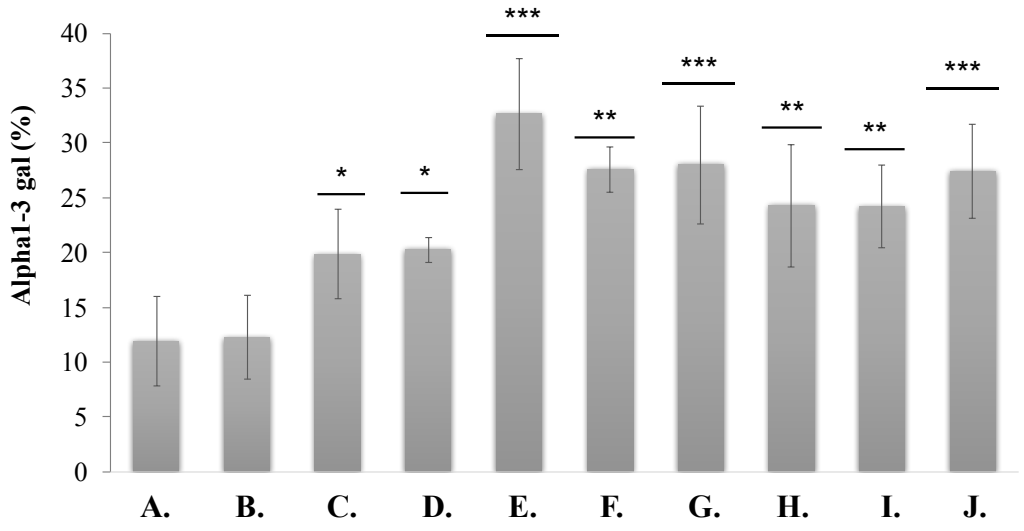
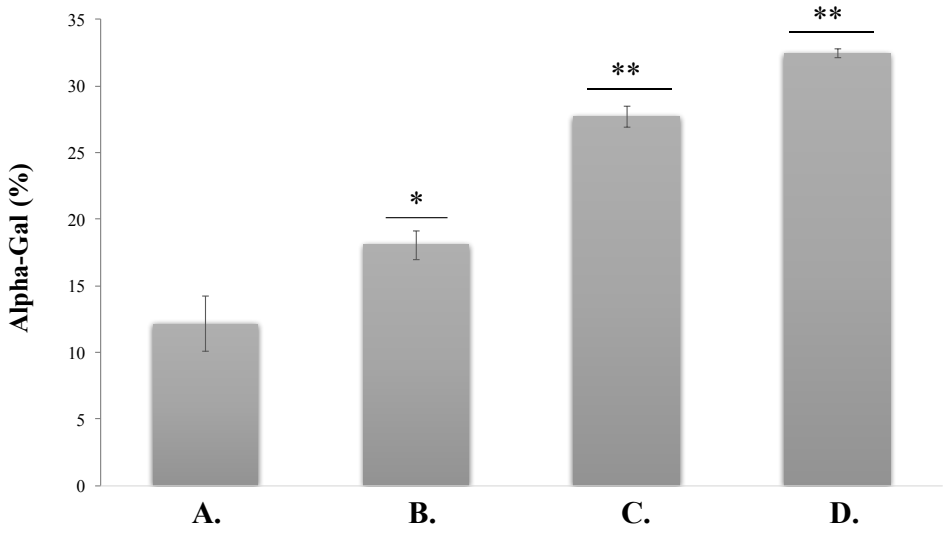


Figure 6.4 shows the effect of M and Gal when added alone or combined (M+Gal) to NS0 cultures. A significantly increase in alpha-Gal residues to up to 49% and 128% was observed when NS0 cells were cultured in the presence of 7 µM M (p-Value 0.0003) and 20 mM Gal (Gal_2; p-Value <0.0001) respectively in comparison to the control. Addition of M along with Gal increased furthermore the relative abundance of alpha-Gal containing glycans to up 167% with respect to the control. This indicates a synergistic effect between M and Gal during the incorporation of alpha-Gal residues.

Figure 6. 4 Relative abundance of glycans containing alpha-1,3 Gal residues from NS0-IgG1Mab.

A. Biogro™ media (control) was supplemented with **B.** 7 μm M; **C.** 20 mM Gal_2 or **D.** 7 μM M and 20 mM Gal_2. Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Values correspond to the mean ± SD of triplicates (n = 3). Pairs of means ± SD significantly different (p-Value 0.0003*; <0.0001**) with respect to the control culture



6.5 Discussion

Mammalian cells commonly used in culture processes have the potential of adding the alpha-Gal epitope, a residue that is not normally found in circulating human IgG (Butler and Spearman 2014). The enzyme α1-3 galactosyltransferase (α1-3GT) forms the alpha-Gal epitope by linking a Gal residue via a α1-3 glycosidic bond to *N*-acetyllactosamine residues (Galβ1 − 4GlcNAc − R) of *N*-glycans (Lin, George *et al.* 2015). The human α1-3GT pseudogene's sequence was found to contain two point mutations, the same that resulted in a frame shift mutation and a

premature stop codon (Huai, Qi *et al.* 2016). The suppression of alpha-Gal epitope expression in Old World monkeys, apes and humans its believed to have taken place less than 28 million years ago probably due to an evolutionary pressure exerted by an infectious agent endemic to the Old World (Galili, Shohet *et al.* 1988, Macher and Galili 2008). In contrast to non-primate mammals and New World monkeys, humans have around 1% of circulating antibodies against the alpha-Gal epitope which have been responsible for rapid hyperacute rejection of pig xenografts and severe anaphylactic reactions to therapeutics containing alpha-Gal residues (Macher and Galili 2008). As a result, different groups have focused on ways to inactivate the α1-3GT. One approach has been the expression of a competing glycosyltransferase such as α1,2 fucosyltransferase (α1,2FT) (Sharma, Okabe *et al.* 1996, Tanemura, Miyagawa *et al.* 1998); *N*-acetylglucosaminetransferase III (Ezzelarab and Cooper 2005, Macher and Galili 2008) and α2,6 and α2,3 sialyltransferase (α2-6ST; α2-3ST) (Tanemura, Miyagawa *et al.* 1998). These enzymes along with α1,3 GalT share the same acceptor substrate *N*-acetyllactosamine (Sharma, Okabe *et al.* 1996). However, this strategy has only been successful in decreasing the levels of alpha-Gal epitope in cells in culture, transgenic mice and pigs but not in completely eliminating this epitope (Sharma, Okabe *et al.* 1996, Tanemura, Miyagawa *et al.* 1998, Ezzelarab and Cooper 2005, Huai, Qi *et al.* 2016). Another more effective approach has been the disruption of the α1,3GT gene which has resulted in the complete elimination of alpha-Gal epitope expression (Huai, Qi *et al.* 2016).

Knowing that mammalian cells have an active α1,3GT gene, there is the need to determine an appropriate host cell system where recombinant glycoproteins are devoid of alpha-Gal epitope to ensure the drug's efficacy and to prevent eliciting immunogenic responses, such as anaphylactic shock in humans. Furthermore, it is important to determine the location in which the

terminal alpha-Gal motif was attached in the glycoprotein. For instance, alpha-Gal residues located on the Fc-linked glycans were found to be hardly recognized by IgE anti alpha-Gal antibodies (e.g. Remicade, Infliximab) in comparison to alpha-Gal residues located at the Fab region (e.g. Cetuximab) (Kuriakose, Chirmule *et al.* 2016). If the mammalian cell line used expresses the alpha-Gal epitope, the implementation of the aforementioned strategies is an option to reduce/eliminate the levels of alpha-Gal (e.g. knock out of α1-3GT gene). Otherwise, it is important to monitor possible environmental sources that can enhance the incorporation of alpha-Gal residues. In this chapter, the content of alpha-Gal was evaluated in IgG1 Mab produced by NS0 cell cultured in media supplemented with glycosylation precursors.

In Chapter 5, a significant improvement in the galactosylation index of up to 8.45%, 22.4%, 33.1% and 27% was observed in M-, M+Urd-, M+Gal- and M+Urd+Gal- containing cultures respectively. However, this increment in GI could also meant an increase in the alpha-Gal epitope. Evaluation of *N*-glycan profiles showed the following alpha-Gal containing glycans: F6A2G1**Ga1**, F6A2G2**Ga1**, F6A2G2**Ga1**S1 and F6A2G2**Ga1**S1/S2. A significant increase of up to 68%, 174% and 135% was estimated in alpha-Gal-containing glycans in IgG1 Mab produced in M+Urd-, M+Gal- and M+Urd+Gal-containing cultures respectively. However, the peaks corresponding to F6A2G2Ga1 and F6A2G2Ga1S1/S2 peaks shared the same GU values of 8.28 and 9.54 with F6A2G2S1 and F6A2G2S2 respectively, which not allowed for an accurate estimate of alpha-Gal content. For this reason, exoglycosidase digestion was used to determine the real contribution of manganese and Gal supplementation on alpha-Gal content in IgG1 Mab. The latter were chosen over M and M+Urd as galactose containing cultures showed the highest increment in Gal-containing glycans. Originally, IgG1 Mabs showed 66.7% and 6.37% of beta-Gal and alpha-

Gal containing glycans respectively. While the content of beta-Gal-containing glycans remained close to the values seen in untreated cultures (66.7-71.9%), the content of alpha-Gal glycans increased from 6.37% to 17.2% and 23.5% in M-, Gal- and M+Gal- supplemented cultures. The latter represents an increase of up to 170% and 269% in the content of alpha-Gal epitope in Gal- and M+Gal- containing cultures respectively. In addition, these results indicate a synergistically effect on the increment of alpha-Gal content in IgG1 Mab when manganese and Gal were supplemented together.

While there is still the need for further investigation regarding the effect of environmental factors on the content of alpha-Gal in recombinant proteins, the results presented in this chapter showed that galactosylation precursor feeding strategies can enhance the content of alpha-Gal in Mabs. Even though the proportion of alpha-Gal epitope required to induce an anaphylactic reaction is not well known, it is suspected that alpha-Gal residues should be sufficiently exposed for IgE binding. For instance, radioimmunoassays (RIAs) have shown that anti alpha-Gal IgE mainly reacted with bi- alpha-Gal epitopes (990 nmol alpha-Gal/μmol IgG) in both Fab arms rather than with the mono-alpha-Gal epitopes from the Fc domain in Cetuximab (140 nmol/ μmol alpha-Gal/μmol IgG in the Fc domain) (van Bueren, Rispens *et al.* 2011). Similarly, another FDA-approved monoclonal antibodies which have shown to contain alpha-Gal residues in the Fc domain failed to react with anti alpha-Gal IgE antibodies (e.g. SP2/0: Remicade, infliximab; NS0: Synagis, CHO: Vectibix, palivizumab; and Orecia: abatacept). Finally, since the main objective of supplementing galactosylation precursors to NS0 cultures was to increase the terminal Gal for further attachment of Sia, next experiments should consider the fact that α1-3GT and

sialyltransferases compete for the same substrate. This can compromise the enhancement of sialylation and instead favor the attachment of alpha-Gal residues.

6.6 Conclusions

- NS0-IgG1 Mab showed a GI of 0.58 where 6.37% corresponded to alpha-Gal glycans in untreated cultures.
- Cultures supplemented with 20 mM Gal showed a significant increase in GI to 0.75 of which 71.9% and 17.2% corresponded to beta-Gal- and alpha-Gal-containing glycans respectively.
- Supplementation of 7 μM M and 20 mM Gal showed a further increase in GI to 0.78 of which 67.2% and 23.5% corresponded to beta-Gal- and alpha-Gal- containing glycans respectively.
- Galactosylation precursors enhanced the addition of alpha-Gal residues into IgG1 Mab, represented by a significant increase in alpha-Gal content of up to 170% and 269% in Gal- and M+Gal- containing cultures respectively.
- Radioimmunoassays (RIAs) are required to establish if the content of alpha-Gal epitopes found in NS0-IgG1 Mab are both, high and exposed enough to react with IgE antibodies. The latter would give information regarding possible immunogenic reactions.
- Further investigation is required regarding environmental factors that can enhance/diminish the attachment of alpha-Gal epitopes

Section C*

Nutrient supplementation influence on sialylation of NS0-IgG1 Mab

Sialylation constitutes the last event of glycosylation which occurs in the *trans*- portion of the Golgi apparatus. Once attached to glycan chains, Sias occupy terminal positions of glycolipids and glycoproteins where they participate in different biological processes (Varki and Schauer 2009). In addition, sialylation has a big impact on the quality of therapeutic glycoproteins (Hossler, Khattak *et al.* 2009). For instance, Sias confer a negative charge which in turn can influence the thermal stability (Mimura, Church *et al.* 2000), resistance to proteases and solubility of glycoproteins (Gu and Wang 1998, Bork, Horstkorte *et al.* 2009, Varki and Schauer 2009). In addition, Sia can strongly impact the efficacy of antibodies via modulation of anti-inflammatory activity (Jedrzejewski PM 2013). Furthermore, Sias can extend the serum half-life of several glycoproteins by masking sub terminal Gal residues and preventing recognition by asialo-glycoprotein receptors of the liver (Varki and Schauer 2009, Savinova, Lobanova *et al.* 2015). For these reasons, several approaches have been made to enhance sialylation of glycoproteins**: 1.** over-expression of enzymes participating in the assembly and transport of Sias and **2.** Knockdown of glycosidases responsible for degradation of glycans and reducing quality of synthesized protein (Gramer and Goochee 1993, Kaneko, Sato *et al.* 2010). However, these two approaches can be

* Data presented in Section C, including Chapter 7-12 have not yet been published.

time- and cost-consuming. Another much simpler alternative than genetic engineering is altering media composition by adding substrates that feed into the Sia biosynthetic pathway and produce CMP-Sia which is the substrate for sialyltrasferases. For example, nucleotide sugar precursors (e.g. glucosamine and *N*-acetylmannosamine) and the addition of nucleosides (Cyt, Urd) have shown to modulate CMP-Sia pools. However, the subsequent changes in sialylation will depend on the cell line, model protein and cultivation methods (Pels Rijcken, Overdijk *et al.* 1995, Gu and Wang 1998, Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001, Clark, Griffiths *et al.* 2005, Wong, Wati *et al.* 2010, Gramer, Eckblad *et al.* 2011).

In this section, experiments were meant to evaluate the effect that Sia precursors have on the glycosylation of IgG1 Mab. Particularly, we aimed to bypass key regulatory steps within the Sia pathway. For instance, Kornfeld *et al.* observed that UDP-GlcNAc prevents further synthesis of itself by inhibiting the enzyme glutamine 6-phosphate transaminidase responsible for the conversion of fructose 6-phosphate to glucosamine 6-phosphate (Kornfeld, Kornfeld *et al.* 1964). In addition, CMP-Neu5Ac strongly inhibits the activity of UDP-*N*-acetylglucosamine 2-epimerase (GNE) which converts UDP-GlcNAc into ManNAc (Kornfeld, Kornfeld *et al.* 1964, Pels Rijcken, Overdijk *et al.* 1995, Rijcken 1995). Knowing that both UDP-GlcNAc and ManNAc are key intermediates of Sia biosynthesis, the following experiments aimed to increase their intracellular concentrations by supplementation of peracetylated glucosamine (Ac_4GlcNAc) and peracetylated mannosamine (Ac_4ManNAc) and evaluate their effect in IgG1 Mab sialylation. In addition, the pyrimidine nucleotides Urd and Cyt were added to increase the Urd and Cyt pools required for the activation of UDP-GlcNAc and CMP- Sia respectively. The latter supplementation of exogenous

precursors was expected to detour the aforementioned regulating steps and therefore attain a higher degree of sialylation in IgG1 Mab.

The Sia biosynthetic pathway shows tolerance for synthetic monosaccharides similar in structure to a natural precursor but bearing an unnatural chemical group (Du, Meledeo *et al.* 2009). Several groups have shown that protection of polar functional groups on sugars can facilitate cellular uptake by passive diffusion through membranes (Luchansky, Goon *et al.* 2004). Acetylation has been used to cover hydroxyl groups and render sugars more hydrophobic (Sarkar, Fritz *et al.* 1995). The latter has allowed the usage of much lower concentrations of Sia precursor derivatives (micromolar range) while achieving the same effect than their free counterparts. For example, analogs of *N*-acetylglucosamine and *N*-acetylmannosamine have been developed to intercept the hexosamine pathway at key points during the biosynthesis of Sias (Almaraz, Tian *et al.* 2012). In our studies, the effect of two analogs of Sia precursors was investigated: peracetylated mannosamine (Ac_4ManNAc) and peracetylated glucosamine (Ac_4GlcNAc). A higher uptake of these non-natural sugars is expected due to the presence of acetyl groups added to the four free hydroxyl groups present in ManNAc and GlcNAc. Once in the cytosol, the acetyl groups of Ac_4ManNAc/Ac_4GlcNAc are released by non-specific acetylases and the sugar analogs can enter the Sia biosynthetic pathway (Yarema 2005). Interestingly, sialyltransferases are known to metabolize artificial Sia intermediates which represents a powerful strategy to improve sialylation in glycoproteins (Bayer, Schubert *et al.* 2013).

Chapter 7*

Effect of glucosamine on the incorporation of sialic acids in NS0-IgG1 Mab

7.1 Introduction

Glycosylation of therapeutic proteins is essential for warranting structural, biological and clinical stability as well as for protecting against proteolytic degradation and rapid removal from circulation (Ficko 2014). In fact, considerable attention has been directed towards a higher content of Sias but a lower content of Neu5Gc to avoid removal of glycoproteins from the circulatory system by asialoglycoprotein receptors as well as immunogenic reactions. In fact, the degree of terminal sialylation has been correlated to glycoproteins serum half-life and *in vivo* bioactivity quality attribute (Werner, Kopp *et al.* 2007).

UDP-*N*-acetylglucosamine (UDP-GlcNAc) constitutes an essential precursor for the synthesis of glycoproteins, glycolipids and proteoglycans. For instance, UDP-GlcNAc is used by the enzyme UDP-GlcNAc:dolichol-P $GlcNAc_1P$ to produce GlcNAc-P-P-dolichol at early stages of *N*-linked glycosylation in the ER (Dan and Lehrman 1997). In addition, UDP-GlcNAc constitutes a substrate for GlcNAc transferases responsible for the elongation of glycan chains in the Golgi apparatus (Yang and Butler 2002). Finally, UDP-GlcNAc serves as a key precursor for

* Data presented in this chapter have not been published. Glucosamine analog was kindly prepared by Evelyn Ang, student from the Dr. Perreault's Lab, Chemistry Department from University of Manitoba.

biosynthesis of Sias. UDP-GlcNAc synthesis starts with the formation of glucosamine-6-phosphate ($GlcN_6P$) from fructose-6-phosphate ($Fruc_6P$) by transamination using glutamine (Gln) or ammonium ions as a nitrogen source (**Figure 7.1**) (Grammatikos, Valley *et al.* 1998). $GlcN_6P$ is then *N*-acetylated via an acetyl-CoA-mediated reaction to form *N*-acetylglucosamine-6-phosphate ($GlcNAc_6P$) which is then isomerized to *N*-acetylglucosamine-1-phosphate ($GlcNAc_1P$) via a 1,6 bi-phosphate intermediate (Yang and Butler 2002, Freeze and Elbein 2009). Finally, $GlcNAc_1P$ reacts with UTP to form UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is in equilibrium with UDP-GalNAc. Alternatively, glucosamine can be phosphorylated to form $GlcNAc_1P$ via a kinase that can use either GlcNAc or *N*-acetylmannosamine (ManNAc)(Freeze and Elbein 2009).

High intracellular levels of UDP-GlcNAc have been observed under high ammonium ion (NH_4^+) concentrations in culture media (Grammatikos, Valley *et al.* 1998). In fact, Ryll *et al.* proposed that NH_4^+ serves as a precursor for UDP-GlcNAc formation by reacting with $Fruc_6P$ to form $GlcN_6P$ (Ryll and Wagner 1992). Previous experiments have shown that treatment with glucosamine also results in an increase in UDP-GNAc levels similar to ammonia's effect. However, the changes induced by glucosamine/ Urd feeding on *N*-linked glycosylation are difficult to predict and depend on the cell line, glycoprotein product and cell culture process (Wong, Wati *et al.* 2010). For example, Wong *et al.* showed an increase in the availability of UDP-GlcNAc via glucosamine feeding which in turn increased the amount of core glycans available for complete sialylation without any change in antennarity (Wong, Wati *et al.* 2010). In contrast, other studies have shown an increase in the activity of branching enzymes like Mgat4 and 5 which results in an increase in glycan antennary along with a decrease in both sialylation and nucleotide triphosphates

(NTP) (Pederson, Knop *et al.* 1992). This decrease in UTP was suggested to limit further increase of the UDP-GNAc pool (Pederson, Knop *et al.* 1992, Gawlitzek, Valley *et al.* 1998, Grammatikos, Valley *et al.* 1998). Opposite to this, Gawlitzek *et al.* showed that incorporation of 5 mM of GlcN didn't have a negative impact on galactosylation and sialylation of TNFR-IgG (Gawlitzek, Ryll *et al.* 2000). Similarly, Grammatikos *et al.* cultured BHK-21 cells in the presence of 12.5 mM GlcN *plus* 2mM Urd and did not observe any reduction in IL-Mu6 sialylation (Grammatikos, Valley *et al.* 1998). In contrast, a higher decrease in PolySia content in NCAM was observed in 10 mM NH_4Cl-fed cultures with respect to GlcN or Urd containing cultures. An increase in UDP-GlcNAc can impair the transport of CMP-Sia into the trans Golgi where sialylation of glycoproteins takes place (Pels Rijcken, Overdijk *et al.* 1993, Pels Rijcken, Overdijk *et al.* 1995). Even though an increase in UDP-GlcNAc was observed in CHO cells, the decrease in sialylation induced by ammonia was related to an increase in the *trans*-Golgi pH and not to an increase in UDP-GlcNAc and UTP depletion (Borys, Linzer *et al.* 1993, Zanghi, Mendoza *et al.* 1998, Gawlitzek, Ryll *et al.* 2000).

Increased levels of UDP-GlcNAc are known to correlate with higher synthesis of branching glycans in the Golgi (Gawlitzek, Valley *et al.* 1998, Grammatikos, Valley *et al.* 1998). This increase of terminal GlcNAc residues would suggest a higher availability of sites for Gal and subsequent Sia addition to oligosaccharides. However, knowing that an increase in antennarity in immunoglobulins is limited to the space available between the CH_2 domains of the heavy chains (Jefferis 2009), the following experiments investigated the effect of UDP-GlcNAc precursor feeding on the *N*-linked glycosylation of IgG1 Mab by culturing NS0 cells in the presence of peracetylated glucosamine (Ac_4GlcNAc, a glucosamine analog) alone or in combination with Urd.

7.2 Objectives.

- Investigate the effect of $Ac_4GlcNAc$ on the degree of terminal sialylation of IgG1 Mab produced by NS0 cells.
- Explore the effect of combining $Ac_4GlcNAc$ with Urd in the glycosylation of IgG1 Mab.

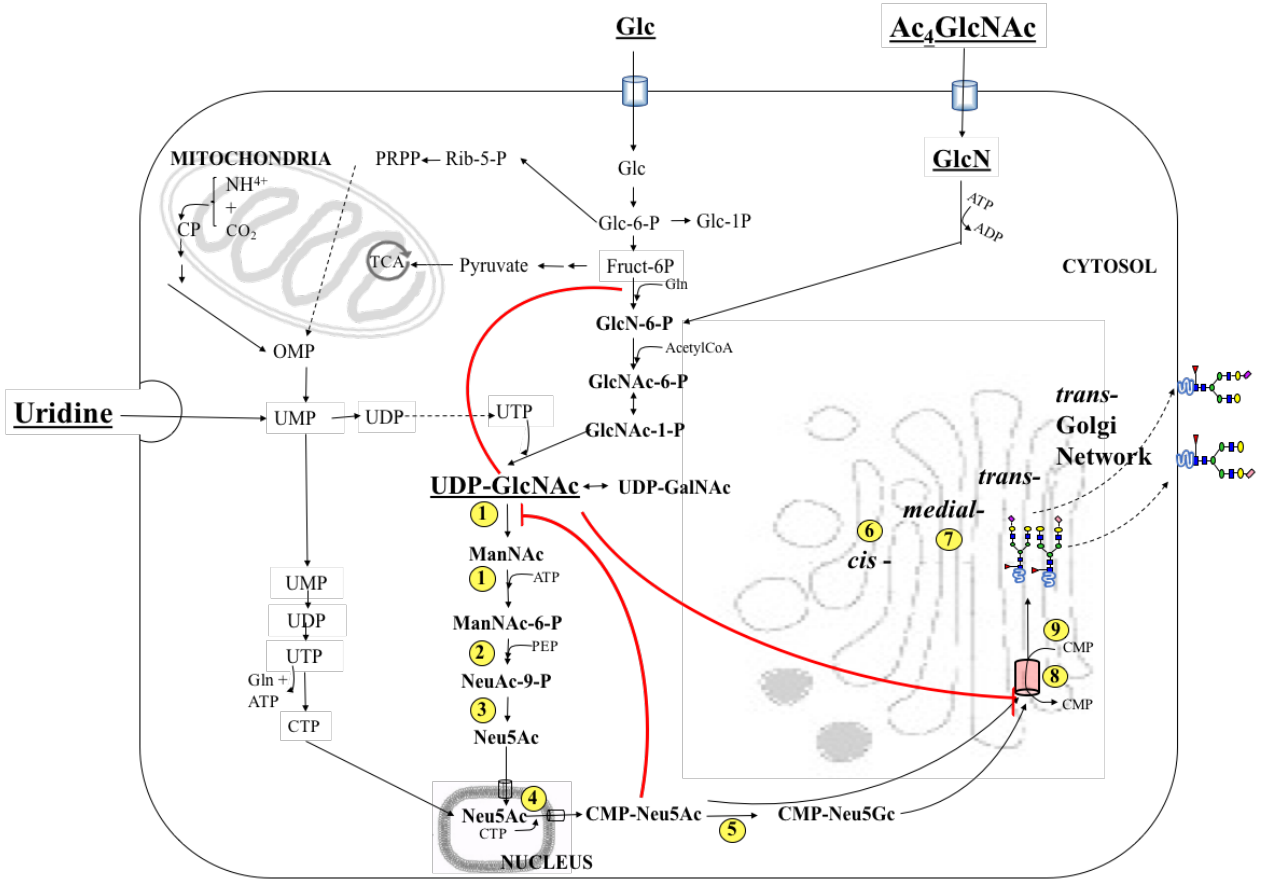
7.3 Experimental set up

7.3.1 Peracetylated glucosamine experiments.

Preliminary experiments were conducted to assess $Ac_4GlcNAc$ optimal concentrations for NS0 cells growth. Concentrations > 100 μM $Ac_4GlcNAc$ (200, 500 and 1000 μM $Ac_4GlcNAc$) showed to have a detrimental effect on growth (< $2.2x10^5$ cells/mL) and viability (< 80%) (data not shown). Thus, a solution of 250 mM of $Ac_4GlcNAc$ was dissolved in 100% ethanol and diluted to reach lower concentrations such as: 10 μM, 25 μM, 50 μM and 100 μM $Ac_4GlcNAc$. The volume of ethanol necessary to reach the mentioned concentrations were added to an empty 125 mL shaker flask, letting ethanol evaporate prior to the addition of media and cells. Culture containing 0 μM $Ac_4GlcNAc$ contained the same volume of ethanol used to dissolve 100 μM of $Ac_4GlcNAc$ but without the precursor and was used as control. Cells were inoculated at a concentration of $2.5x10^5$ cells/mL and shake flasks were placed in a rotary shaker at 120 rpm in an incubator at 37°C and 10% CO2 for four days.

Figure 7. 1 Nucleotide sugar precursor feeding used to fine-tune N-linked glycoslation of NS0-IgG1 Mab.

$Ac_4GlcNAc$ was added at the beginning of a batch culture alone or in combination with Urd. Enzymes and key steps of hexosamine pathways are: **1.** UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE); **2.** *N*-acetylneuraminic acid-9-phosphate synthase; **3.** *N*-acetylneuraminic acid-9-phosphatase; **4.** CMP-*N*-acetylneuraminic acid synthase; **5.** CMP-*N*-acetylneuraminic acid hydroxylase; **6.** mannosidase I (cis-Golgi); **7.** GlcNAc transferase I and mannosidase II (medial-Golgi); **8.** Sia transporter; **9.** Gal transferase and sialyltransferases (trans-Golgi). Red flat arrows indicate sites of feedback inhibition.



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7.3.2 Peracetylated glucosamine and Urd experiments.

The effect of adding pyrimidine-nucleotide precursors Urd alone or in combination with $Ac_4GlcNAc$ was evaluated on the sialylation of IgG1 Mab. For this purpose, NS0 cells were seeded at 2.5 x 10^5 cells/mL in Biogro™ media supplemented with 1mM, 2.5 mM and/or 5 mM Urd alone or in combination with 25 μM $Ac_4GlcNAc$. Control sample had no supplement addition. Each condition was performed in duplicates (n=2) During the experiments, culture shake flasks were placed in a shake platform at 120 rpm were incubation took place for four days at 37°C with 10% CO_2. For both set of experiments, daily sampling (900 μL) was performed to determine cell growth and viability of each condition by trypan blue exclusion method and for metabolite analysis and protein production. On day four, cell culture was terminated and supernatants were collected, filtered, concentrated and Mab was protein A purified before further analysis.

7.4 Results

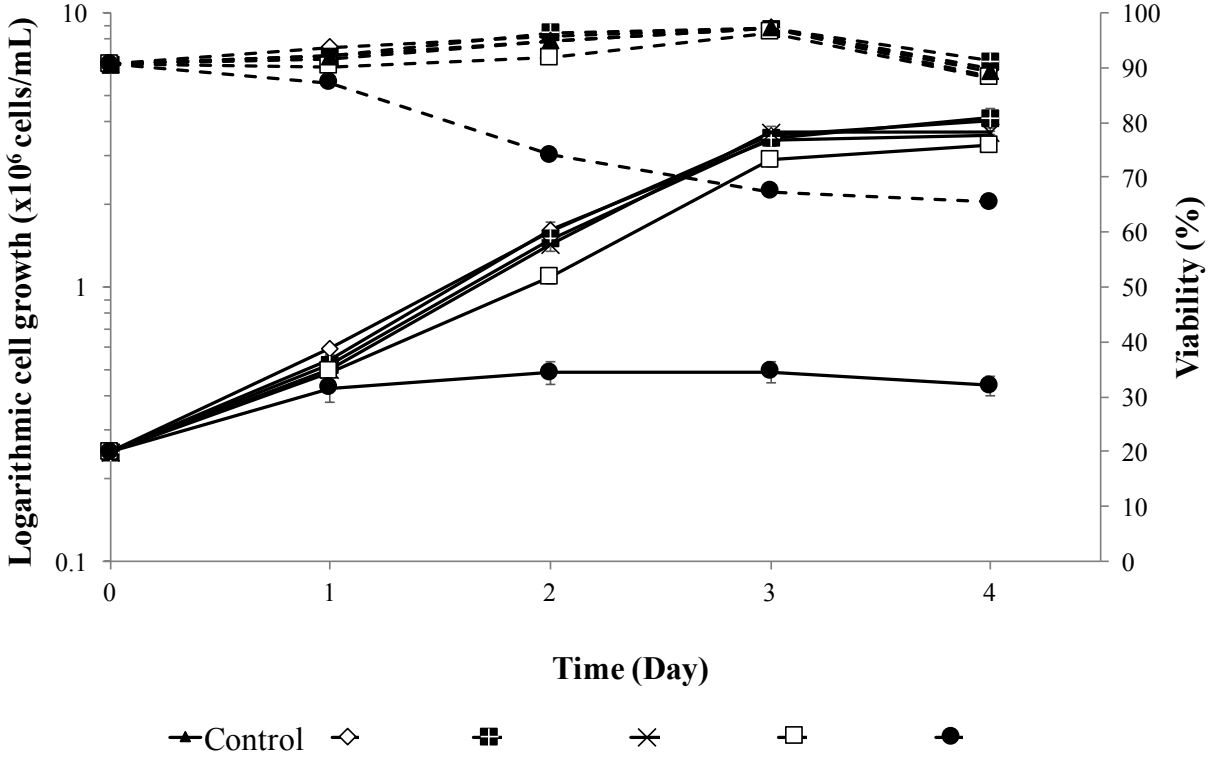
7.4.1 Effect of peracetylated glucosamine and Urd on NS0 cell growth and viability

Preliminary experiments tested the effect that increasing concentrations of $Ac_4GlcNAc$ had on NS0 cells growth and viability **(Figure 7.2).** Cultures containing 0-50 μM $Ac_4GlcNAc$ showed evidence of cell growth from day one until day three where cells with a specific growth rate of ~0.03-0.04 h^{-1} when cells entered a stationary phase. On day four, the MCDs reached in control cultures (4.06 ± 0.36 x10^6 cells/mL) were comparable with cultures supplemented with 10-25 μM $Ac_4GlcNAc$ but significantly higher than cultures containing >50 μM $Ac_4GlcNAc$ (0.44 ± 0.04 x10^6 cells/mL; p-Value <0.003). Furthermore, 100 μM $Ac_4GlcNAc$ cultures showed a decline

of about 29%, 87% and 100% in specific growth rates on day one, two and three respectively when compared to the control. Cell viability declined as early as day one in cultures containing 100 μM Ac_4GlcNAc to 87.2 ± 3.42% and further decrease to 65.4% ± 3.22 on day four. Viability in concentrations <100 μM Ac_4GlcNAc were maintained over 96% during the first three days of culture and then slightly declined to about ~88% on day four.

Figure 7. 2 The effect on cell growth and viability of NS0 cells when cultured in Biogro™ media containing increasing concentrations of Ac_4GlcNAc.

Cells were seeded at $2.5x10^5$ cells/mL in shaker flasks containing 0 μM, 10 μM, 25 μM, 50 μM and 100 μM Ac_4GlcNAc. Control culture did not contain any supplement. Data points represent mean ± SD of duplicates for each condition (n=2). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.



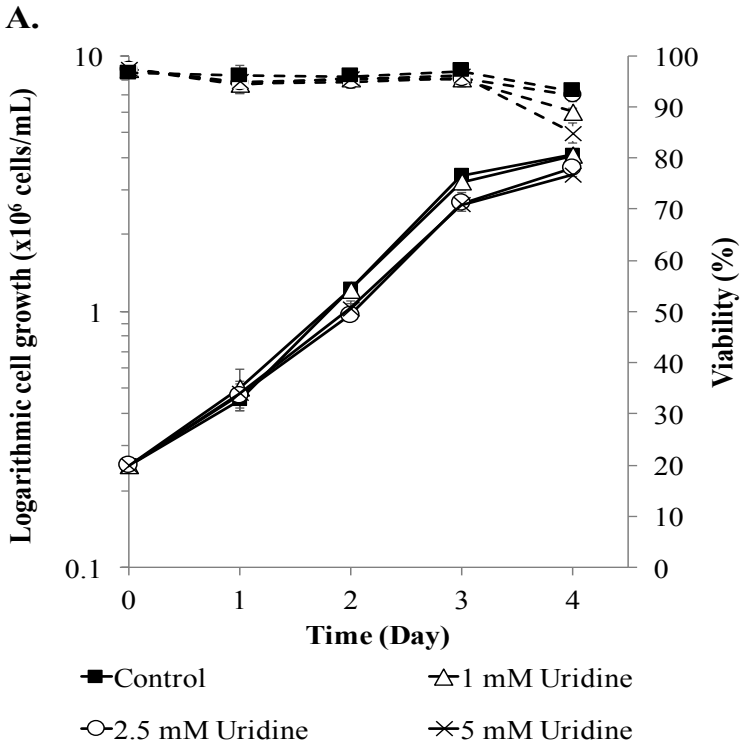
A separate set of experiments focused on evaluating the effect of Urd alone (1 mM, 2.5 mM or 5 mM) or in combination with 25 μM Ac_4GlcNAc. The concentration of Ac_4GlcNAc (25 μM) was chosen since NS0 cell growth was not affected at this concentration when compared to the control (**Figure 7.2**). **Figure 7.3 A and B** show that there were no significant differences in overall growth rates (day 1-2) between the precursor-fed and untreated control cultures. However, cultures containing 25 μM Ac_4GlcNAc and Urd showed a significantly higher growth rates (0.026 h^{-1}) on day four in comparison to the control culture (0.01 h^{-1}) (p-Value 0.0085). MCDs were reached on day four in all culture conditions, with control cultures showing the highest MCD (4.09 ± 0.05 x 10^6 cells/mL) in comparison to cultures containing Urd alone or in combination with Ac_4GlcNAc **(Table 7.2)**. MCDs and cell viabilities declined by up to 16% and 10% respectively as the concentration of Urd increased from 1 mM to 5 mM on day four. The latter shows that uridine has a negative effect on cell growth in NS0 cells. Cultures supplemented with 25 μM Ac_4GlcNAc showed a slight reduction in cell density of about 10% (3.69 ±0.21 x10^6 cells/mL) in comparison to the control. MCDs were compromised even more when Ac_4GlcNAc was combined with uridine **(Figure 7.3A and B).** For instance, a significant decrease of about 8%, 13% and 26% in MCDs was observed in cultures containing 1 mM, 2.5 mM and 5 mM Urd combined with 25 μM Ac_4GlcNAc respectively (p-Value <0.03). NS0 cells' viability remained over 94% until day three in all culture conditions. In contrast, viability decreased significantly to up to 9% and 6% in cultures containing 5 mM Urd and 5 mM Urd with 25 μM Ac_4GlcNAc respectively (**Figure 7.3A and B**).

These results show that addition of Ac_4GlcNAc in concentrations higher than 50 μM had an inhibitory effect on cell growth reflected by a decrease of up to 29% in growth rates since day

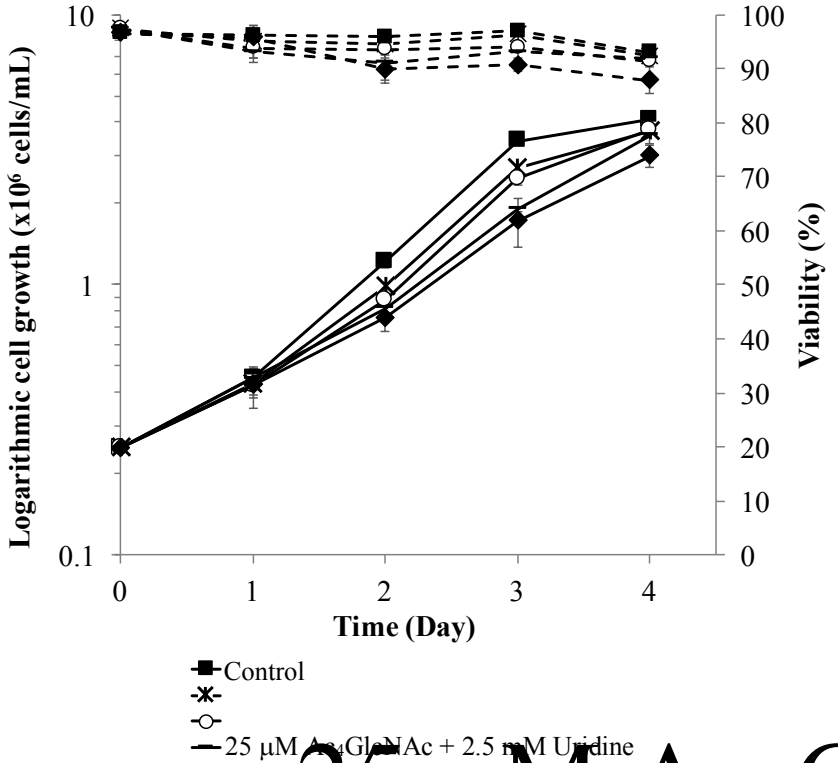
one. Furthermore, the lowest viability (65%) was observed in cultures with 100 μM Ac_4GlcNAc. With respect to Urd supplementation, an increase in concentrations from 1mM to 5 mM caused a decreased of up to 16% on MCDs on day four. Urd's negative effect on cell growth was enhanced when incorporated along with 25 μM Ac_4GlcNAc, with a decrease in MCDs of about 8-26% in 1-5 mM Urd *plus* 25μM Ac_4GlcNAc on day four.

Figure 7. 3 The effect on cell growth and viability of NS0 cells when cultured in Biogro™ media containing increasing concentrations of uridine and Ac_4GlcNAc.

A. uridine: 1mM, 2.5mM and 5mM and **B.** 25μM Ac_4GlcNAc alone or in combination with 1mM, 2.5mM and 5mM uridine. Cells were seeded at $2.5x10^5$ cells/mL in shaker flasks. Control culture did not contain any supplement. Data points represent mean ± SD of duplicates for each condition (n=2). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.



B.



25 µM Ac_4GlcNAc

25 µM Ac_4GlcNAc +

7.4.2 Effect of Ac_4GlcNAc feeding in NS0 cells metabolism.

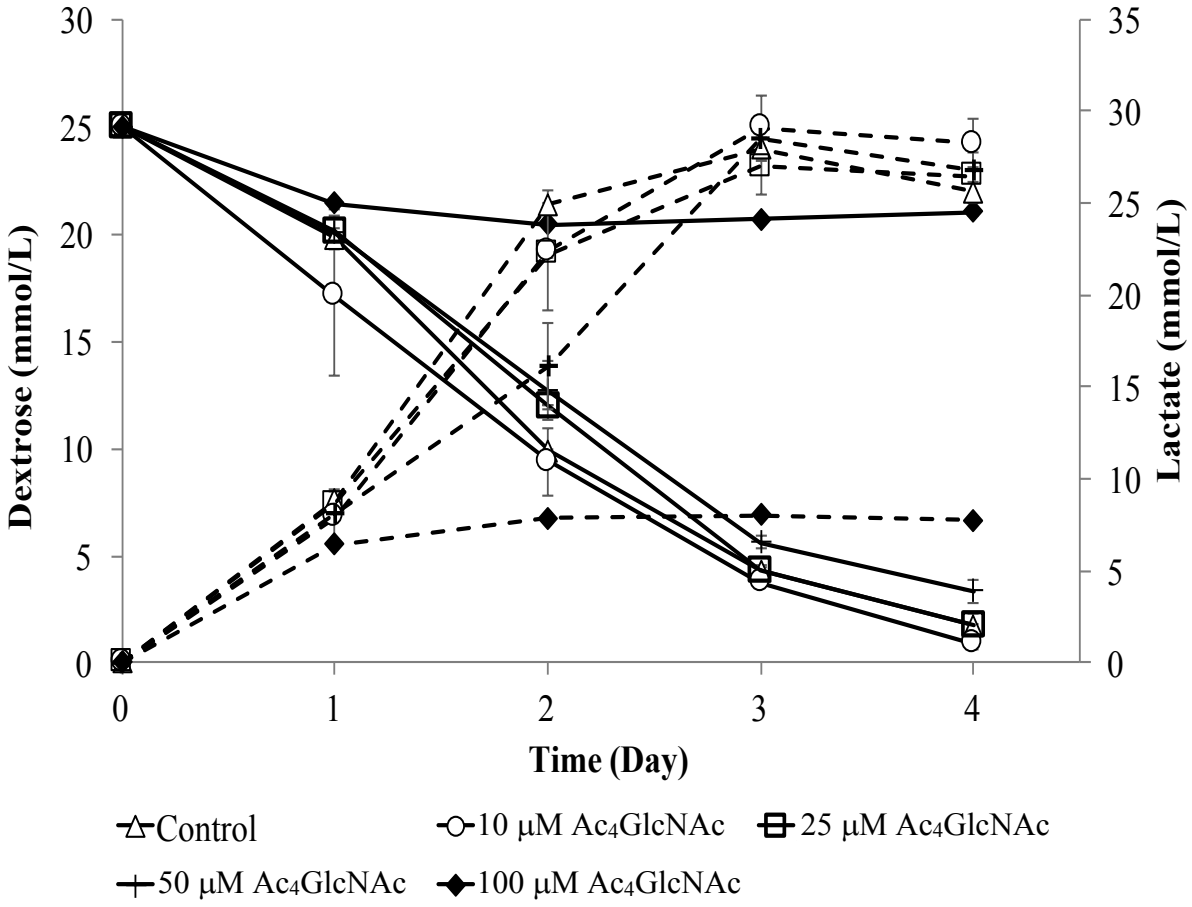
25 µM Ac_4GlcNAc -

The effects of Ac_4GlcNAc on NS0 cells metabolism were assessed by measuring the specific consumption rates of glucose (q_{Glc}) as well as the specific production rates of lactate (q_{Lac}). For this purpose, glucose consumption and lactate production were quantified daily by a biochemistry analyzer (**Figure 7.4**). The initial glucose concentration in Biogro™ media (25mM) declined to 9.89 mM and 1.72 mM on day two and four respectively in control cultures. In contrast, Ac_4GlcNAc containing cultures (10-50 µM) did not show a significant difference in glucose and lactate concentrations with respect to the control. Increasing Ac_4GlcNAc to 100 µM caused a

significant reduction in glucose consumption (q_{Glc}) of about 77% in comparison to 0-50 μM Ac_4GlcNAc cultures (**Table 7.1**; p-Value <0.05). The latter is consistent with the amount of residual glucose (21 mM) found on the media since day two (**Figure 7.4**).

Figure 7. 4 The effect of Ac_4GlcNAc on the cell metabolism of NS0 cells.

Glucose (solid lanes) and lactate (dashed lanes) concentrations (mM) were determined during a four-day batch culture. NS0 cells were seeded at $2.5x10^5$ cells/mL in shaker flasks containing media with increasing concentrations of Ac_4GlcNAc (10-100 μM). Control culture did not contain any supplement. Data points represent mean ± SD of duplicates samples for each condition (n=2) of one set of experiments.



With respect to lactate production, cultures containing 0-50 μM Ac_4GlcNAc showed the highest amount with up to 29.1 mM on day three, which slightly decreased to 25.6 mM on day four. In cultures containing 100 μM Ac_4GlcNAc, lactate production reached plateau after day two with 8.04 mM. This represented a significant reduction in q_{Lac} of about 79% in 100 Ac_4GlcNAc cultures with respect to the control on day 2 (**Table 7.1**). However, the $Y_{Lac/Glc}$ yield was much higher in 100 μM Ac_4GlcNAc cultures with 1.93 than cultures containing 0 to 50μM Ac_4GlcNAc with a $Y_{Lac/Glc}$ between the range 1.17-1.24. Thus, 0 to 50μM Ac_4GlcNAc-containing cultures showed a much more efficient metabolism with a much lower amount of glucose transformed into lactate.

A significantly higher residual glucose concentration was observed in uridine-fed cultures (6.5 mmol/L) and in Ac_4GlcNAc+uridine-fed cultures (7.75 mmol/L) when compared to untreated cultures (2.5 mmol/L; p-Value <0.0001). However, the final lactate concentration observed in control cultures (27.8 mmol/L) was not significantly affected by the precursor alone nor by the combination of precursor and nucleoside. Control cultures showed a q_{Glc} of 0.77 ± 0.11 μmol/10^6 cells.day, which decreased in uridine-fed cultures by up to ~68% (p-Value 0.04) and slightly increased by ~73% in 25 μM Ac_4GlcNAc+5mM uridine-fed cultures (**Table 7.2**; p-Value 0.03). The $Y_{Lac/Glc}$ varied between 1.29 and 1.45 mol/mol in precursor ± nucleoside-fed cultures in comparison to the control with 1.69 mol/mol; which suggests a metabolic shift from a high to low-lactate producing metabolism.

These results show that Ac_4GlcNAc affected NS0 cell's metabolism. For instance, a significant reduction in q_{Glc} of about 77% was observed in cultures containing 100 μM Ac_4GlcNAc

with respect to 0-50 μM Ac_4GlcNAc. These decrease in q_{Glc} was consistent with the accumulation of glucose at 21mM in 100 μM Ac_4GlcNAc cultures since day two. In addition, a significant reduction in q_{Lac} of about 79% was observed in 100 Ac_4GlcNAc-supplemented cultures with respect to the control on day 2, where lactate reached plateau at 8 mM. Incorporation of uridine alone reduced q_{Glc} by 68% with respect to the control, opposite to an increase of up to 73% in q_{Glc} in cultures containing 2.5-5 mM uridine+25 μM Ac_4GlcNAc when compared to the untreated cultures. A metabolic shift from a high to low-lactate producing metabolism was observed in cultures supplemented with 25 μM Ac_4GlcNAc ± 1-5 mM uridine where the $Y_{Lac/Glc}$ decline to 1.29 and 1.45 mol/mol in comparison to the control with 1.69 mol/mol.

7.4.3 Influence of peracetylated glucosamine and uridine on IgG1 production by NS0 cells.

With respect to NS0-IgG1 Mab production, cultures containing between 10-50 μM Ac_4GlcNAc show comparable volumetric productivity to untreated cultures (99.7 μg/mL; **Table 7.1)**. In contrast, 100 μM Ac_4GlcNAc-containing cultures accumulated significantly lower concentrations (~88%) over the four-day culture period in comparison to the control. With regards to cell-specific productivity, the q_{Mab} was determined from the slope of the plot of NS0-IgG1 Mab concentration and the integral viable cell density (IVCD) values**.** A slight but significant increase in q_{Mab} was observed in >50 μM Ac_4GlcNAc concentrations in comparison to the control (**Table 7.1**). With respect to uridine containing cultures, uridine alone did not influence the total amount of Mab produced when compared with untreated control cultures (**Table 7.2**). However, cultures containing 25 μM Ac_4GlcNAc and 25 μM Ac_4GlcNAc plus 1 mM uridine showed a slightly lower volumetric productivity of 90.1±3.72 μg/mL and 93.1±2.97 μg/mL respectively (p-Value <0.005).

Comparable q_{Mab} were observed between control cultures (15.4 ± 0.18 μg/10^6 cell.day) and cultures containing 1mM uridine (16.5 ± 0.13 μg/10^6 cell.day) and 25 μM Ac_4GlcNAc (15.9 ± 0.14 μg/10^6 cell.day). However, a significant increase in q_{Mab} of about 17% and 19% was observed in 2.5 mM and 5 mM uridine-supplemented cultures respectively (p-Value ≤0.002). Combination of 25 μM Ac_4GlcNAc with 1mM, 2.5 mM and 5 mM uridine also showed a significant increase of about 14%, 41%, 55% respectively when compared to the control (p-Value ≤0.008).

These results show that concentrations >50 μM Ac_4GlcNAc significantly reduced the total amount of Mab produced by NS0 cells by ~88%). However, q_{Mab} a slight but significant increase of about 25% was observed in >50 μM Ac_4GlcNAc in comparison to the control. Incorporation of uridine alone did not influence the final Mab yield observed on day with respect to control cultures. However, a significant increase in q_{Mab} of about 17% and 19% was observed in 2.5 mM and 5 mM uridine-supplemented cultures respectively. Furthermore, 25 μM Ac_4GlcNAc + 1-5 mM uridine also showed a significant increase of up to 55% when compared to the control.

7.4.4 Effect of Ac_4GlcNAc and uridine on *N*-glycosylation of NS0-IgG1 Mab.

The effect of the Sia precursor peracetylated glucosamine (Ac_4GlcNAc) on NS0-IgG1 Mab glycosylation was evaluated in a four-day batch culture (**Figure 7.5A)**. Released 2-AB labeled *N*-glycans derived from IgG1 produced by NS0 cells were analyzed quantitatively using normal-phase chromatography.

Table 7. 1 Culture parameters of NS0 cells cultured in media containing increasing concentrations of $Ac_4GlcNAc$.

Volumetric productivity, cell-specific productivity, cell-specific glucose uptake (q_{Glc}) and lactate formation (q_{Lac}) were determined during a four-day batch culture. The $Y_{Lac/Glc}$ corresponds to the moles of lactate produce per mole of glucose. Daily samples were taken to measure cell density, glucose consumption, lactate production and cell productivity. The control sample did not include any supplement. The data shows the average and standard deviation corresponding to two samples (n=2) of one set of experiments.

Ac4GlcNAc (μM)	Mab yield (μg/mL)[a]	q_{Mab} (μg/106 cells.day)[b]	q_{Glc} (mmol/10^6 cells.day)[c]	q_{Lac} (mmol/10^6 cells.day)[c]	$Y_{lac/glc}$
0 †	99.7 ± 0.00	12.3 ± 0.45	-9.01 ± 0.04	14.6 ± 0.36	1.17 ± 0.04
10	100 ± 0.01	13.2 ± 0.18	-7.64 ± 5.21	14.4 ± 4.80	1.17 ± 0.04
25	94.2 ± 0.00	12.7 ± 0.68	-8.45 ± 0.07	14.1 ± 0.36	1.14 ± 0.03
50	82.4 ± 0.01	13.8 ± 0.40*	-9.30 ± 0.81	10.1 ± 2.64	1.24 ± 0.04
100	**12.4 ± 0.00*** **	**15.4 ± 0.33****	**-2.07 ± 0.46***	**3.07 ± 0.22****	**1.93 ± 0.06*****

a) Measured on day four

b) Specific cell productivity during four-day batch culture

c) Over the period of time before glucose concentration reached plateau (day 2)

d) Pairs of means ± SD significantly different (p-Value <0.05*; <0.005**;<0.0001**) with respect to the control

e) Control sample †

Table 7. 2 Culture parameters of NS0 cells cultured in Biogro™ media supplemented with uridine and $Ac_4GlcNAc$.

A. without any supplement (Control), **B.** 1 mM Uridine (Urd), **C.** 2.5 mM Urd; **D.** 5 mM Urd; **E.** 25 μM $Ac_4GlcNAc$; **F.** 25 μM $Ac_4GlcNAc$ + 1 mM Urd; **G.** 25 μM $Ac_4GlcNAc$ + 2.5 mM Urd; **H**. 25 μM $Ac_4GlcNAc$ + 5 mM Urd. MCDs were reached on day four in all culture conditions. Mab yield corresponds to the volumetric productivity during the four days of cell culture. Specific glucose uptake (q_{Glc}), by-product formation (q_{Lac}) and productivity (q_{Mab}) determined from day zero up to day four. The $Y_{Lac/Glc}$ corresponds to the amount of lactate produce per mole of glucose. Daily samples were taken to measure cell density, glucose consumption, lactate production and cell productivity. The data shows the average and standard deviation corresponding to two samples (n=2)

Culture condition	MCD (x10^{6}cells/mL)	Mab yield (μg/mL)	q_{Mab} (μg/10^6 cells.day)	q_{Glc} (μmol/10^{6}cells.day)	q_{Lac} (μmol/10^{6}cells.day)	$Y_{lac/Glc}$ (mol/mol)
A.	4.09 ± 0.05	107 ± 4.54	15.4 ± 0.18	-0.77 ± 0.11	-0.37 ± 0.00	1.69 ± 0.00
B.	4.07 ± 0.38	109 ± 13.0	16.5 ± 0.13	-0.51 ± 0.16	-1.34 ± 1.11	1.45 ± 0.16
C.	3.64 ± 0.06	113 ± 12.5	**18.0 ± 0.47***	**-0.25 ± 0.32***	0.45 ± 1.22	**1.32 ± 0.05***
D.	3.41 ± 0.06	103 ± 9.94	**18.4 ± 0.82****	-0.45 ± 0.04	-0.68 ± 0.07	**1.37 ± 0.02***
E.	3.69 ± 0.21	**90.1 ± 3.72**[c)]	15.9 ± 0.14	-0.52 ± 0.21	0.19 ± 0.93	**1.34 ± 0.06***
F.	3.75 ± 0.25	**93.1 ± 2.97**[b)]	17.6 ± 0.62*	-0.60 ± 0.37	0.51 ± 0.68	**1.31 ± 0.00***
G.	3.58 ± 0.28	108 ± 9.72	**21.7 ± 1.24*****	-1.24 ± 0.18	0.58 ± 0.31	**1.29 ± 0.06***
H.	3.01 ± 0.17	109 ± 11.3	**23.8 ± 0.45*****	**-1.33 ± 0.16***	1.79 ± 0.39*	1.42 ± 0.30

Pairs of means ± SD significantly different with respect to the control culture (n=2; p-Value <0.05*; <0.001**; <0.0001***)

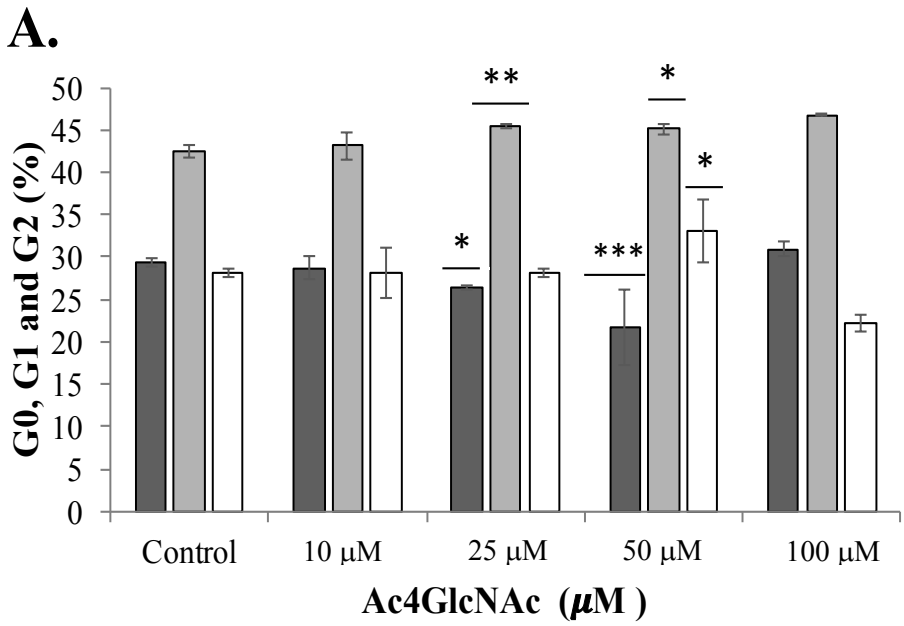
Glycan assignments were performed by comparison of GU values for unknown glycans with glycan standard GU values within the database from NIBRT (GlycoBase). Addition of 10 μM Ac_4GlcNAc did not influence Mab's glycosylation, showing comparable profiles with the control culture with 29.4%, 42.5% and 28.1% of glycans containing zero (G0), one (G1) and two (G2) Gal residues respectively. Cultures containing 25μM and 50 μM Ac_4GlcNAc showed a slight but significant decrease of up to ~26% (p-Value 0.0001) in G0 glycans along with an increase of up to 10% in G1 glycans (p-Value <0.05). In addition, an increase of about 18% in G2 containing glycans was observed in 50 μM Ac_4GlcNAc. In contrast, 100 μM Ac_4GlcNAc induced a significant decrease of about 20% in G2 glycans when compared to the control. The relative abundance of Gal containing glycans was used to calculate the galactosylation index in IgG1 Mabs (**Figure 7.5B.**) Control cultures showed a GI of 0.49 ± 0.01 which increase in cultures containing 50 μM Ac_4GlcNAc to up to 0.56 ± 0.04; which represents a significant ~12.8% increase (p-Value 0.0006). Finally, cultures containing 100 μM Ac_4GlcNAc showed the lowest GI with 0.46 ± 0.01; which represents a significant decrease of 7% with respect to the control (p-Value 0.009).

As a Sia precursor, Ac_4GlcNAc`s effect on the amount of sialylated glycans was evaluated in IgG1 Mabs (**Figure 7.6**). In control cultures, 9.52% ± 1.19 of the total glycan pool corresponded to sialylated glycans, with a ratio of mono- to di-sialylated glycans of 7:3. Comparable amounts were observed in cultures supplemented with 10 μM, 25 μM and 50 μM Ac_4GlcNAc with 9.91% ± 2.64, 8.75% ±1.68 and 11.57% ±1.27 respectively. Sialylation significantly decreased to 6.34 % ± 0.50 in cultures containing 100 μM Ac_4GlcNAc; which represented a decrease of about 33% with respect to untreated cultures (p-Value 0.03). The latter corresponded to a ratio of 5:1 of mono- to di- sialylated glycans in cultures with 100 μM

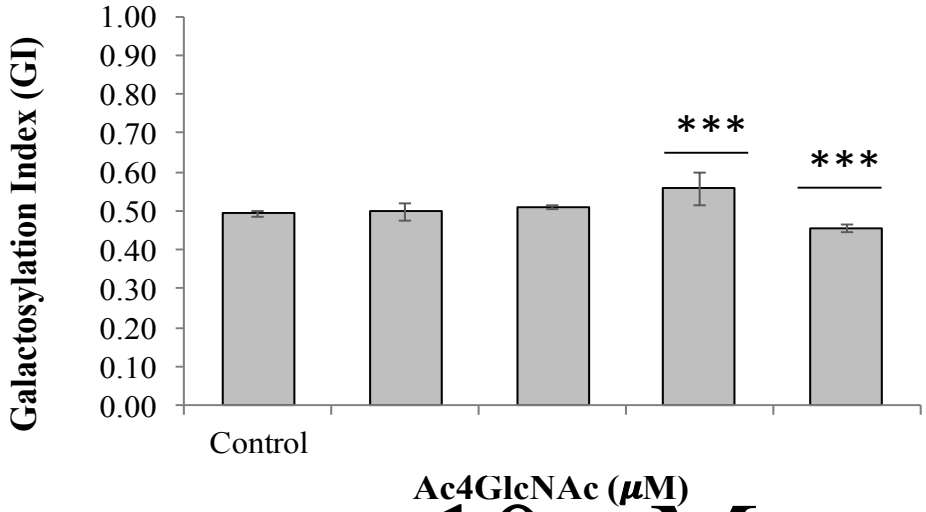
$Ac_4GlcNAc$. Incorporation of uridine significantly improved galactosylation of NS0-IgG1. For instance, an increase of up to 27% in G2 glycans along with a significant decrease of about 37% in G0 glycans was observed in ≥2.5 mM uridine-containing media (p-Value <0.05) (**Figure 7.7A**). As a result, the GI increased significantly by up to 15% when compared to the control (**Figure 7.7B**, p-Value < 0.05).

Figure 7. 5 Effect of $Ac_4GlcNAc$ on the N-glycosylation of NS0-IgG1 Mabs.

A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in media supplemented with 0, 10, 25 and 100 $Ac_4GlcNAc$. **B.** Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). Values correspond to the mean ± SD of duplicates of two independent experiments (n = 4). Pairs of means ± SD significantly different (p-Value <0.05*; <0.01**; <0.001***) with respect to the control culture



B.

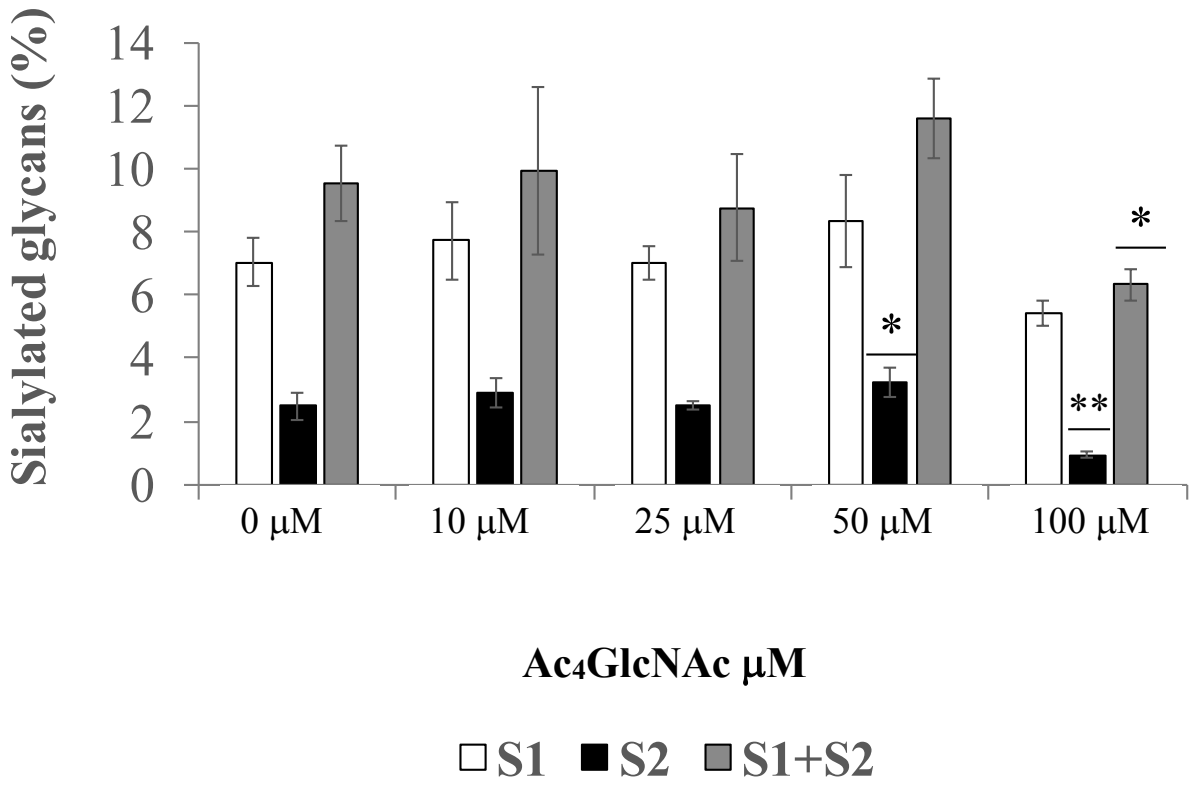


10 μM 25 μ

In contrast, there were no differences in the glycosylation profiles in Mabs produced in 25 μM $Ac_4GlcNAc$ +/- 1 mM uridine when compared to the control (**Figure 7.8A**). On the other hand, cultures containing 25 μM $Ac_4GlcNAc$ + 2.5-5 mM uridine showed a significant decrease in G0 glycans of up to 43% along with a significant increase in G2 glycans of up to 31% (p-Value <0.05). Thus, an increase in the GI of up to 16% was observed when 25 μM $Ac_4GlcNAc$ were combined with 2.5-5mM uridine (**Figure 7.8B**, p-Value <0.05). However, combination of peracetylated glucosamine and uridine (**Figure 7.8A** and **B**) made no significant difference with respect to cultures with either just a precursor or a nucleoside (**Figure 7.7A** and **B and 7.8A** and **B**). The effect of uridine on IgG1 Mab sialylation is shown in **Figure 7.9A.** Control cultures showed 12.2% ±5.68 of glycans terminating with Sias. Even though there was an increase in galactosylated glycans in cultures containing uridine (2.5-5 mM) alone or in combination with 25μM $Ac_4GlcNAc$, sialylation was not significantly influenced in these cultures. However, a slight but not significant decrease was seen in uridine cultures (**Figure 7.9A**) and uridine plus $Ac_4GlcNAc$ (**Figure 7.9B**).

Figure 7. 6 Effect of $Ac_4GlcNAc$ on the amount of glycan structures containing one (S1) or two (S2) Sias.

The total amount of sialylation corresponds to S1+S2. Values correspond to the mean ± SD of duplicates of two independent experiments (n = 4). Pairs of means ± SD significantly different (p-Value <0.05*; <0.0005**) with respect to the control culture



These results show that $Ac_4GlcNAc$ did not influence *N*-linked glycosylation of IgG1 Mabs when concentrations below 50 μM were supplemented to NS0 cells. A slight but significant increase in galactosylated glycans of about 13% was observed in cultures containing 50 μM $Ac_4GlcNAc$. More importantly, cultures containing 100 μM $Ac_4GlcNAc$ showed a significant decrease in galactosylation of 7% which in turn affected the addition of Sias. In fact, a significant reduction of about 33% was observed in 100 μM $Ac_4GlcNAc$. In contrast, uridine containing

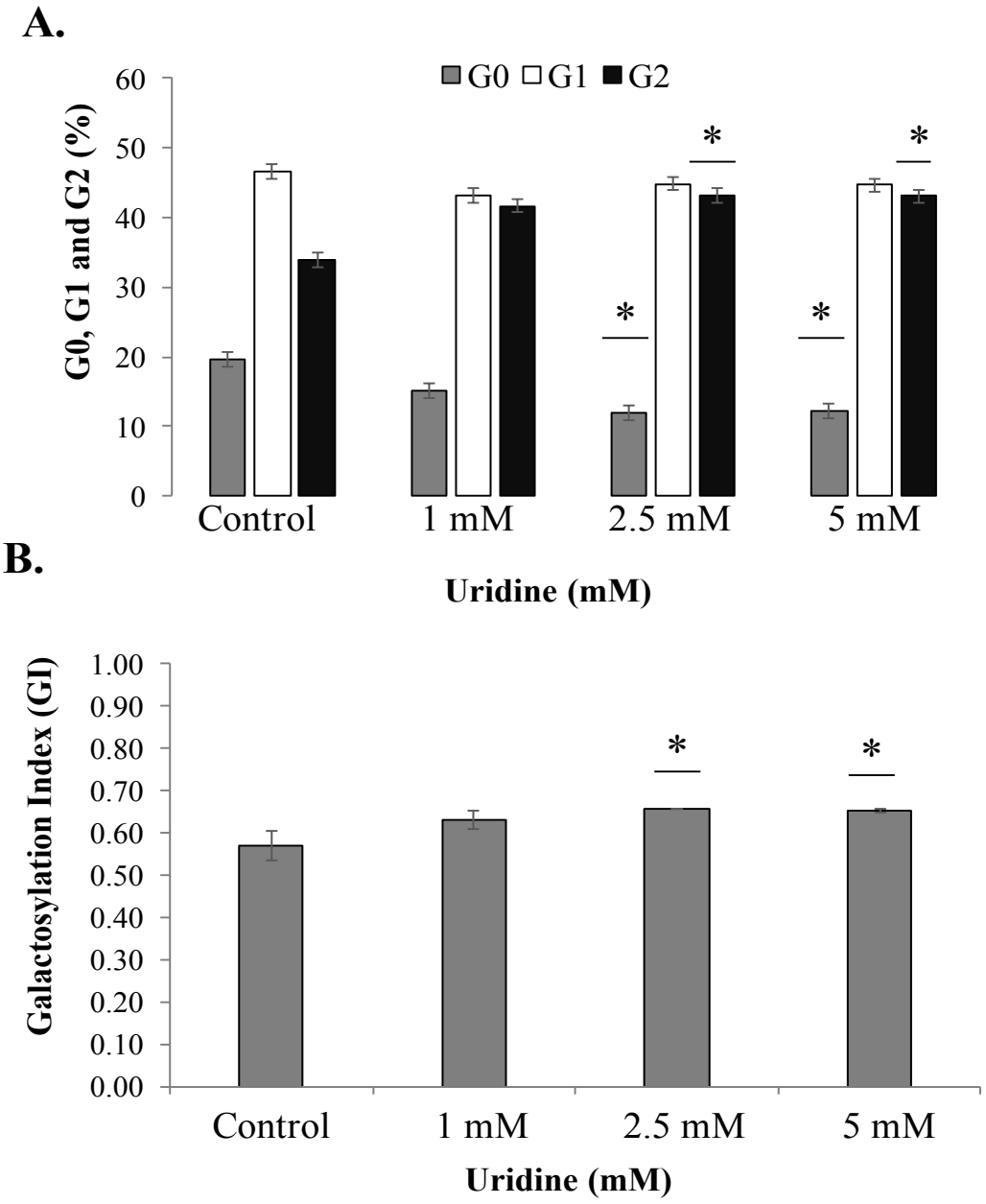
culture showed an increase in GI of 15% when concentrations increased to 5 mM in comparison to the control. An increment in proportion of galactosylated glycans (16%) was also observed in cultures containing both 2.5-5 mM uridine plus 25μM Ac_4GlcNAc. However, the increment in galactosylation observed in uridine- and Ac_4GlcNAc cultures was not enough to improve the sialylation of IgG1 Mab.

7.5 Discussion

Previous studies in metabolic labeling of Sias have shown that non-natural Sias containing *N*-acyl groups had a higher uptake efficiency than their monosaccharide counterparts (Sarkar, Fritz *et al.* 1995, Luchansky, Goon *et al.* 2004, Doi K. 2010, Chen and Liang 2012, Savinova, Lobanova *et al.* 2015). Once inside, cells will remove the acyl groups by action of esterases and convert these compounds into their active forms (Sarkar, Fritz *et al.* 1995). In this chapter, one Sia precursor analog Ac_4GlcNAc was used to evaluate its effect in sialylation of IgG1 Mab produced by NS0 cells. An inhibition in cell growth and viability in Ac_4GlcNAc was observed when concentrations reached >50 μM. The inhibitory effect of exogenous glucosamine on cell growth was consistent with previous reports (>3.5 mM GlcN), even though the concentrations used in this chapter were in the micromolar range (Bekesi and Winzler 1969, Krug, Zweibaum *et al.* 1984, Pederson, Knop *et al.* 1992, Pels Rijcken, Overdijk *et al.* 1995, Gawlitzek, Valley *et al.* 1998, Zanghi, Mendoza *et al.* 1998, Baker, Rendall *et al.* 2001, Wong, Wati *et al.* 2010).

Figure 7. 7 Effect of the nucleoside uridine on the N-glycosylation of NS0-IgG1 Mabs.

A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in media supplemented with 0, 1, 2.5 mM and 5 mM uridine.
B. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). Values correspond to the mean ± SD of duplicates (n = 2) of one set of experiments. Pairs of means ± SD significantly different (p-Value <0.05) with respect to the control cultures



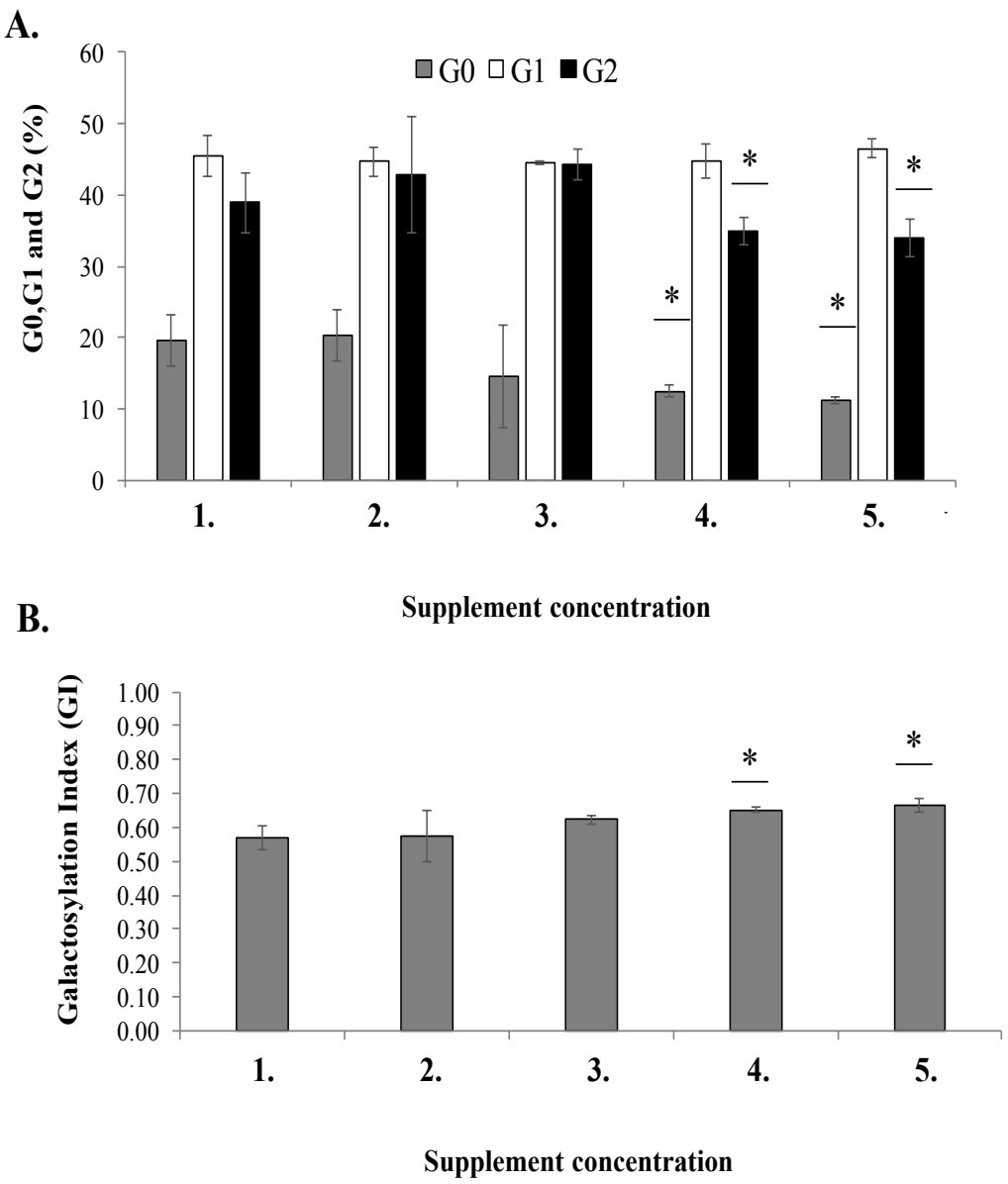


Figure 7. 8 Effect of combining the non-natural Sia precursor $Ac_4GlcNAc$ along with the nucleoside uridine on the N-glycosylation of NS0-IgG1 Mabs.

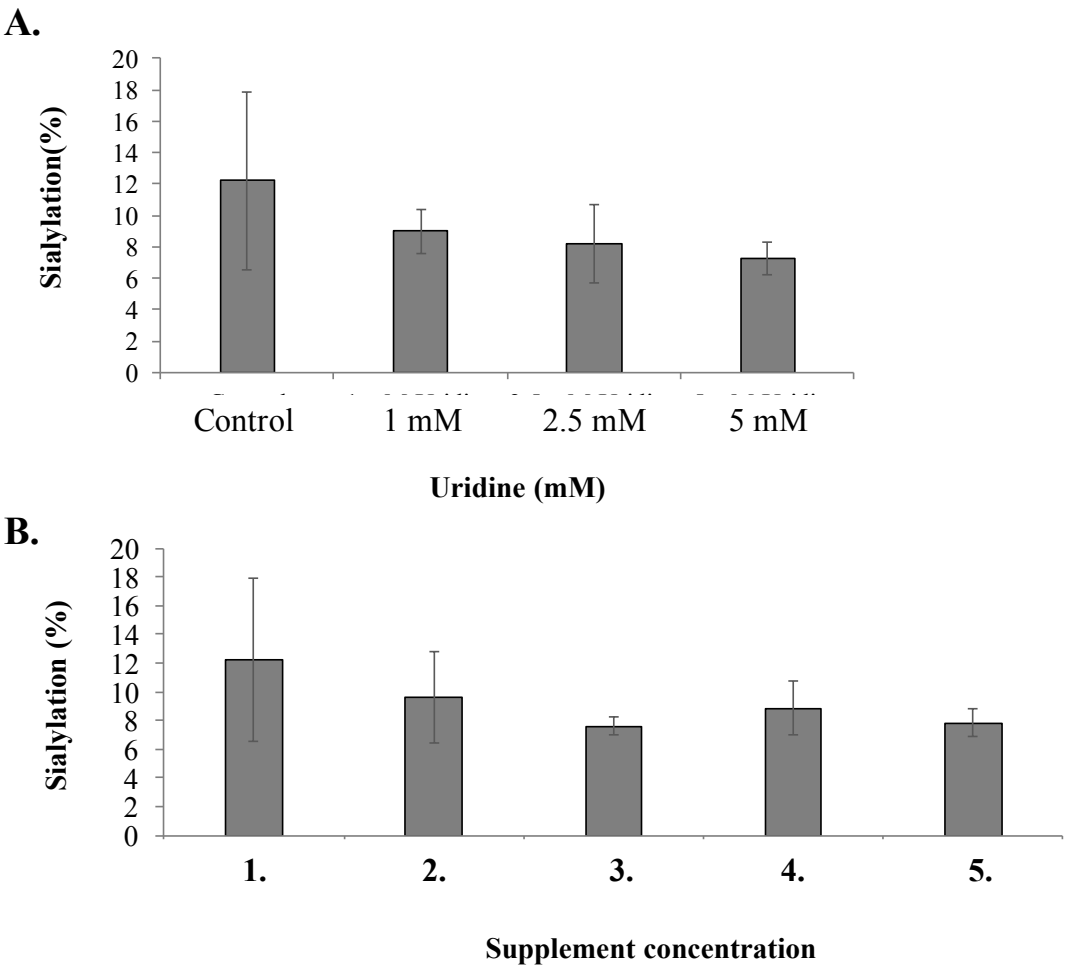
A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in Biogro™ media: **1.** without supplements (control); or supplemented with **2.** 25 μM $Ac_4GlcNAc$; **3.** 1mM Urd plus 25 μM $Ac_4GlcNAc$; **4.** 2.5 mM Urd plus 25 μM $Ac_4GlcNAc$; **5.** 5 mM Urd plus 25 μM $Ac_4GlcNAc$.

B. Galactosylation index (GI) was defined as: (G2+0.5*G1)/(G0+G1+G2).

Values correspond to the mean ± SD of duplicates (n = 2) of one independent experiment. Pairs of means ± SD significantly different (p-Value <0.05) with respect to the control culture.

Figure 7. 9 Effect of uridine and Ac_4GlcNAc on sialylation.

A. Increasing concentrations of the nucleoside uridine alone or **B**. in combination with 25 μM Ac_4GlcNAc. **1.** Control cultures did not show any supplement; **2.** 25 μM Ac_4GlcNAc; **3.** 1 mM Urd plus 25 μM Ac_4GlcNAc; **4.** 2.5 mM Urd plus 25 μM Ac_4GlcNAc; **5.** 5 mM Urd plus 25 μM Ac_4GlcNAc on the percentage of sialylated glycans in NS0-IgG1 Mabs. Values correspond to the mean ± SD of duplicates (n = 2) of one set of experiments. Pairs of means ± SD significantly different (p-Value <0.05) with respect to the control cultures.



The biochemical basis for these decrease in cell growth are poorly understood and has been attributed to different factors: **1.** Depletion of uracil pool caused by a diversion of UTP towards the activation of UDP-GlcNAc which in turn can result in a decrease in UDP-hexoses. **2.** Burden in cellular energy metabolism due to a decrease in adenine nucleotides involved in the phosphorylation of GlcN into GlcN6-P and synthesis of UTP via *de novo* and salvage pathways; **3.** Increase in pH caused by unionized ammonia (NH_3) accumulation from GlcN sugar deamination; **4.** Interference with normal glycosylation processing and **5.** Decrease in glycolysis due to competition for glucose transporters (Kornfeld, Kornfeld *et al.* 1964, Bekesi and Winzler 1969, Krug, Zweibaum *et al.* 1984, Doyle and Butler 1990, Pederson, Knop *et al.* 1992, Grammatikos, Tobien *et al.* 1999). Indeed, in addition to the decrease observed in cell growth, 100 μM Ac_4GlcNAc cultures also show a significant decrease of about 77% and 79% in q_{Glc} and q_{Lac} respectively. It is known that glucosamine competes with glucose for transport by three members of the glucose transporters family (GLUT1, GLUT2 and GLUT4) which differ in their affinity for glucose and glucosamine (Uldry, Ibberson *et al.* 2002, Yarema 2005). This competition of GlcN for sugar transport with glucose might be responsible for the reduction in cell growth and for the accumulation of exogenous glucose observed in cultures with 100 μM Ac_4GlcNAc. Thus, concentrations <100 μM Ac_4GlcNAc are desired to avoid adverse effects such inhibition of growth and glucose starvation due to the presence of glucosamine (Grammatikos, Valley *et al.* 1998, Zanghi, Mendoza *et al.* 1998).

Knowing that depletion of UTP might be responsible for the reduction in growth in GlcN-treated cultures, the non-natural Sia Ac_4GlcNAc was combined with increasing concentrations of uridine. However, the inhibitory effect of Ac_4GlcNAc on NS0 cell growth was not reversed by

addition of Urd, instead growth was further reduced by about 8-26% in 1-5 mM uridine *plus* 25μM Ac_4GlcNAc on day four. This is consistent with other studies where a decrease in viable cells was observed in GlcN or GlcN+Urd containing media (Zanghi, Mendoza *et al.* 1998). In addition, NS0 cells cultured in media containing 25 μM Ac_4GlcNAc+2.5-5 mM Urd showed a significant increase in *qGlc* (77%) with respect to the control. Interestingly, even though cultures containing 100 μM Ac_4GlcNAc; 2.5-5 mM Urd and 2.5-5 mM Urd- 25 μM Ac_4GlcNAc showed the lowest MCDs on day four, these cultures showed a significant increase in q_{Mab} of about ~25%, 17% and 40% respectively. These results are consistent with Zanghi *et al.* which also observed an increase in NCAM membrane protein production in GlcN and GlcN plus uridine treated cultures (Zanghi, Mendoza *et al.* 1998).

Modulating UDP-GlcNAc pool content by GlcN-feeding has shown different effects on the *N*-linked glycosylation depending on the cell line used (e.g. CHO, BHK), glycoprotein produced and cell culture process (e.g. FCS, media composition, T-flasks vs. bioreactors) (Gawlitzek, Valley *et al.* 1998, Grammatikos, Valley *et al.* 1998, Baker, Rendall *et al.* 2001, Yang and Butler 2002). Previous studies have distinguished the production of more complex oligosaccharides with low sialylation at higher concentrations of UDP-GlcNAc induced by high concentrations of ammonia (NH^{4+}) as well as by NH_4Cl- or GlcN-supplementation (Gawlitzek, Valley *et al.* 1998, Grammatikos, Valley *et al.* 1998, Baker, Rendall *et al.* 2001). In fact, a few studies suggest that the greatest the amount of UDP-GlcNAc, the greatest the decrease in sialylation (Zanghi, Mendoza *et al.* 1998). A decrease in a dose-dependence manner in Sia content was also observed in NCAM protein when small cell lung cancer (SCLC) and CHO cells were cultured in media with increasing concentrations of uridine (0-3.5 mM). This decrease in

sialylation was further intensified in GlcN *plus* uridine cultures (Zanghi, Mendoza *et al.* 1998). Similarly, treatment with 100 μM Ac_4GlcNAc showed the highest decrease (~6 and ~33%) in galactosylation and sialylation of NS0-IgG1 Mab. The latter was caused by a reduction in the ratio of mono- to di-sialylated (5:1) glycan structures with respect to the control (7:3). In contrast, Grammatikos *et al.* observed an increase in tri- and tetra-antennary structures in IL-Mu6 produced by BHK cells cultured in the presence of GlcN+Urd. Even though the UDP-GlcNAc pool increased by 35-41%, there was no change in the sialylation of IL-Mu6 produced in GlcN+Urd treated cultures (Grammatikos, Valley *et al.* 1998). In NS0-IgG1 Mab, incorporation of uridine slightly improved galactosylation to up to ~18% with respect to the control. However, the increase in Gal residues was not enough to improve sialylation in uridine-supplemented cultures. The latter is consistent with the results previously showed in Chapter 6, where M+U, M+G and M+U+G cultures showed an equivalent increase in GI but uridine containing cultures showed a lower sialylation in comparison to M+G. In addition, combination of 25 μM Ac_4GlcNac with 1-5 mM uridine also failed to affect sialylation in NS0-IgG1 Mab.

Due to the limited availability of Ac_4GlcNAc, further experimentations involving the evaluation of intracellular concentrations of UDP-GlcNAc were not possible in Ac_4GlcNAc +/- uridine-treated NS0 cells. Nevertheless, NS0 cells seemed to accumulate UDP-GlcNAc at an intracellular concentration of about 7.49 ± 1.99 fmole/cell which was not influenced by uridine, Gal or manganese supplementation (see Chapter 5). Interestingly, low concentrations of UDP-GlcNAc have been associated with a more homogeneous bi-antennary glycosylation; which is characteristic of immunoglobulins (Grammatikos, Valley *et al.* 1998, Raju, Briggs *et al.* 2000).

However, the possibility of higher UDP-GlcNAc concentrations by Ac_4GlcNAc+Urd treatment cannot be discarded and need to be confirmed by nucleotide sugar analysis.

In addition of serving as a Sia precursor, UDP-GlcNAc is used by *N*-acetylglucosaminyl transferases to assemble branched glycans. As previously mentioned, treatment with GlcN+/- uridine can result in more complex and highly branched oligosaccharides due to an increase in UDP-GlcNAc pools (Grammatikos, Valley *et al.* 1998). However, glycosylation in immunoglobulins is restricted to mainly bi-antennary glycans due to steric hindrance and limited accessibility in between the heavy chains by glycosyltransferases (Majid, Butler *et al.* 2007). Thus, an increase in UDP-GlcNAc concentrations might have a different effect on NS0-IgG1 Mab glycosylation than an increase in antennarity. Rijcken *et al.* suggested that higher accumulations of UDP-GlcNAc in the cytosol could be responsible for inhibiting transport of CMP-Sia to the Golgi apparatus (Pels Rijcken, Hooghwinkel *et al.* 1990, Pels Rijcken, Overdijk *et al.* 1995). As a result, CMP-Sia could accumulate and inhibit further synthesis of Sia by feedback inhibiting UDP-*N*-acetylglucosamine epimerase (Figure 7.1) (Pels Rijcken, Overdijk *et al.* 1995). Thus, further analysis of NS0 nucleotide sugar pool is necessary to confirm if any increase in CMP-Sia and UDP-GlcNAc pool has occurred in Ac_4GlcNAc+/- uridine treated cultures. In addition, it has been observed that GNE / MNK enzyme shows a specificity for physiological UDP-GlcNAc which renders chemically modified glucosamine analogues as poor candidates for improving sialylation (Bayer, Schubert *et al.* 2013)

Decreases in sialylation can also be a result of damage caused by released sialidase into the culture supernatant. Other studies have shown that sialidase, β-galactosidase and fucosidase

can be secreted by CHO cells. However, the presence of exoglycosidases was not investigated in NS0 cultures but it cannot be discarded, especially in cultures containing 100 μM Ac_4GlcNAc were viability decreased significantly. In cultures with both Ac_4GlcNAc *plus* Urd, higher viabilities than 100 μM Ac_4GlcNAc-fed cultures (~90%) were observed as well as the lack of changes in sialylation suggests that sialidase were not present. This could be confirmed by measuring sialidase concentrations in NS0 cell culture supernatant. Decrease in sialylation has also been associated with limited nucleotide sugar's transport to the Golgi as well as with a decrease in GalT and SiaT activity due to alkalinization of the *trans*- Golgi compartment induced by ammonium accumulation (optimal pH 6.5) (Gawlitzek, Ryll *et al.* 2000). Thus, it would be interesting to compare the effect that Ac_4GlcNAc and elevated ammonium concentrations have on NS0-IgG1 glycosylation.

7.6 Conclusions.

- Ac_4GlcNAc was inhibitory to cell growth at 100 μM, competing for the same transport system as glucose which resulted in a significant reduction in q_{Glc} of about 77%.
- Uridine had a dose-dependent negative effect of cell growth (MCDs ↓16%) which was intensified in Urd + 25μM Ac_4GlcNAc (MCDs ↓26%). However, specific productivity increased at 2.5 mM and 5 mM Uridine +/- 25 μM Ac_4GlcNAc (qMab ↑17 to 40%). In addition, 25 μM Ac_4GlcNAc+2.5-5 mM Urd showed an increase in q_{Glc} by 77% with respect to the control.
- Uridine and Ac_4GlcNAc failed to increase sialylation in NS0-IgG1 Mab. In accordance to previous reports a decrease in sialylation was observed >50 μM Ac_4GlcNAc.

- Uridine improved galactosylation by up to 18% at ≥2.5 mM when incorporated alone or in combination with 25 μM Ac_4GlcNAc.
- In contrast to other reports, antennarity of *N*-linked oligosaccharides of NSO-IgG1 Mab was not affected by Ac_4GlcNAc+/-Urd supplementation.
- Further experiments should investigate the levels of nucleotides and nucleotide sugars in Ac_4GlcNAc+/-Urd to further explain the changes observed in NS0 cells metabolism and sialylation. The latter would allow to explain if the decrease in cell growth observed in Ac_4GlcNAc cultures was an effect of nucleotide depletion (e.g. ATP, UTP) as well as if the decrease in sialylation was a response to UDP-GlcNAc accumulation inhibiting transport of Sia to the Golgi.

Chapter 8*

Effect of *N*-acetylmannosamine on the sialylation of IgG1 Mab

8.1.Introduction

N-acetylmannosamine (ManNAc) has been considered as an important linkage between the hexosamine and Sia pathways, which makes ManNAc the first specific precursor in the biosynthesis of Neu5Ac. **Figure 8.1** shows Sia biosynthesis in mammalian cells, which starts with the cytosolic nucleotide sugar UDP-GlcNAc which is converted to *N*-acetylmannosamine-6-P (ManNAc-6-P) by action of the bifunctional enzyme Uridine-diphospho-*N*-acetylglucosamine-2-epimerase / *N*-acetylmannosamine kinase (GNE/MNK) in two consecutive steps (Freeze and Elbein 2009). Then, condensation of ManNAc-6-P with phosphoenolypyruvate (PEP) results in Neu5Ac-9-P, which after dephosphorylation by a specific phosphatase yields free Neu5Ac (Martinez-Duncker and Tiralongo 2013, Yorke 2013). Free Sia derived from biosynthesis along with Sias recycled/recovered from the lysosome are activated into the nucleotide donor CMP-Sia by CMP- Sia synthetase (CSS) and then transported to the Golgi apparatus were the sugar is transferred to nascent glycan chains by sialyltransferases (Gu and Wang 1998). Consequently, sialylation depends on the availability of the nucleotide sugar precursor CMP-Sia as well as the adequate sialyltransferase activity to transfer Sia to terminal Gal residues (Gu and Wang 1998).

* Data presented in this Chapter have not been published. Sugar analogs were kindly prepared by Dr. Ed Bodnar from the Dr. Perreault's Lab from the Chemistry Department University of Manitoba.

Capping of terminal Gal residues by Sias helps glycoproteins escape from recognition by hepatocyte asialoglycoprotein receptors. Thus, high levels of terminal sialylation are desired in therapeutic glycoproteins to improve their serum half-life, quality and *in vivo* activity (Bork, Horstkorte *et al.* 2009, Yin 2017). In **Chapter** 7, the Sia precursor analog Ac_4GlcNAc was tested in its ability to increase terminal sialylation in IgG1 Mab produced by NS0 cells. However, sialylation was not further improved in Ac_4GlcNAc supplemented cultures and instead a decrease in terminal sialylation was observed at >50 μM. This chapter investigates the effect of another Sia precursor: ManNAc. Different studies have suggested that ManNAc supplementation can improve product sialylation by increasing the intracellular pools of CMP-Sia in the trans-Golgi (Gu and Wang 1998, Wong, Wati *et al.* 2010, Yorke 2013). However, in mammalian cells only minor proportions of the millimolar levels of UDP-GlcNAc is converted to ManNAc, indicating that ManNAc production is kept under tight control through allosteric feedback inhibition of GNE by CMP-Neu5Ac (Pels Rijcken, Overdijk *et al.* 1995, Rijcken 1995, Du, Meledeo *et al.* 2009). Thus, ManNAc feeding has been an useful tool to evade this checkpoint (Bayer, Schubert *et al.* 2013). For this reason, the following experiments tested the effect of ManNAc, in the form of peracetylated mannosamine (Ac_4ManNAc), on sialylation of NS0-IgG1 Mab (**Figure 8.1**). As previously mentioned, the incorporation of acetyl groups was intended to increase the hydrophobicity of ManNAc and thus increase the passive diffusion of Ac_4ManNAc into NS0 cells (Sarkar, Fritz *et al.* 1995, Luchansky, Goon *et al.* 2004, Doi K. 2010, Chen and Liang 2012, Savinova, Lobanova *et al.* 2015). In addition, Cyt supplementation has been suggested to help increasing the intracellular concentrations of CTP which in turn may enhance the conversion of Neu5Ac to CMP-Neu5Ac (Seppala, Tietze *et al.* 1991). However, an increase in the nucleotide

sugar pool has not always guaranteed an increase in sialylation and supplementation of key intermediates of the Sia pathway need to be evaluated in each cell line individually.

8.2.Objective.

- Evaluate the effect that the artificial analog Ac_4ManNAc alone or in combination with increasing concentrations of Cyt has on the *N*-glycosylation of NS0-IgG1 Mab, particularly their effect on sialylation.

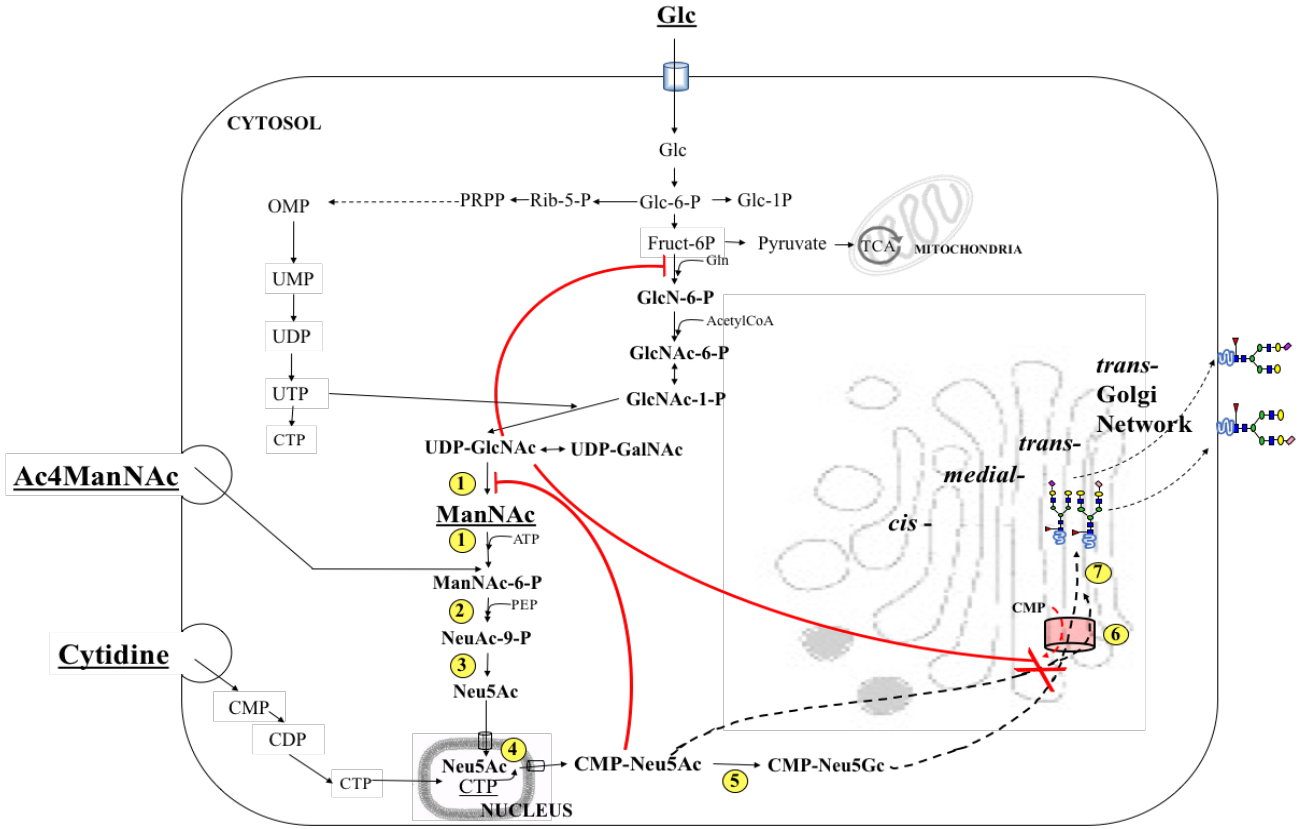
8.3.Experimental set up

8.3.1 Peracetylated mannosamine experiments

The effect of peracetylated mannosamine (Ac_4ManNAc) on the glycosylation of NS0-IgG1 Mab was determined by using a solution of 250 mM of Ac_4ManNAc which was dissolved in 100% ethanol and diluted with the required volume to reach: **a)** 100 μM, 200 μM, 500 μM and 1000 μM Ac_4ManNAc. Ac_4ManNAc dilution was then added to an empty 125 mL shaker flasks, where ethanol was allowed to evaporate prior to the addition of media and cells. The culture 0 μM Ac_4ManNAc contained the same volume of ethanol used to dissolve 1000 μM of Ac_4ManNAc but without the precursor to evaluate ethanol's effect if any in cell growth and viability.

Figure 8. 1 Representation of mammalian Sia metabolism.

Sia analog Ac_4ManNAc was used as a metabolic precursor for the biosynthesis of CMP-Neu5Ac in NS0 cells. Ac_4ManNAc was added at the beginning of a batch culture alone or in combination with Cyt. Enzymes and key steps of hexosamine pathways are: **1**. UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE); **2.** *N*-acetylneuraminic acid-9-phosphate synthase; **3.** *N*-acetylneuraminic acid-9-phosphatase; **4.** CMP-*N*-acetylneuraminic acid synthase; **5.** CMP-*N*-acetylneuraminic acid hydroxylase; **6**. Sia transporter; **7.** Sialyltransferases (trans-Golgi). Red flat arrows and cross indicate sites of feedback inhibition and trasnport inhibition respectively.



8.3.2 Peracetylated mannosamine *plus* Cyt experiments

The effect of increasing pyrimidine-nucleotide precursors Cyt alone or in combination with Ac_4ManNAc was evaluated on the sialylation of IgG1 Mab. Preliminary studies were focused on establishing the optimal Cyt concentrations that will promote cell growth. For this purpose, NS0 cells were seeded at 2.5 x 10^5 cells/mL in Biogro™ media supplemented with 0.5 mM, 1mM, 2.5 mM, 5 mM and/or 10 mM Cyt alone or in combination with 100 μM Ac_4ManNAc. Control samples had no supplement added. This set of experiments was done twice which resulted in a total of 4 samples per condition (*n=4*)

During the experiments, culture shake flasks were placed in a shake platform at 120 rpm were incubation took place for four days at 37°C with 10% CO_2. Daily sampling (900 μL) was performed to determine cell growth and viability of each condition by trypan blue exclusion method and for metabolite analysis and protein production. On day four, cell culture was terminated and supernatants were collected, filtered, concentrated and Mab was protein A purified before further analysis.

8.4.Results

8.4.1 Culture of NS0 cells in media containing Ac_4ManNAc

The following experiments focussed on evaluating the effect of the artificial Sia precursor Ac_4ManNAc on NS0 cells growth. This synthetic sugar was added at the beginning of a four-day batch culture and its effect on NS0 cell growth and viability are shown in **Figure 8.2**. NS0 cells

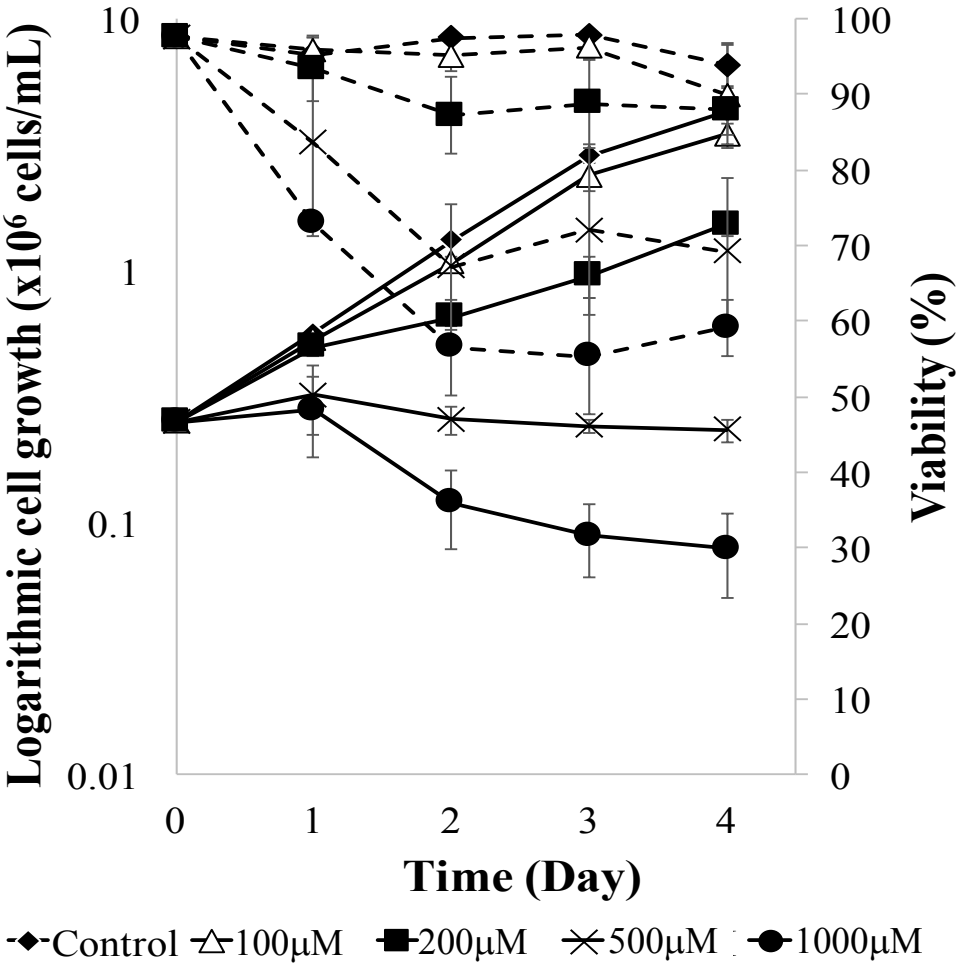
cultured in media containing 100 μM Ac_4ManNAc showed comparable growth rates (μ) with control cultures all along the culture period. In contrast, 200 μM Ac_4ManNAc-containing cultures showed a slightly lower but significant decrease in μ (≤0.02 h^{-1}) on day two and three when compared to the control (0.03 h^{-1}; p-Value ≤0.0009) (**Figure 8.2**) NS0 cells cultured in media with 500-1000 μM Ac_4ManNAc showed the lowest growth rate (μ=≤0.01 h^{-1}) since day one in comparison to the control (p-Value 0.04). MCDs were reached on day four in cultures containing <200 μM Ac_4ManNAc, with significantly lower MCDs by up to by 20% and 65% in 100 μM and 200 μM respectively when compared to the control (4.31 x 10^6 cells/mL; p-Value <0.0001). In contrast, cultures with 500 and 1000 μM reached their MCDs on day one (0.32± 0.1 x10^6 cells/mL and 0.28± 0.1 x10^6 cells/mL), which corresponds to a decrease of about 48% and 54% with respect to the control (0.61± 0.03 x10^6 cells/mL; p-Value ≤0.0025). Cell viabilities were over 90% in cultures containing ≤100 μM Ac_4ManNAc. In contrast, increasing Ac_4ManNc concentrations from 200 to 1000 μM compromised NS0 cells viabilities since day one (<80%). Consequently, it was decided to continue further experiments with 100 μM Ac_4ManNAc.

Finally, the effect of adding Cyt alone or in combination with Ac_4ManNAc on NSO cell growth is shown in **Figure 8.3A** and **8.3B** respectively. On day one, cultures containing 5-10 mM Cyt showed a significant decrease in cell viability to 83% and 74% respectively as well as the lowest cell densities (~67%) with respect to the control (93%) (p-Value <0.0001). A slight but still significant decrease in cell growth of about 21% was observed in cultures with 2.5 mM Cyt but cell viability was high as 97%. Incorporation of Ac_4ManNAc decreased significantly decrease in MCDs from 84% to up to 92% when combined with 0.5-2.5 mM Cyt respectively (p-Value

<0.0001). These cultures also showed a significant decrease in cell viability with 86% in comparison to the control with 97% on day one.

Figure 8. 2 The effect of Ac_4ManNAc on cell growth and viability of NS0 cells.

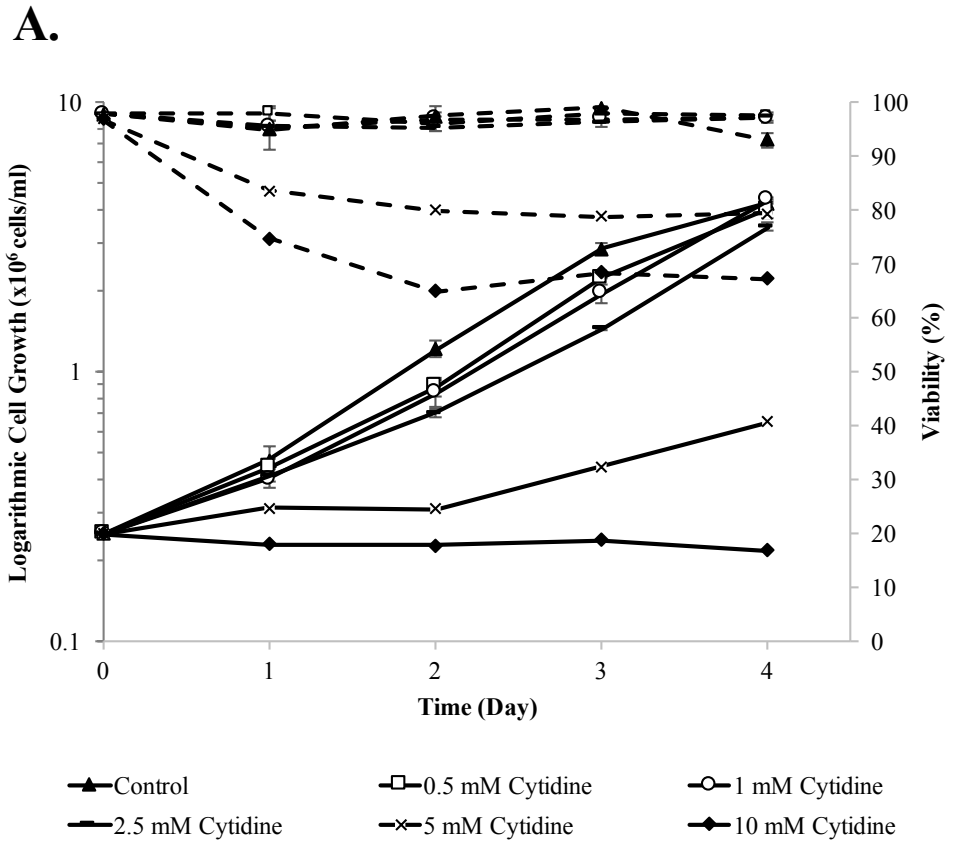
NS0 cells when cultured in Biogro™ media containing increasing concentrations of Ac_4ManNAc (0μM, 100 μM, 200 μM, 500 μM, 750 μM and 1000 μM). Control culture did not contain any supplement. Data points represent mean ± SD of duplicates of two set of independent experiments (n=4). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.



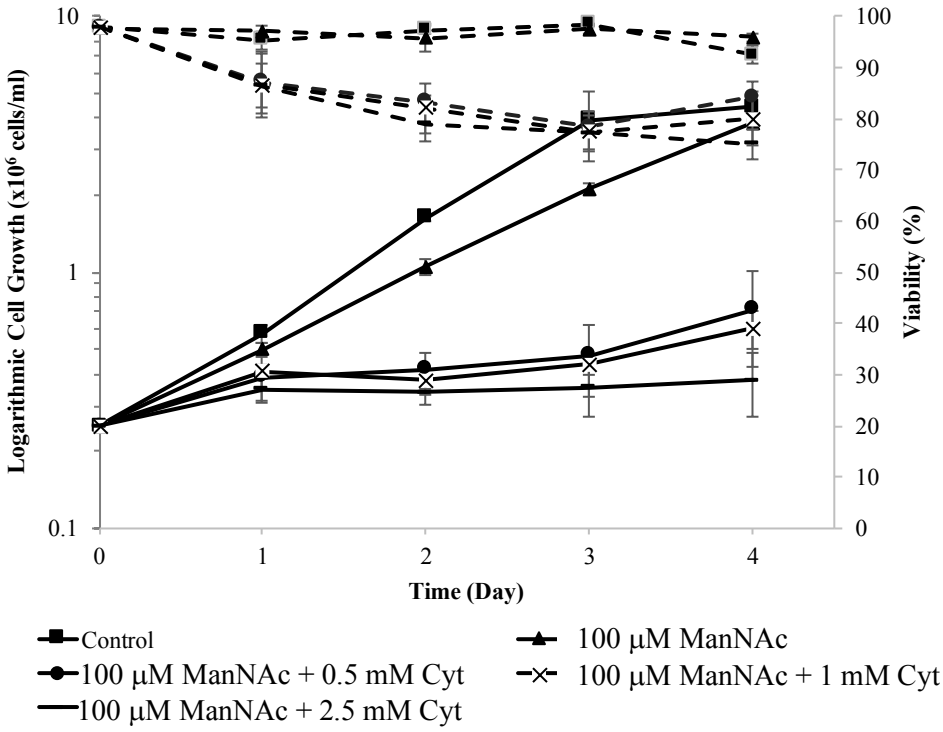
These results show that the analog sugar Ac_4ManNAc inhibited NS0 cell growth when concentrations reached >200 μM with a significant decrease of up to 98% and 37% in MCDs and viability respectively on day four. Finally, incorporation of Cyt alone slightly affected cell growth but in combination with Ac_4ManNAc viability and MCDs significantly decreased by up to 86% and 92% respectively when combined with 2.5 mM Cyt.

Figure 8. 3 The effect of Cyt on cell growth and viability of NS0 cells.

A. Cyt: 0.5 mM, 1 mM, 2.5 mM, 5 mM, and 10 mM and **B.** 100 μM Ac_4ManNAc alone or in combination with 0.5 mM, 1 mM and 2.5 mM Control culture did not contain any supplement. Data points represent mean ± SD of duplicates for each condition (n=2). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay.



B.



8.4.2 Effect of peracetylated mannosamine on NS0 cells' metabolism.

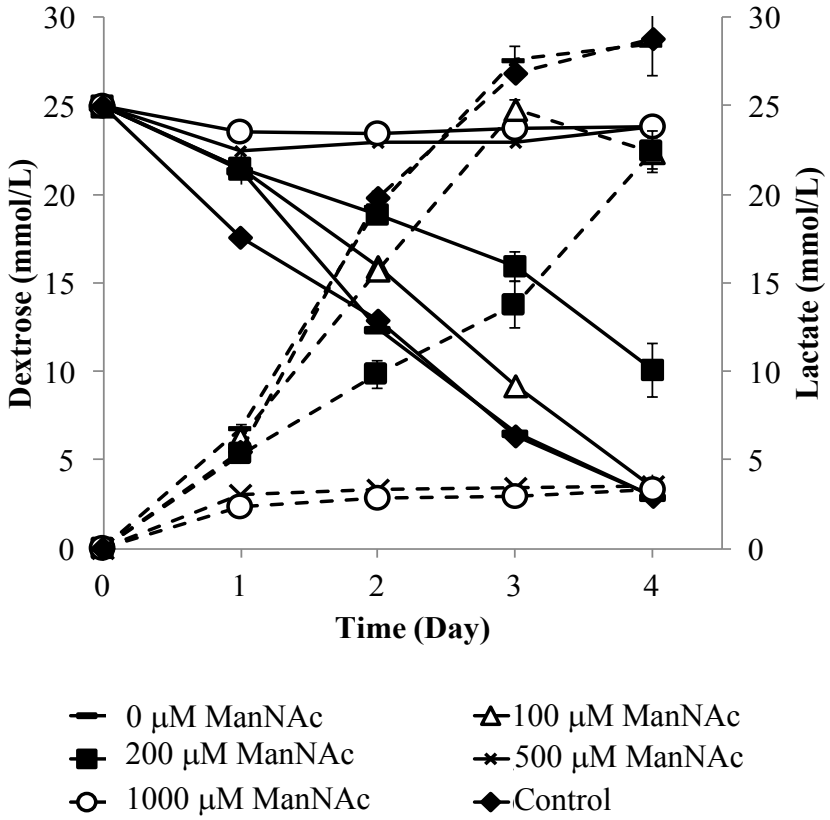
Ac_4ManNAc influenced NS0 cells growth when concentrations >100 μM Ac_4ManNAc were added to the media. To evaluate Ac_4ManNAc effect on NS0 cells' metabolism, supernatant samples were daily taken to monitor glucose consumption and lactate production. Specific rates were calculated by plotting glucose and lactate concentrations against the integral values of NS0 cell's growth curve. The specific glucose consumption (q_{Glc}) in cultures containing 100 μM Ac_4ManNAc where comparable to control cultures throughout the culture period. However, NS0 cultures containing 200 μM Ac_4ManNAc showed a significant decrease of about 52% in q_{Glc} on day two (p-Value 0.0025), along with a higher amount of residual glucose on day four (~10mM) in comparison to the control (~2.93 mM) (p-Value 0.0002). NS0 cultures containing ≥ 500 μM

Ac_4ManNAc showed a significant decrease on q_{Glc} of about 50% on day one (p-Value 0.006), after which glucose consumption stopped, accumulating glucose in the media at a concentration of ~24 mM until day four (**Figure 8.4**). Thus, a positive q_{Glc} was observed since day two (1.32 μmol/10^6 cells.day; p-Value <0.0001). With respect to specific lactate production, the control and 100 μM Ac_4ManNAc-containing cultures showed high production rates during the first two days with up to 14.3 μmol/10^6 cells.day. In contrast, cultures containing 200 μM Ac_4ManNAc showed a significantly lower q_{Lac} by 47% on day two when compared to the control (p-Value 0.0001). The least amount of lactate was produced by cells cultured in media containing 500 and 1000 μM Ac_4ManNAc with a q_{Lac} of 8.67 μmol/10^6 cells.day and 7.60 μmol/10^6cells.day on day one compared to the control with 15.32 μmol/10^6 cells.day (p-Value <0.005). After day one, 500 and 1000 μM Ac_4ManNAc-containing cultures' q_{Lac} decreased (<1.85 μmol/10^6cells.day (p-Value <0.0001). Thus, lactate accumulated in cell culture media to up to 29 mM in cultures containing 0-200 μM Ac_4ManNAc in comparison to >500 μM Ac_4ManNAc with 3 mM (**Table 8.1**). The $Y_{Lac/Glc}$ was estimated from the slope of a plot of q_{Lac} versus q_{Glc}, which did not show any significant difference between Ac_4ManNAc-containing cultures and the control culture.

These results show that Ac_4ManNAc affected significantly NS0 cells metabolism when used at >100 μM. These was reflected by a significant decrease in q_{Glc} of up to 96 % in cultures with 1000 μM Ac_4ManNAc which lead to glucose accumulation in the media at 24 mM. In accordance to these results, lactate production was significantly lower by 94% in cultures containing 500-1000 μM Ac_4ManNAc with respect to the control.

Figure 8. 4 The effect of Ac_4ManNAc on the cell metabolism of NS0 cells.

Glucose (solid lanes) and lactate (dashed lanes) concentrations (mM) were determined during a four-day batch culture. NS0 cells were seeded in media with increasing concentrations of Ac_4ManNAc (0-1000 μM). Control culture did not contain any supplement. Data points represent mean ± SD of duplicates samples for each condition (n=4) of two set of experiments.



8.4.3 Effect of Ac_4ManNAc and Cyt on the production of IgG1 Mab by NS0 cells.

Knowing that Ac_4ManNAc affected NS0 cell growth and metabolism, the next experiments focused on evaluating the effect of Ac_4ManNAc on IgG1 production. Mab concentration decreased from about 11% to up to 96% as Ac_4ManNAc concentration increased from 100 μM to 1000 μM respectively on day 4 (p-Value <0.0001); **Table 8.1)**. In particular, cultures with >500 μM Ac_4ManNAc showed a significant lower volumetric product since day one

which did not improve in the remaining culture period, showing a comparable *qMab* to control cultures with 4.53±0.93 μg/10^6 cells.day (p-Value <0.0001). **Table 8.1** shows the specific cell productivity calculated by plotting NS0-IgG1 concentration (μg/mL) against the integral viable cell density (IVCD). Specific cell productivities were not affected in Ac_4ManNAc containing cultures with respect to the control with 4.95-7.16 μg/10^6 cells.day. A more significant reduction (36%-62%) in volumetric productivity was observed in cultures with >500 μM Ac_4ManNAc on day one (p-Value <0.0001). **Figure 8.5A** shows Cyt's influence in IgG1 Mab productivity in NS0 cells. IgG1 production decreased significantly from 12% to up to 28% as the concentration of Cyt increased from 1 mM to 2.5 mM respectively when compared to untreated cultures (p-Value <0.05). However, specific cell productivity was not affected by Cyt supplementation (**Figure 8.5B**). With respect to Ac_4ManNAc cultures, a significant decrease of about 45% in Mab yield was observed at 100 μM. Further impact was observed when Ac_4ManNAc was combined with 0.5 mM, 2.5 mM and 5 mM Cyt, with a significant decrease of about 56%, 69% and 77% respectively (p-Value <0.0001). However, there was not an observable trend in specific cell productivity.

These results show that a significant decrease Mab yield was observed in cultures supplemented with increasing concentrations of Ac_4ManNAc of up to 96%. Combining Cyt with Ac_4ManNAc had a more pronounced negative effect in volumetric productivity than when Cyt or Ac_4ManNAc where supplemented on their own. However, none of the supplements affected the specific cell productivity of NS0 cells.

8.4.4 Effect of Ac_4ManNAc on *N*-glycosylation of NS0-IgG1 Mab.

The effect of Ac_4ManNAc feeding on *N*-linked glycosylation of NS0-IgG1 Mabs produced in Biogro™ media supplemented with 0, 100, 200, 500 and 1000 μM Ac_4ManNAc is shown in **Figure 8.6** and **8.7.** Control cultures and Ac_4ManNAc supplemented cultures showed the same three main peaks corresponding to F6A2G0, F6A2G1 and F6A2G2 (**Figure 8.7**); however, the proportions of Gal-containing glycans changed depending on the concentration of Ac_4ManNAc added to the media **(Figure 8.6A)**. For instance, cultures containing 100 μM Ac_4ManNAc showed the same glycan distribution (G0<G1<G2) as non-supplemented cultures. However, a significantly higher proportion of G1 glycans (47.2%±2.76) was observed in 100 μM Ac_4ManNAc cultures (p-Value <0.05). An increase of about 38% and 12% was observed in G0 and G1-containing glycans in 200 μM Ac_4ManNAc-fed cultures with respect to the control (p-Value <0.01).

The highest increase in non-galactosylated glycans of up to 74% along with a significant decrease of about 36% in G2-glycans was observed in cultures containing >500 μM Ac_4ManNAc with respect to the control (p-Value <0.004). As a result, the GI significantly decreased by up to 18%, 23% and 17% respectively in cultures supplemented with 200, 500 and 1000 μM Ac_4ManNAc respectively (p-Value ≤0.008; **Figure 8.6B** and **8.7).** As previously described, NS0-IgG1 Mabs showed the following sialylated glycans: A2G2S1, F6A2G1S1, F6A2G2Ga1, F6A2G2S1, F6A2G2S2 and F6A2G2Ga1S1/S2 (**Figure 8.8**).

Table 8. 1. Culture parameters of NS0 cells cultured in media containing increasing concentrations of Ac_4ManNAc.

Control sample did not include any supplement. Daily samples were taken to measure glucose consumption, lactate production and cell productivity. Cell-specific glucose uptake (q_{Glc}) and by-product formation (q_{Lac}). The $Y_{Lac/Glc}$ corresponds to the amount of lactate produce per mole of glucose. The data shows the average and standard deviation corresponding to duplicates from two independent experiments (n=4)

Ac4ManNAc (μ M)	Glc[a] (day)	Residual Glc (mM)	Residual lactate (mM)	q_{Glc} [b] (μ mol/10 ^{6} cell.day)	q_{Lac} [b] (μ mol/10 ^{6} cell.day)	$Y_{Lac/Glc}$ [c] (mol/mol)	Mab Yield[d] (μ g/mL)	q_{Mab} [c] (μ g/10 ^{6} cell.day)
0	4	2.93 ± 0.08	28.6 ± 1.10	-9.21 ± 0.37	14.3 ± 1.30	1.30 ± 0.05	38.8 ± 6.60	4.95 ± 0.72
100	4	3.48 ± 0.70	22.3 ± 0.00	-7.16 ± 0.82	**12.2 ± 0.80*****	1.04 ± 0.03	**34.3 ± 1.85***	5.94 ± 0.22
200	4	10.0 ± 1.51	22.4 ± 1.13	**-4.38 ± 0.35****	**7.51 ± 1.32*****	1.50 ± 0.08	**18.8 ± 0.77*****	6.10 ± 0.84
500	2	23.9 ± 0.35	3.54 ± 0.43	**1.32 ± 1.49*****	**0.84 ± 0.15*****	1.19 ± 0.60	**2.21 ± 0.28*****	7.16 ± 1.89
1000	2	23.8 ± 0.00	3.35 ± 0.00	**-0.37 ± 2.62*****	**1.85 ± 0.00*****	1.54 ± 0.00	**1.71 ± 0.68*****	4.53 ± 0.93

a) Day at which NS0 cells stopped consuming glucose

b) Measured on day two where all cultures where consuming glucose.

c) Over the period of time where glucose was still being consumed a)

d) Measured on day 4.

e) Pair of means ± SD significantly different (p-Value 0.01*; 0.002**; <0.0001***) with respect to the control are shown in bold

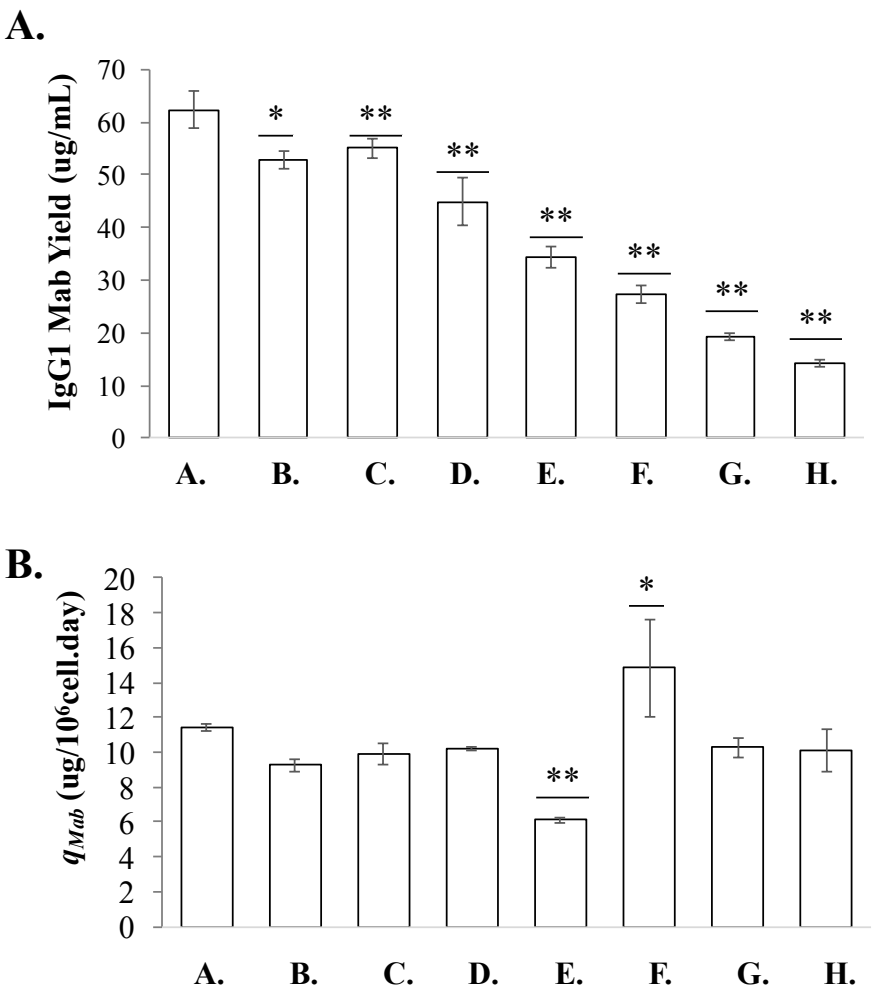


Figure 8. 5 IgG1 Mab production by NS0 cells cultured in media containing Cyt and Ac_4ManNAc.

A. culture media supplemented with **B.** 0.5 mM Cyt; **C.** 2.5 mM Cyt; **D**. 5 mM Cyt; **E.** 100 μM Ac_4ManNAc; **F.** 0.5 mM Cyt + 100 μM Ac_4ManNAc; **G**. 2.5 mM Cyt + 100 μM Ac_4ManNAc and **H.** 5 mM Cyt + 100 μM Ac_4ManNAc. **B**. Specific cell productivity was determined on NS0 cells from the slope of the plot of IgG1 Mab yield and the integral viable cell density (IVCD) values. The data shows the average and standard deviation corresponding to duplicates from one independent experiment (p-Value= *<0.05; **≤0.0001; n=2)

A significant decrease in sialylation was observed as Ac_4ManNAc concentration increased from 0 μM (total sialylation = 14.7% ± 3.23) to 100, 200, 500 and 1000 μM (total sialylation = 11.1% ± 2.86; 5.27% ± 0.62; 6.66% ± 2.03; and 8.83% ± 3.41 respectively) (**Figure 8.7**). However, a slight increase in sialylation was observed between 200 and 1000 μM Ac_4ManNAc. The latter could have resulted from low signal intensities observed in cultures containing 500-1000 μM Ac_4ManNAc. Since, sialylated glycans eluted after 38 minutes and corresponded to the smallest peaks detected in the chromatograph, the high variability between sialylated peak areas could have resulted from the low signal intensities (high baseline) observed at >200μM Ac_4ManNAc in comparison to the control (**Figure 8.7**).

Ac_4ManNAc supplementation was expected to increase sialylation by enhancing Neu5Ac biosynthesis. However, sialylation was not improved and probably higher concentrations of CTP were also required for Sia activation prior to attachment to oligosaccharides. For this reason, Cyt was evaluated alone (**Figure 8.9**) and then in combination with Ac_4ManNAc (**Figure 8.10**) on their effect on glycosylation and sialylation. Control cultures showed 19.6% ± 3.50, 46.5% ± 2.95 and 3.9% ± 4.22 of G0, G1 and G2 glycans respectively. In contrast, 2.5 mM Cyt containing cultures showed a significantly decrease of up to 37% in G0 glycans along with an increase of up to 28% in G2 glycans when compared to the control (p-Value <0.05; **Figure 8.9A**). Consequently, a significant increase in the galactosylation index (GI) of 15% was observed in 2.5 mM Cyt - supplemented cultures (**Figure 8.9 B**; p-Value 0.002).

Figure 8. 6 Effect of Ac_4ManNAc on the N-glycosylation of NS0-IgG1 Mabs.

A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in media supplemented with 0, 100, 200, 500 and 1000 Ac_4ManNAc.Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. **B.** Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). Values correspond to the mean ± SD of duplicates from three different set of experiments (n = 6). Pairs of means ± SD significantly different (p-Value <0.05*; <0.01**; <0.0001**) with respect to the control culture.

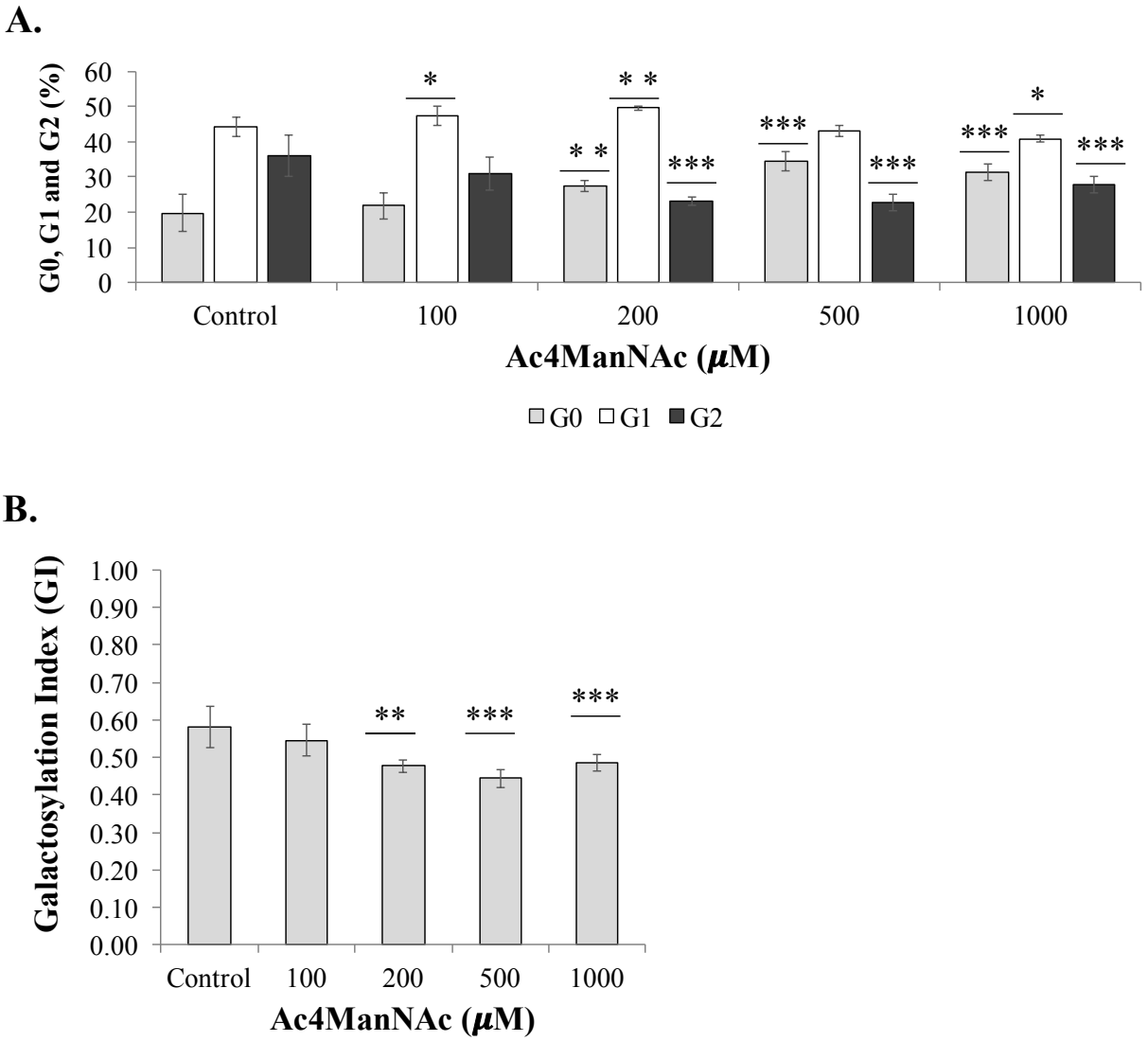


Figure 8. 7 Relative abundance of glycans containing Sias (%) from NS0-IgG1 samples cultured in media supplemented Ac_4ManNAc.

Values correspond to the mean ± SD of duplicates of three independent experiments (n = 6).

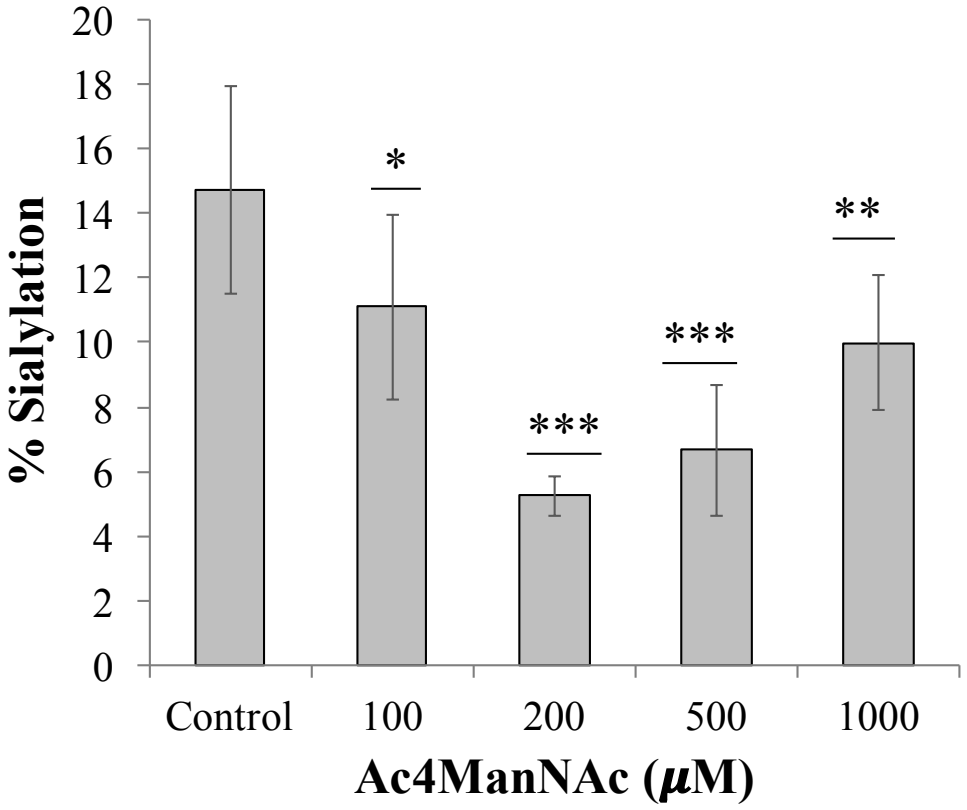
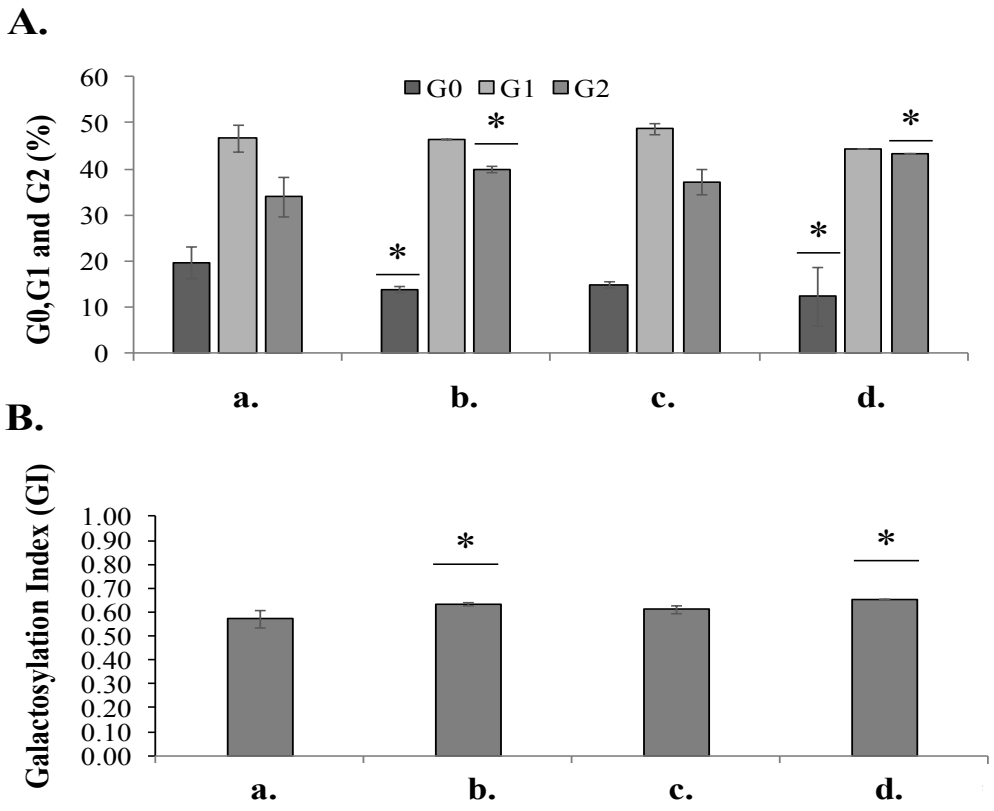


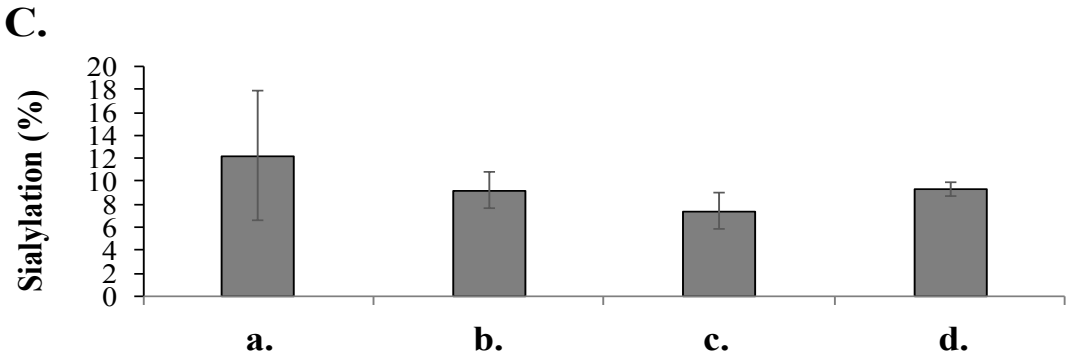
Figure 8.9A. shows the relative abundance of Gal containing glycans when 100 μM Ac_4ManNAc was combined with 0.5, 1 and 2.5 mM Cyt. No differences were observed in cultures with 0.5-1 mM Cyt and 100 μM Ac_4ManNAc with respect to the control. A slight change in Gal -containing glycans distribution was observed in cultures with 100 μM Ac_4ManNAc+2.5 mM Cyt, with a significant increase (36%) in G0 glycans along with a significant decrease in G2 glycans when compared to the untreated cultures. The presence of more highly non-galactosylated glycans in Ac_4ManNAc+2.5 mM Cyt cultures caused a decrease in GI of 12% when compared to the control **(Figure 8.9B**). With respect to branches terminating in Sia constituted 12.2% ± 5.68 of the

total glycan pool in control cultures (**Figure 8.9C**). A slight decrease in sialylated glycans were observed in cultures containing Cyt alone, but this decrease was not significant when compared to the control. However, combination of Cyt with 100 μM Ac_4ManNAc caused a more significant decrease in sialylation of up to 76% when compared to untreated cultures (p-Value <0.05, **Figure 8.9C**).

Figure 8. 8 Effect of Cyt on the N-glycosylation of NS0-IgG1 Mabs.

A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in media supplemented with **a.** 0 mM; **b.** 0.5 mM, **c.** 1 mM and **d.** 2.5 mM Cyt. Galactosylation index (GI) was defined as (G2+0.5*G1)/(G0+G1+G2). C. Percentage sialylation from IgG1 Mabs. Pairs of means ± SD significantly different (p-Value <0.05*; n = 2) with respect to the control culture.





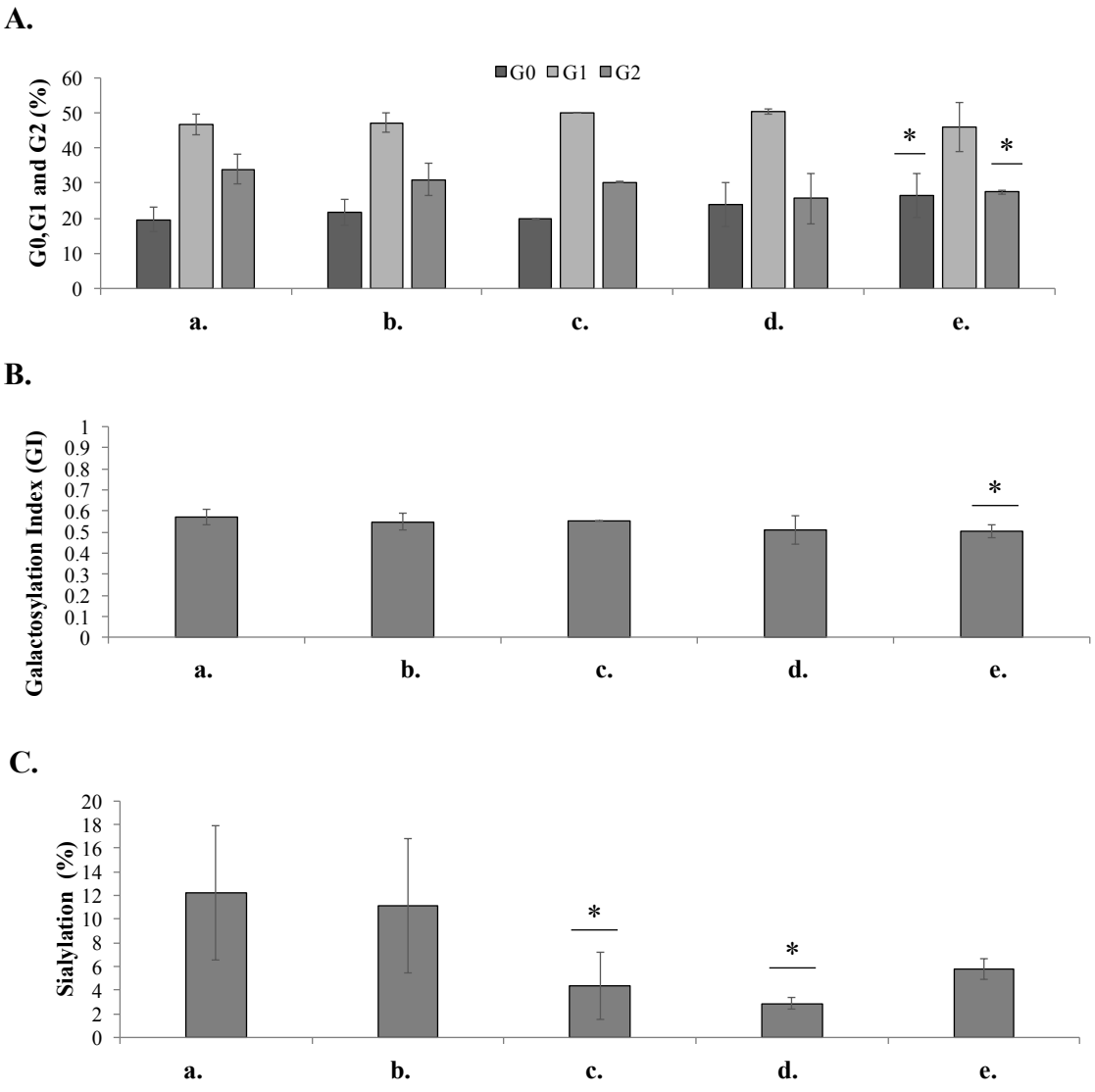
These results show that Ac_4ManNAc impacted negatively NS0-IgG1 Mab *N*-glycosylation. Galactosylation decreased significantly by up to 17%-23% in cultures containing 200-1000 μM Ac_4ManNAc. In the absence of Gal residues, sialyltransferases were not able to add Sias to oligosaccharides which led to a decrease by up to 64% in sialylation in cultures with >200 μM Ac_4ManNAc. Cyt incorporation in culture media at 2.5 mM had a positive effect on galactosylation, reducing G0 and increasing G2 glycans by up to 37% and 28 % respectively. The latter further improved the GI by 15% but without any effect on sialylation. When combined with 100 μM Ac_4ManNAc, Cyt had a negative effect in sialylation that was not previously observed at 100 μM Ac_4ManNAc.

8.5.Discussion

Several studies have focused in nucleotide sugar precursor feeding strategies to increase the intracellular concentrations of CMP-Sia to improve recombinant proteins sialylation (Pels Rijcken, Overdijk *et al.* 1995, Gu and Wang 1998, Savinova, Lobanova *et al.* 2015).

Figure 8. 9 Effect of Ac_4ManNAc and Cyt on the N-glycosylation of NS0-IgG1 Mabs.

A. Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2) from NS0-IgG1 samples cultured in media supplemented with **a.** 0 mM; b. 100 μM Ac_4ManNAc; **c.** 0.5 mM Cyt + 100 μM Ac_4ManNAc; **d.** 1 mM Cyt + 100 μM Ac_4ManNAc; **e**. 2.5 mM Cyt + 100 μM Ac_4ManNAc. **B.** GI was defined as (G2+0.5*G1)/(G0+G1+G2). **C.** Percentage sialylation from IgG1 Mabs. Values correspond to the mean ± SD of duplicates from duplicates (n = 2). Pairs of means ± SD significantly different (p-Value <0.05*) with respect to the control culture



In previous chapters, an increase in both galactosylation and sialylation was observed when Gal, Urd and M were added to NS0 cultures (**Chapter 3**). In contrast, a decrease in sialylation was observed in IgG1 Mab produced in media containing Ac_4GlcNAc+/-Urd (**Chapter 7**). In this chapter, the next approach was to test the influence that the immediate Sia precursor ManNAc had on sialylation of IgG1 Mab. Supplementation with ManNAc +/- Cyt has shown to successfully increase the intracellular pools of CMP-Sia the immediate substrate for sialyltransferases (Seppala, Tietze *et al.* 1991, Pels Rijcken, Overdijk *et al.* 1995, Gu and Wang 1998, Doi K. 2010, Wong, Wati *et al.* 2010). However, most reports have used high concentrations to induce a change in sialylation due to ManNAc poor cell permeability (Pels Rijcken, Overdijk *et al.* 1995, Luchansky, Goon *et al.* 2004, Wong, Wati *et al.* 2010). As an alternative, mannosamine bearing non-natural chemical substituents (e.g. Ac_4ManNAc, Prop4ManNAc, But4ManNAc) has been used to selectively tune the sialylation of individual surface glycans (Sarkar, Fritz *et al.* 1995, Kim, Sampathkumar *et al.* 2004, Luchansky, Goon *et al.* 2004, Chen and Liang 2012, Savinova, Lobanova *et al.* 2015). For instance, analog-treated pancreatic cancer cells (SW1990) showed a significant higher sialylation, enhancing their ability to migrate across the extracellular matrix. In contrast, 1,3,4-O-Bu_3ManNAc -treated cell adhesion molecules (e.g. CD44 and integrin $\alpha 6$) showed a more modest increase in sialylation, indicating that the activities of these proteins is sensitive to sialylation. Thus, the effect that ManNAc analogs can have on sialylation will depend on the initial degree of sialylation and type of cell. In this chapter, an analog of ManNAc: Ac_4ManNAc was used in an attempt to improve sialylation in IgG1 Mab produced by NS0 cells as is has shown to have 600 times higher uptake efficiency than ManNAc(Kim, Sampathkumar *et al.* 2004, Chen and Liang 2012). However, addition of ≥200 μM Ac_4ManNAc had a negative impact on IgG1 Mab galactosylation and sialylation. Particularly, an increase in non-

galactosylated glycans was observed with a significant decrease in GI by up to 48% in comparison to untreated control cultures. As a result, in the absence of terminal Gal glycans, Sias attachment declined by up to 25-64% in cultures containing ≥100 μM Ac_4ManNAc. In contrast, a slightly (3-16%) but significant increase in galactosylation was observed in 0.5-2.5 mM Cyt containing cultures but without any change in sialylation. In the same way, combining Cyt with 100 μM Ac_4ManNAc did not have an impact on IgG1 Mab sialylation. Feeding of Ac_4ManNAc +/- Cyt was expected to favor Sia biosynthesis by overpassing the well-known feedback inhibition of GNE by CMP-Sia, which impedes further conversion of UDP-GlcNAc into ManNAc. However, the concentration of intracellular CMP-Sia is not known in these experiments due to limited amounts of Ac_4ManNac. The protocol used for quenching and extraction of metabolites requires at least $1x10^7$ cells for analysis, which was difficult to obtain specially in cultures with such low final cell densities (≥200 μM Ac_4ManNAc). In any case, even if an increase in CMP-Sia is considered, sialylation was not improved by Ac_4ManNAc nor by Cyt. A lack of improvement in sialylation even after an increase in CMP-Sia has been related to an inhibition in transport of the Sia precursor across the Golgi membrane. In accordance with this, Cacan *et al.* described the existence of an intraluminal pool (CMP-Neu5Ac and CMP) which regulates/inhibits the translocation of CMP-Sia into Golgi vesicles (Cacan, Cecchelli *et al.* 1984, Capasso and Hirschberg 1984, Pels Rijcken, Overdijk *et al.* 1995) (Figure 8.1).

A possible explanation for the decrease in sialylation observed in IgG1 Mab could be the toxicity of Ac_4ManNAc at ≥ 200 μM Ac_4ManNAc shown by the significant decrease (up to 71%) in cell viability and glucose consumption (up to 52%) in comparison to the control. In fact, a correlation was observed between a decrease in viability as the concentration of Ac_4ManNAc

increased from 0 to 1000 μM (R^2=0.95; data not shown). Kim *et al.* related the growth inhibition and loss of cell viability in ManNAc-analog treated Jurkat cells to apoptosis initiated by these compounds (Kim, Sampathkumar *et al.* 2004). Chen *et al.* also observed a decrease in cell growth but at >1000 μM Ac_4ManNAc indicating that accumulation of high concentrations of acetic acids could be responsible for decreasing the intracellular pH of cells. However, the same group observed an increase of up to 2-3-fold in sialylation at <500 μM Ac_4ManNAc after 12 hours of incubation and a rapid decline after 24 hours. This increase in sialylation was correlated with higher cell growth and cell viability (Chen and Liang 2012). In this chapter, sialylation was evaluated after four days of incubation in media supplemented with Ac_4ManNAc. At this point, viability and cell growth were already compromised which could suggest the release of sialidase in culture media by lysed cells. In accordance to this, previous reports have shown that lysosomal and cytosolic sialydases activity was upregulated in ManNAc +/- Cyt containing cultures (Wong, Wati *et al.* 2010). This is in addition to the previously seen downregulation of sialyltransferase genes in cells experiencing ManNAc analog-induced apoptosis (Kim, Sampathkumar *et al.* 2004) . With respect to Cyt, there was not a decrease in cell growth and viability at concentrations below 5 mM; however, combination of Cyt with100 μM Ac_4ManNAc seemed to affect cell growth and viability in NS0 cells.

Further experimentation is needed to confirm if Ac_4ManNAc is indeed causing apoptosis and consequently reducing sialylation in NS0 cells (e.g. DNA fragmentation analysis, Anexin V binding assays). In addition, culture media should be monitored daily for the presence of sialidase to discard the possibility that the decrease in sialylation was in fact a result of exoglycosidases.

8.6.Conclusions

- A decline in cell viability was correlated with increasing concentrations of Ac_4ManNAc.

- A significant decrease in galactosylation index of up 48% was observed in cultures with ≥200 μM Ac_4ManNAc.

- A significant decrease of up to 25-64% was observed in sialylation in cultures with ≥100 μM Ac_4ManNAc.

- Increasing concentrations of Cyt induced a decrease in IgG1 Mab yield of up to 28% which was intensified by combination with 100 μM Ac_4ManNAc (up to 77%).

- Cyt increased galactosylation by up to 16% without any effect on sialylation

- There was no synergistic effect on glycosylation by combining Ac_4ManNAc and Cyt.

- The lack of influence of Cyt and Ac_4ManNAc on sialylation can be further explained by analyzing CMP-Sia pools which will tell if Ac_4ManNAc+Cyt indeed caused an increase in this nucleotide sugar

- Presence of sialidase in supernatant could be responsible for the decline /no change in sialylation in these experiments

- Viability tests will be useful to see if this ManNAc analog is inducing apoptosis in NS0 cells and thus sialylation.

- Multiple feedback inhibition steps are present in the Sia pathway which make difficult to improve sialylation in mammalian recombinant proteins. Impaired transport of CMP-Sia across the Golgi membrane could have resulted as a response of CMP or CMP-Sia accumulation inside the Golgi.

Section C

Nutrient supplements effect on the proportion of Neu5Gc in NS0-IgG1 Mab

In preceding chapters, different feeding strategies were used to increase the degree of sialylation of two monoclonal antibodies. However, an increase in sialylation could also mean an increase in Neu5Gc which is undesirable during the production of therapeutic glycoproteins. Neu5Gc cannot be produced by human cells due to a genetic mutation in the CMAH gene. The latter encodes for the cytidine mono-phosphate-*N*-acetylhydroxylase (CMAH) that converts Neu5Ac to Neu5Gc in the cytosol of most animal species (Muchmore EA 1989). Despite of Neu5Gc deficiency in humans, Neu5Gc has been found at high concentrations in certain tumors and at small concentrations in normal human epithelial and endothelial blood vessels (Samraj, Laubli *et al.* 2014). It has been suggested that exogenous Neu5Gc can be metabolically incorporated into human cells from dietary sources (e.g. red meats, dairy products) (Tangvoranuntakul, Gagneux *et al.* 2003). The latter was confirmed in oral ingestion studies of Neu5Gc in human volunteers where traces were incorporated into newly synthesized glycoproteins and/or eliminated in urine (Tangvoranuntakul, Gagneux *et al.* 2003). This was not different in *in vitro* studies, where human epithelial cells, neuroblastomas and human carcinomas were able to incorporate exogenous free-Neu5Gc and Neu5Gc from animal products such as fetal calf serum (Tangvoranuntakul, Gagneux *et al.* 2003, Varki, Hedlund *et al.* 2013, Naito-Matsui, Davies *et al.* 2017). However, for exogenous Neu5Gc to be incorporated, cells must contain, the lysosomal transporter as well as of the lysosomal sialidase (Varki, Hedlund *et al.* 2013). After lysosomal

degradation, Neu5Gc is transported to the cytosol where it can be recycled and converted into its respective CMP-Sia form (Ajit Varki 2009). Thus, Sia transporters and sialyltransferases can transport or utilize either CMP-Neu5Ac or CMP-Neu5Gc without any strong preference.

Knowing the immunogenicity and clearance issues related to Neu5Gc, it is important to minimize the expression of Neu5Gc in protein therapeutics for human use. For instance, efforts have focused on choosing a mammalian expression host that can produce highly sialylated glycoproteins but with low content of Neu5Gc. However, most of the cell lines used to produce human therapeutics are not typically of human origin and are known to contain Neu5Gc at different levels. For example, the levels of Neu5Gc produced in CHO cells has shown to vary between 1-15%, different from murine cell lines like NS0 which have shown levels over 60% (Baker, Rendall *et al.* 2001, Chenu, Gregoire *et al.* 2003, Lalonde and Durocher 2017). In fact, some of the monoclonal antibodies already approved by the FDA, which are produced mainly by CHO and NS0 cells, have shown to contain traces of Neu5Gc (Diaz, Padler-Karavani *et al.* 2009). Disruption of CMAH gene in mouse has been used to obtain glycoproteins free from Neu5Gc. However, it has been shown that the uptake mechanism of Neu5Gc previously described is not specific for human cells but it can also take place in other mammalian cells like CHO and NS0 cells with a non-functional CMAH gene (Varki, Hedlund *et al.* 2013). All the above suggests that choosing an animal cell line with low or no expression of Neu5Gc does not guarantee that further contamination cannot arise from Neu5Gc present in the culture media supplements. In the following Chapters, NS0 and CHO cells were chosen to examine Neu5Gc-content after supplementation with different precursors. These two cell lines are known for their significantly different ratio between Neu5Gc:Neu5Ac.

The approach taken in this chapter was to evaluate Neu5Gc content in NS0 cells when cultured in the presence of well-known supplements used to increase sialylation in recombinant glycoproteins. These supplements included galactosylation precursors (Gal, Urd and M, see **Chapter 9**) as well as sialylation precursors (Ac_4ManNAc +/- Cyt, Ac_4GlcNAc +/-Urd. In addition, the effect of free Sias was evaluated on the sialic acid proportions of NS0 and CHO cells. In addition, since both cell lines showed different proportions of Neu5Gc:Neu5Ac, incorporation of free Neu5Ac, Neu5Gc or Neu5Ac *plus* Neu5Gc were evaluated on their effect on sialic acid proportions (see **Chapter 10**). Finally, knowing that animal derived supplements are a well-known source of Neu5Gc contamination, NS0 cells were cultured in media containing serum and its influence was evaluated in IgG1Mab (**Chapter 11**). Finally, the effect of CMAH enzyme's cofactor iron was evaluated on its effect over the amount of Neu5Gc was also evaluated in NS0-IgG1 Mab (**Chapter 12**). Understanding the impact that culture media supplements have on the conversion of Neu5Ac to Neu5Gc will provide with new insights on possible ways to control Neu5Gc content during human therapeutics production.

C.1 Hypothesis of Research

N-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the immediate precursors for sialylation reactions occurring at the *trans*-portion of the Golgi. Incorporation of free sialic acids can enhance sialylation of monoclonal antibodies. Free Neu5Gc is expected to increase the content of Neu5Gc-containing glycoproteins, including EG2 cells which are characteristic for producing negligible amounts of Neu5Gc. The latter can also be observed in cells exposed to serum which is known to be a source of contamination of Neu5Gc. Thus, NS0

cells and EG2 cells cultured in the presence of serum are expected to show an increase in Neu5Gc-containing glycoproteins. On the opposite site, incorporation of free Neu5Ac can metabolically compete with intracellular Neu5Gc, reducing the amount of NS0's predominant sialic acid, Neu5Gc. Finally, an increase in sialylation can be observed by adding iron which acts as a cofactor of the enzyme CMAH responsible for the conversion of CMP-Neu5Ac to CMP-Neu5Gc. The latter is expected to increase the content of Neu5Gc-containing glycoproteins with respect to Neu5-Ac glycoproteins in both, NS0 and EG2 cells.

Chapter 9

Nucleotide sugar precursors effect on sialic acid proportions

9.1.Introduction

Among higher animals, humans and chicken lack a functional cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH), enzyme responsible to produce Neu5Gc from CMP-Neu5Ac (Schauer, Srinivasan *et al.* 2009, Varki 2010).This irreversible mutation on the human gene *CMAH* is believed to have occurred 2.5-3 mya., shortly before the time when brain expansion began in humankind's ancestry (Chou, Hayakawa *et al.* 2002). Neu5Gc loss could have conferred a survival advantage by avoiding recognition from microbial pathogens (e.g. influenza viruses, *E. coli*K99) and may have affected interactions with receptors for Sias such as B-lymphocyte-associated glycoprotein (CD22), myelin associated glycoprotein (MAG), selectins, sialoadhesins, lectins (Schauer 1995, Irie, Koyama *et al.* 1998, Muchmore EA 1998).

Even though there is no evidence of an alternative biosynthetic pathway, trace amounts of Neu5Gc have been detected in human carcinomas, fetal tissues, blood vessel walls and cultured human cells (e.g. embryonic stem cells). The latter suggests the existence of an alternate source of Neu5Gc (Pham, Gregg *et al.* 2009, Banda, Gregg *et al.* 2012). Tangvoranuntakul *et al.* proposed that exogenous Neu5Gc, presumably from dietary sources (e.g. red meat and dairy products), can

enter human cells via clathrin-independent endocytic pathways (pinocytosis) and then is transported into the cytosol where it will fuse with endosomes/lysosomes (Tangvoranuntakul, Gagneux *et al.* 2003, Varki, Hedlund *et al.* 2013). Then, Neu5Gc is transported to the cytosol where it is activated to CMP-Neu5Gc prior to be metabolically incorporated and expressed on the surface of human cells as if it was made in the same cell (Tangvoranuntakul, Gagneux *et al.* 2003, Pham, Gregg *et al.* 2009, Varki, Hedlund *et al.* 2013). In accordance to this, it has been shown that sialyltransferases are unable to discriminate between Neu5Ac and Neu5Gc, which differ by one oxygen atom (Banda, Gregg *et al.* 2012).

Humans produce a serum-sickness like reaction when encountering Neu5Gc residues, where anti-Neu5Gc antibodies (0.1 - 0.2% of circulating IgG) are expressed, also known as anti-Hanganutziu-Deicher (H-D) antigen antibodies (Muchmore EA 1989, Varki 2001). The latter were first described in patients receiving therapeutic injections of animal serum (Morito, Kano *et al.* 1982, Higashi, Hirabayashi *et al.* 1985, Ghaderi, Taylor *et al.* 2010). Thus, there is a big concern regarding exposure of humans to therapeutics produced in animal cells (e.g CHO, NS0, BHK cells) (Baker, Rendall *et al.* 2001, Borys, Dalal *et al.* 2010, Hossler 2011) (Ghaderi, Taylor *et al.* 2010), in media containing possible Neu5Gc-containing additives (e.g. FBS), in animal organs considered for transplantation (e.g. pig livers) and in transplantation of human embryonic stem cells- derived grafts (Furukawa K 1988 , Ajit Varki 2009, Varki, Hedlund *et al.* 2013). In addition, the presence of Neu5Gc in other mammals limit the use of glycosylated therapeutics produced in milk of animals. Even though it is not entirely known the fate of Neu5Gc in patients who receive therapeutics with high content of Neu5Gc in terms of clearance, absorption and re-expression on endogenous cell surface glycolipids, it is certain that Neu5Gc can be metabolically incorporated

in human glycans and can direct an immunologic attack against "self". For this reason, Neu5Gc is also known as the first human "xeno-auto-antigen" (Varki 2001) (Ghaderi, Zhang *et al.* 2012)

Most of studies found in literature have focused on the effect of Sia precursors on the total amount of sialylated glycans. However, there is not a lot of information regarding the effect of glycosylation precursors on the content of Neu5Gc vs. Neu5Ac in recombinant glycoproteins produced by mammalian cells. Here, the effect that galactosylation precursors (e.g. MGU, **Chapter 3**) have on the proportion of Neu5Gc vs. Neu5Ac was evaluated in NS0 and CHOEG2 cells. In addition, the effect that the Sia precursors analogs (e.g. Ac_4GlcNAc, Ac_4ManNAc, **Chapter 7** and **Chapter 8**) and pyrimidine nucleotides (e.g. Cyt and pyrimidine) have on the proportions of each Sia. The latter will provide useful information regarding supplements that can increase / reduce the content of Neu5Gc.

9.2.Objectives.

- The effect of galactosylation precursors such as Gal, M and Urd on the proportion of Sias, particularly on the content of Neu5Gc.
- The influence of Sia precursors such as Ac_4ManNAc and Ac_4GlcNAc was evaluated on their effect on Sia proportions
- Combination of Sia precursors and pyrimidine nucleotides used to increase CMP-Sia pool were tested on their ability to influence on Neu5Ac: Neu5Gc content.

9.3.Experimental set up

9.3.1 Effect of MGU on the Sia proportions of NS0-IgG1 and EG2-hFc Mabs

To determine whether nucleotide sugar precursor feeding influenced Sias proportions, NS0 cells expressing IgG1 were cultured in media containing 7 μM M; 2.5mM Urd_1 and 5 mM Urd_2; and 10 mM Gal_1 and 20 mM Gal_2 alone or a combination of them as previously specified in **Chapter 3** (**Section 5.2**, **Figure 5.1**). These concentrations were chosen based on previous reports by Baker *et al*.(Baker, Rendall *et al.* 2001), Grammer *et al.* (Gramer, Eckblad *et al.* 2011), Markely (Markely 2011), and Hills *et al.* (Hills, Patel *et al.* 2001) where galactosylation precursors increased glycosylation of recombinant glycoproteins. However, the effect of these supplements on Sia proportions were not explored in these publications. Each condition was performed in duplicates and the results shown are the average of two independent experiments (n=4). A separate set of experiments included the CHO cell line expressing the EG2-hFc Mab which were cultured in media containing 7 μM M, M+Urd_2, M+Gal_2, 5 mM uridine and M+Urd_2+Gal_2 (**Chapter 3, Section 5.2**). Each condition was done in duplicates and results were averaged (n=2).

9.3.2 Effect of Ac_4ManNAc and Cyt on the Sia proportions of NS0-IgG1 and EG2-hFc Mabs

The effect of Ac_4ManNAc on the proportion of Neu5Gc:Neu5Ac was evaluated in NS0 cells when cultured in Biogro™ media containing 100 μM, 200 μM, 500 μM and 1000 μM Ac_4ManNAc. For more details about experimental set up refer to **Chapter 8 (Section 8.2.1)**. In addition, the influence of increasing pyrimidine-nucleotide precursors Cyt at 0.5 mM, 1mM, 2.5

mM, 5 mM and/or 10 mM Cyt alone or in combination with 100 μM Ac_4ManNAc on Sia proportions was also investigated in NS0 cells producing IgG1 Mab (See **Chapter 8, Section 8.2.2)**. Control samples had no supplement added. This set of experiments was done twice which resulted in a total of 4 samples per condition (*n=4*)

9.3.3 Effect of Ac_4GlcNAc and Urd on the Sia proportions of NS0-IgG1 and EG2-hFc Mabs.

The effect of Ac_4GlcNAc on the Sia proportions of NS0-IgG1 Mab was evaluated in Biogro™ media containing: 10 μM, 25 μM, 50 μM and 100 μM Ac_4GlcNAc (For further details see **Chapter 7, Section 7.1.1**). A separate set of experiments looked at the effect of 10 μM Ac_4GlcNAc plus 1 mM, 2.5 mM and 5 mM uridine (see **Chapter 7, Section 7.2.2**). Control sample had no supplement addition. Each condition was performed in duplicates and the average of one independent experiment is shown (n=2). Culture shake flasks of 250 mL containing 80 mL of culture media were placed in a shake platform at 120 rpm were incubation took place for four days at 37°C with 10% CO_2. On day four, cell culture was terminated where culture media was collected and spun down to recover supernatants containing the final IgG1 product. Supernatant was then filtered and concentrated 10X before further analysis. Then, antibodies were purified using protein A columns, from which 100 μg of Mab was used for Sia analysis. Pre purified Mabs were subjected to mild hydrolysis with 2M acetic acid and then DMB-derivatization prior to RP-HPLC analysis (See Methods, **Chapter 2,** section 2.11.9)

9.4.Results

9.4.1 Proportions of Neu5Gc and Neu5Ac in Mabs produced by NS0 and EG2 cells.

Previous reports have shown that murine cell lines have higher levels of *N*-glycolylneuraminic while hamster cell lines contain lower levels of this immunogenic Sia. Thus, the content of each Sia was evaluated in NS0-IgG1 Mab and compared to CHO EG2-hFc Mab. For this purpose, NS0 cells and EG2 cells were cultured for four days and the accumulated product was subjected to Sia analysis. Sia were released and labeled with a fluorescent dye 1,2-diamino-4,5-methylenodioxybenzene (DMB). The latter is non-selective, meaning that Neu5Gc and Neu5Ac are equally labelled. The derivate of the DMB-labelled Sia were then separated using an isocratic elution by RP-HPLC.

Figure 9.1 shows a comparison of IgG1 Mab's Sia proportion with respect to EG2-hFc Mab. The total amount of Sias was determined as the ratio of moles of Sia/mole Mab. Thus, Neu5Gc content was three times higher than that of Neu5Ac in NS0 cells cultured in Biogro™ media IgG1 Mab's total amount of Sia was 0.38 mole Sia/mole Mab. In contrast, CHO EG2 cells strongly preferred Neu5Ac over Neu5Gc, showing negligible amounts of Neu5Gc (3%) in Biogro™ media. This marked difference between cell lines is shown in **Figure 9.1.**

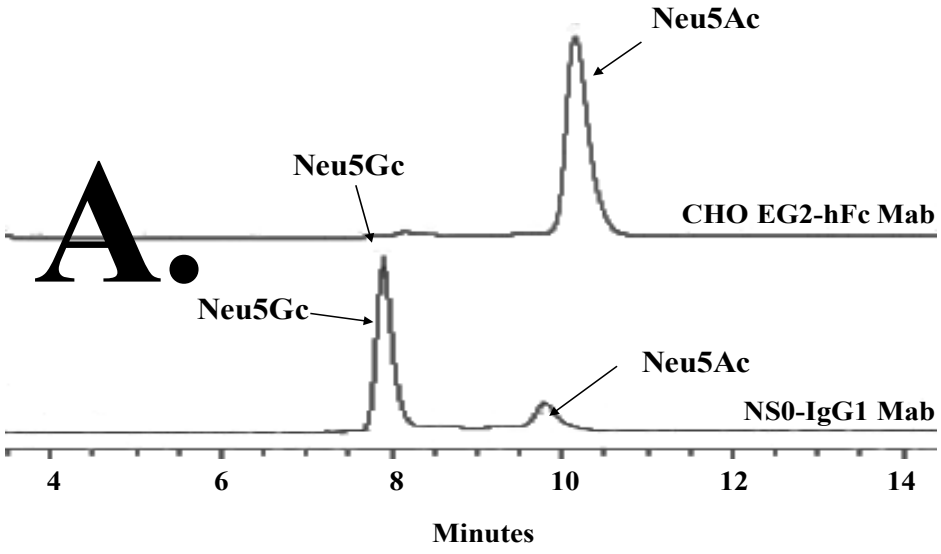
9.4.2 Effect of MGU cocktail in Sia proportions of IgG1 Mab.

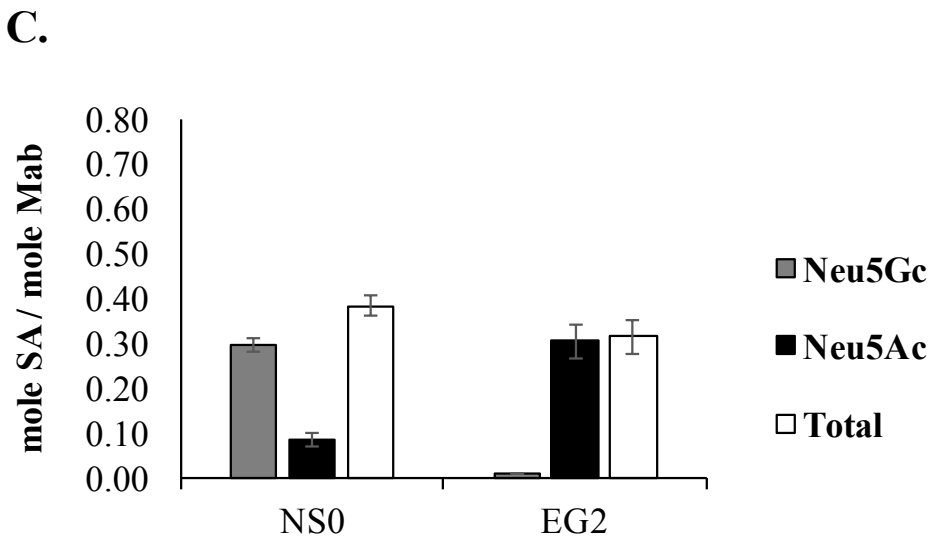
In **Chapter 3**, M, Urd and Gal showed an impact on galactosylation and sialylation of NS0-IgG1 and EG2-hFc Mabs. An increase in sialylated glycans of about 20%, 24%, 27% and

35% was observed in IgG1 Mabs produced in media containing Mn^{2+}, M+Urd, Mn^{2+}+Urd+Gal, M+Gal respectively.

Figure 9. 1 Analysis of Sias proportions associated with CHO EG2 and NS0 cells.

A. CHO EG2-hFc and **B.** NS0-IgG1 Mab produced in untreated Biogro™ media. Sias were released from Mabs by mild acid hydrolysis, labeled with DMB and separated by reverse-phase HPLC analysis. Arrows are indicating the peak corresponding to Neu5Ac and Neu5Gc. **C.** Relative proportions of Neu5Gc and Neu5Ac in NS0-IgG1and EG2-hFc Mabs.





The effect of these supplements on sialylation was further explored by measuring the content of both Neu5Ac and Neu5Gc in previously protein-A purified NS0-IgG1 (**Chapter 3**). The total amount of Sias in NS0-IgG1 from cultures containing M, Urd and Gal is shown as a ratio mole Sia/mole IgG1 Mab**.** When NS0 cultures were incubated in the presence of M, a slightly but significant increase of 45% was observed in the total amount of Sia in comparison to the control (p-Value 0.01). From this total amount of Sia (0.55 mole Sia/mole IgG1 Mab), a 12% decrease in Neu5Gc-containing glycans was observed with respect to the control. Thus, the initial ratio of Neu5Gc:Neu5Ac from control cultures changed from 8:2 to 7:3 (**Table 9.1**). In contrast, EG2-hFc Mabs' Sia content was not affected by M; however, EG2-hFc Mabs showed a significant lower amount of total Sia in comparison to NS0 cells (p-Value 0.0004).

A significant decrease of about 19% and 38% in Neu5Gc content along with a significant increase of up to 66% and 131% in Neu5Ac content was observed in cultures containing M+Urd_1 and M+Urd_2 respectively with respect to NS0 control cultures. In addition, the total amount of Sias increased by up to 45% M+Urd_1 and M+Urd_2 (p-Value 0.03). Furthermore, a change in Neu5Gc to Neu5Ac ratio from 8:2 observed in control cultures to 6:4 and 5:5 was observed in NS0 cultures containing M+Urd_1 and M+Urd_2 respectively (**Table 9.1**). A similar effect was observed in M+Urd+Gal containing cultures, where a ratio of 1:1 ratio resulted from a significant decrease of up to 42% in Neu5Gc along with a significant increase of up to 147% in Neu5Ac (p-Value $\leq$0.008). However, the total content of Sias in M+U+G cultures remain close to the value found in the control, except for M+U1+G1 culture where the total Sia was 1.8 times higher in comparison to the control (p-Value <0.0001). In contrast, EG2-hFc Mabs showed a significant decrease of up to

67% in Neu5Ac when cultured in media containing uridine (e.g. Urd, Mn^{2+}+Urd, Mn^{2+}+Urd+Gal) (p-Value <0.0001) (**Figure 9.2**).

A significant increase of up to 15.7%, 78% and 47% was observed in the total Sia pool of Gal_2, M+Gal_1 and M+Gal_2- containing NS0 cultures respectively (p-Value ≤0.004). The latter shows that incrementing Gal from 10 mM to 20 mM does not result in a further increase in Sia pool. However, the combination of Gal with M decreased by up to 17% the content of Neu5Gc, which represents a change in control's Neu5Gc:Neu5Ac (8:2) to 6:4 and 7:3 (**Table 9.1**). The latter may suggest that M and Gal acted together, decreasing Neu5Gc content as well as increasing Neu5Ac (by up to 58%) to a bigger extent than when Gal was added alone. Nevertheless, Gal containing cultures showed that Neu5Gc was still two times higher than the content of Neu5Ac (p-Value ≤0.007). In EG2 cells, Sia proportions were not affected by Gal supplementation when compared to the control, keeping Neu5Ac as the Sia of preference.

These results show that M, Gal and Urd had a different effect on the Sia proportions of each cell line. EG2 cells showed a higher proportion of Neu5Ac in control cultures with negligible amounts of Neu5Gc. Media containing M, Gal and M+Gal cultures did not significantly influence the content of Sias in EG2 cells. This scenario changed when Urd was supplemented, lowering Neu5Ac amount by up to 67% in EG2 cells. In contrast, NS0-IgG1 Mabs had 3 times more glycans containing Neu5Gc than Neu5Ac. This proportion changed depending on the glycosylation precursor added to NS0 cultures. Incorporation of M induced a significant increase of about 45% in the total Sia pool in comparison to the control.

Figure 9. 2 Effect of MGU cocktail on Sia proportions of EG2-hFc Mab.

Neu5Gc (■) and Neu5Ac (■) content and total Sia pool (□) (mole Sia/mole Mab) in EG2-hFc Mab produced in A. Biogro™ media (control); B. M=7μM; C. Urd = 5mM; D. Gal= 20 mM; E. M + Urd= 5 mM; F. M + Gal= 20 mM); G. M+Urd+Gal.. Neu5Gc/Neu5Ac content was analyzed quantitatively by reverse-phase HPLC of DMB-labeled Sias released by mild acid hydrolysis. Error bars correspond to the SD ± from the average of duplicates from two independent experiments (n =4). *Data significantly different (p-Value <0.05*; <0.001**; <0.0001***) with respect to the control sample.

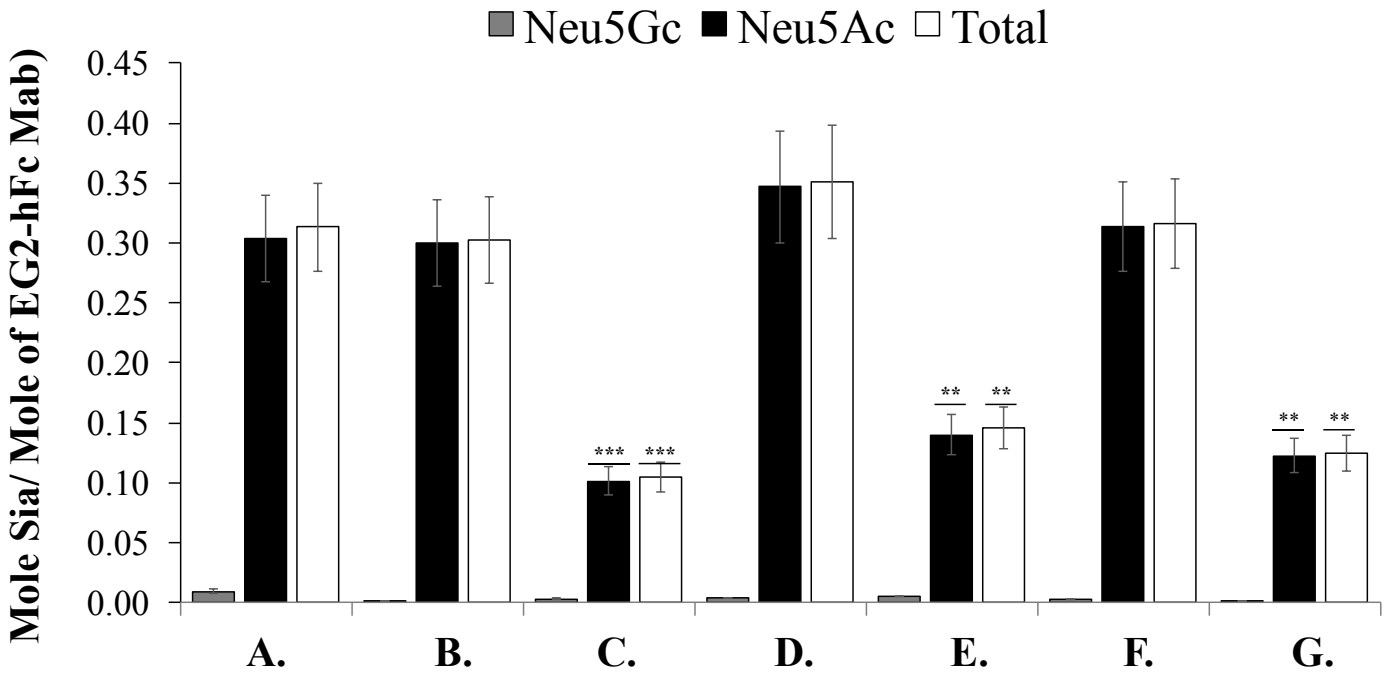


Table 9. 1 Effect of galactosylation precursors on the N-glycosylation of IgG1 Mab.

Changes in Sia proportions are expressed as mole Sia/mole Mab and as a ratio of Neu5Gc vs. Neu5Ac. Error bars correspond to the SD ± from the average of duplicates from two independent experiments (n =4).

Culture supplement	Glycosylation Analysis		Sialic Acid Analysis			Ratio Sia
	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Neu5Gc : Neu5Ac
Control	0.58 ± 0.03	14.4 ± 5.27	0.30 ± 0.01	0.09 ± 0.01	0.38 ± 0.02	8 : 2
M	0.63 ± 0.01	20.2 ± 0.73	0.38 ± 0.03	0.18 ± 0.03	0.55 ± 0.05	7 : 3
M+Urd_1	0.70 ± 0.02	23.4 ± 0.41	0.34 ± 0.03	0.20 ± 0.03	0.53 ± 0.06	6 : 4
M+Urd_2	0.72 ± 0.01	24.6 ± 0.03	0.27 ± 0.04	0.29 ± 0.04	0.55 ± 0.08	5 : 5
Gal_2	0.75 ± 0.00	31.7 ± 0.63	0.38 ± 0.02	0.06 ± 0.01	0.44 ± 0.03	9 : 1
M+Gal_1	0.78 ± 0.01	32.8 ± 3.92	0.44 ± 0.04	0.24 ± 0.03	0.68 ± 0.06	6 : 4
M+Gal_2	0.78 ± 0.01	34.9 ± 1.03	0.37 ± 0.02	0.18 ± 0.03	0.56 ± 0.05	7 : 3
M+Urd_1+Gal_1	0.74 ± 0.01	26.9 ± 0.80	0.37 ± 0.04	0.35 ± 0.03	0.72 ± 0.07	5 : 5
M+Urd_1+Gal_2	0.73 ± 0.02	25.2 ± 1.85	0.19 ± 0.02	0.24 ± 0.03	0.43 ± 0.05	4 : 6
M+Urd_2+Gal_1	0.71 ± 0.04	19.6 ± 6.01	0.25 ± 0.04	0.26 ± 0.08	0.52 ± 0.11	5 : 5
M+Urd_2+Gal_2	0.74 ± 0.02	24.4 ± 1.82	0.24 ± 0.04	0.21 ± 0.03	0.44 ± 0.06	5 : 5

More importantly, Neu5Gc-containing glycans decreased by up to 12% with respect to the control which resulted in a change in Neu5Gc:Neu5Ac from control cultures from 8:2 to 7:3. A more significant change in Sias proportions was observed in uridine containing cultures, changing the ratio of Neu5Gc:Neu5ac to 1:1. In fact, Neu5Gc content was reduced up to 38% while Neu5Ac content increased up to 131% in comparison to the control. Finally, the highest increase (up to 78%) in the total Sia pool was observed in Gal -fed cultures. However, the amount of Neu5Gc was still over Neu5Ac with a ratio of 2:1. Cultures containing M+Urd+Gal showed the lowest amount of Sia in both cell lines. Finally, none of the supplements seem to have an effect over Neu5Gc content in EG2 cells

9.4.3 Effect of Ac_4ManNAc and Cyt on Sia proportions in NS0-IgG1 Mab.

First, the effect of Ac_4ManNAc alone was evaluated on the Sia proportions of IgG1 Mabs. For this purpose, NS0-IgG1 Mab produced in media containing increasing concentrations of Ac_4ManNAc (100, 200, 500,1000 μM) were purified and then subjected to acid-mild hydrolysis for Sia release. The released Sias were DMB-labelled prior to identification by RP-HPLC. Control cultures showed a total amount of 0.30 mole Sia/mole Mab of which ~77% corresponded to Neu5Gc. Cultures containing 100 μM Ac_4ManNAc showed amount of total Sias pool to the control, with a slightly but not significant decrease in Neu5Gc. However, a significant decrease of about 52%, 65% and 78% was observed in the content of Neu5Gc in cultures containing 200 μM, 500 μM and 1000 μM Ac_4ManNac respectively (p-Value $\leq$0.006). The latter changed the ratio of Neu5Gc:Neu5Ac ratio seen in the control to 1:1 in cultures containing 200 μM and 500 μM Ac_4ManNAc (**Table 9.2)**. With respect to Neu5Ac content, no significant differences were

observed between the control and Ac_4ManNAc containing cultures. Finally, the total amount of Sia found in control cultures (0.30 ± 0.22) decreased by up to 43% in cultures containing Ac_4ManNAc. However, these decrease was considered as not significant as there was a great variation between replicates.

Table 9.3 shows the effect of Cyt alone or in combination with 100 μM Ac_4ManNAc. Cyt had no effect over the content of Neu5Ac on IgG1 Mab; however, a significant decrease in the amount of Neu5Gc of up to 55-65% was observed as Cyt increased from 0.5 mM to 2.5 mM with respect to untreated cultures (p-Value <0.001). Furthermore, control cultures' Neu5Gc:Neu5Ac of 8:2 changed to 5:5 in 0.5-2.5 mM Cyt containing cultures (**Table 9.3**). The latter represented a decrease of about 32-39% of the total amount of Sia / mole of IgG1 Mab. Combination of Cyt and 100 μM Ac_4ManNAc also affected the amount of Neu5Gc with a significant decrease of up to 75% with respect to the control (p-Value <0.05). However, this decrease in Neu5Gc was not significantly different to cultures containing Cyt or Ac_4ManNAc alone. The total amount of Sias significantly decrease from 40% to up to 60% in cultures containing Ac_4ManNAc plus Cyt when compared to the control.

These results show that the addition of Ac_4ManNAc influenced the ratio of Neu5Gc:Neu5Ac found in NS0-IgG1 control cultures. NS0-IgG1 Mab originally showed a Neu5Gc:Neu5Ac ratio of 8:2. This content of Neu5Gc decreased up to ~78% in cultures containing 1000 μM Ac_4ManNac. As a result, the Neu5Gc:Neu5Ac ratio was changed to 1:1 in cultures containing 200 μM and 500 μM Ac_4ManNAc. Cultures with 1000 μM Ac_4ManNAc had a slightly higher Neu5Ac content but this was not significant. Incorporation of Cyt also helped reduce the

content of Neu5Gc in NS0-IgG1 Mab showing a ratio of Neu5Gc:Neu5Ac of 1:1. Incorporation of 100 μM Ac_4ManNAc along with Cyt resulted in a significant decrease of Neu5Gc of up to 75% in 100μM Ac_4ManNAc plus 2.5 mM Cyt. Thus, Ac_4ManNAc and/or Cyt could be used as a tool to help "humanise" glycoproteins produced in mammalian cells by decreasing the Neu5Gc content. However, the total Sia pool may be compromised when Ac_4ManNAc is supplemented at high concentrations (>200 μM) or when combined with Cyt (0.5-2.5 mM Cyt+100 μM Ac_4ManNAc.

9.4.4 Effect of peracetylated glucosamine on the conversion of Neu5Ac to Neu5Gc.

Table 9.4 shows the effect of increasing concentrations of peracetylated glucosamine (10, 20, 50 and 100 μM Ac_4GlcNAc) on the proportion of Neu5Gc and Neu5Ac in NS0-IgG1 Mab. Control cultures showed 0.67±0.06 moles of total Sia per mole of IgG1, where 90% corresponded to Neu5Gc. Cultures containing 10 to 50 μM Ac_4GlcNAc showed slightly lower but not significant content of Neu5Gc with respect to the control. However, cultures containing 100 μM Ac_4GlcNAc showed a significant decrease of about 73% in Neu5Gc content with respect to the control (p-Value ≤0.0004). The amount of Neu5Ac was not affected by 100 μM Ac_4GlcNAc. As a result, 100 μM Ac_4GlcNAc showed a Neu5Gc:Neu5Ac ratio of 4:1 in comparison to cultures containing 0 to 50 μM Ac_4GlcNAc with 9:1 (**Table 9.4**).

Table 9. 2 Effect of the sialylation precursors: Ac_4ManNAc on the N-glycosylation of IgG1 Mab.

Changes in Sias proportions are expressed as mole Sia/mole Mab and as a ratio of Neu5Gc:Neu5Ac. Values correspond to the mean ± SD of duplicates of one independent experiments (n = 6).

Culture supplement	**Glycosylation Analysis**		**Sialic Acid Analysis**			**Ratio**
Ac4ManNAc	**GI**	**% Sialylation**	**Neu5Gc**	**Neu5Ac**	**Total**	**Neu5Gc : Neu5Ac**
0 μM	0.58± 0.05	14.7± 3.23	0.23 ± 0.05	0.07 ± 0.01	0.30 ± 0.06	8 : 2
100 μM	0.55± 0.04	11.1± 2.86	0.16 ± 0.04	0.12 ± 0.04	0.28 ± 0.07	6 : 4
200 μM	0.49± 0.06	5.19± 3.11	0.11 ± 0.02	0.12 ± 0.03	0.23 ± 0.05	5 : 5
500 μM	0.44± 0.02	6.66± 2.03	0.08 ± 0.01	0.08 ± 0.02	0.16 ± 0.02	5 : 5
1000 μM	0.47± 0.02	8.83± 2.07	0.05 ± 0.01	0.12 ± 0.05	0.17 ± 0.06	3 : 7

Table 9. 3 Effect of the sialylation precursors Ac_4ManNAc and Cyt on the N-glycosylation of IgG1 Mab.

Changes in Sias proportions are expressed as mole Sia/mole Mab and as a ratio of Neu5Gc:Neu5Ac. Values correspond to the mean ± SD of duplicates of one independent experiments (n = 3).

Supplement	Glycosylation Analysis		Sialic Acid Analysis			Ratio
	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Neu5Gc : Neu5Ac
Control	0.57 ± 0.04	12.2 ± 5.68	0.49 ± 0.02	0.14 ± 0.04	0.63 ± 0.06	8 : 2
0.5 mM Cyt (Cyt_1)	0.63 ± 0.01	9.22 ± 1.58	0.22 ± 0.01	0.21 ± 0.02	0.43 ± 0.02	5 : 5
1 mM Cyt (Cyt_2)	0.61 ± 0.02	7.36 ± 1.58	0.21 ± 0.00	0.18 ± 0.03	0.39 ± 0.03	5 : 5
2.5 mM Cyt (Cyt_3)	0.66 ± 0.00	9.35 ± 0.58	0.17 ± 0.07	0.21 ± 0.02	0.38 ± 0.05	5 : 5
100 μM Ac4ManNAc	0.55 ± 0.04	11.1 ± 2.86	0.16 ± 0.04	0.12 ± 0.04	0.28 ± 0.07	6 : 4
Cyt_1 + 100 μM Ac4ManNAc	0.55 ± 0.00	4.37 ± 0.48	0.24 ± 0.04	0.14 ± 0.02	0.38 ± 0.04	6 : 4
Cyt_2+ 100 μM Ac4ManNAc	0.51 ± 0.07	2.88 ± 0.89	0.26 ± 0.02	0.14 ± 0.01	0.40 ± 0.02	6 : 4
Cyt_3 + 100 μM Ac4ManNAc	0.50 ± 0.03	5.77 ± 3.08	0.12 ± 0.03	0.14 ± 0.01	0.25 ± 0.03	5 : 5

Table 9. 4 Effect of the sialylation precursors: peracetylated glucosamine (Ac_4GlcNAc) on the N-glycosylation of IgG1 Mab.

Changes in Sia proportions are expressed as mole Sia/mole Mab and as a ratio of Neu5Gc vs. Neu5Ac

	Glycosylation Analysis		Sialic Acid Analysis			Ratio
Ac4GlcNAc	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Neu5Gc : Neu5Ac
0 μM	0.49 ± 0.01	9.52 ± 1.19	0.60 ± 0.12	0.07 ± 0.03	0.67 ± 0.12	9 : 1
10 μM	0.50 ± 0.02	9.91 ± 2.64	0.58 ± 0.01	0.05 ± 0.00	0.63 ± 0.01	9 : 1
25 μM	0.51 ± 0.00	8.75 ± 1.68	0.55 ± 0.02	0.04 ± 0.00	0.59 ± 0.02	9 : 1
50 μM	0.56 ± 0.04	11.6 ± 1.27	0.56 ± 0.00	0.05 ± 0.00	0.61 ± 0.00	9 : 1
100 μM	0.46 ± 0.01	6.34 ± 0.50	0.16 ± 0.04	0.04 ± 0.01	0.20 ± 0.05	8 : 2

Ac_4GlcNAc supplementation (<100 μM) was not successful on significantly decrease the content of Neu5Gc in NS0-IgG1 Mabs. Thus, the effect of Ac_4GlcNAc was further explored when combined with uridine. Cultures containing 1-2.5 mM uridine alone showed a slight decrease in Neu5Gc content but was not significant when compared to the control. However, a significantly decrease of ~50% was observed in Neu5Gc content was in 5 mM Urd cultures with respect to the control (p-Value <0.0001). The content of Neu5Ac was not affected by uridine, falling between 0.16-0.20 mole Sia/mole Mab. In contrast, a significant decrease in the amount of Neu5Gc was observed when 25 μM Ac_4GlcNAc was combined with 1, 2.5 and 5 mM uridine (50%; 46%; 60% respectively; p-Value <0.0001). As a result, control cultures' Neu5Gc:Neu5Ac ratio of 8:2 changed to 6:4 in Urd_{1-3}+25 μM GlcNAc (**Table 9.5**). There was not influence on Neu5Ac content from Ac_4GlcNAc alone nor when combined with uridine. As a result, the total amount of Sia found in control cultures (0.63 ± 0.15) significantly decreased (29%-45%) in cultures with Ac_4GlcNAc alone or when combined with uridine (p-Value <0.0001).

In summary, Ac_4GlcNac supplementation at 10 to 50 μM didn't show any significant difference in the amount of Neu5Gc nor Neu5Ac. However, when the concentration was increased to 100 μM Ac_4GlcNA, a significant reduction of about 73% was observed in the amount of Neu5Gc. With respect to Urd, a significant reduction in the amount of Neu5Gc by up to 50% was observed at 5 mM with respect to the control. Neu5Gc content significantly decreased by up to 60% when 25 μM Ac_4GlcNAc was combined with 1-5 mM uridine when compared to the control. These changes in Neu5Gc content changed the original Neu5Gc:Neu5Ac ratio of 8:2 in control cultures to 6:4.

Table 9. 5 Effect of the sialylation precursors: uridine (Urd) and peracetylated glucosamine (Ac_4GlcNAc) on the N-glycosylation of IgG1 Mab.

Changes in Sia proportions are expressed as mole Sia/mole Mab and as a ratio of Neu5Gc vs Neu5Ac. Values correspond to the mean ± SD of duplicates of one independent experiments (n = 2).

Supplement	Glycosylation Analysis		Sialic Acid Analysis			Ratio
	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Neu5Gc : Neu5Ac
Control	0.57 ± 0.04	12.2 ± 5.68	0.49 ± 0.07	0.14 ± 0.02	0.62 ± 0.05	8 : 2
1 mM Urd (Urd_1)	0.63 ± 0.02	8.99 ± 1.42	0.38 ± 0.01	0.20 ± 0.01	0.59 ± 0.03	7 : 3
2.5 mM Urd (Urd_2)	0.66 ± 0.00	8.22 ± 2.49	0.44 ± 0.05	0.16 ± 0.02	0.60 ± 0.03	7 : 3
5 mM Urd (Urd_3)	0.65 ± 0.00	7.23 ± 1.02	0.25 ± 0.01	0.17 ± 0.01	0.42 ± 0.02	6 : 4
25 uM Ac4GlcNAc	0.57 ± 0.07	9.63 ± 3.18	0.25 ± 0.00	0.12 ± 0.01	0.36 ± 0.01	7 : 3
Urd_1 + 25 uM Ac4GlcNac	0.62 ± 0.01	7.60 ± 0.66	0.24 ± 0.00	0.15 ± 0.01	0.40 ± 0.01	6 : 4
Urd_2 + 25 uM Ac4GlcNac	0.65 ± 0.01	8.86 ± 1.82	0.27 ± 0.01	0.19 ± 0.01	0.45 ± 0.01	6 : 4
Urd_3 + 25 uM Ac4GlcNac	0.67 ± 0.02	7.81 ± 0.98	0.20 ± 0.00	0.15 ± 0.01	0.34 ± 0.00	6 : 4

9.5.Discussion

Different approaches have been taken to diminish the content of biotherapeutic products with the immunogenic Neu5Gc. For instance, disrupting the CMAH gene by gene knock-out, random mutagenesis and antisense oligonucleotide strategies has allowed the reduction of the activity of CMAH enzyme responsible for the conversion of CMP-Neu5Ac to CMP-Neu5Gc (Chenu, Gregoire *et al.* 2003, Varki, Hedlund *et al.* 2013). However, animal cells lacking a functional CMAH gene have also shown to incorporate Neu5Gc into their glycoproteins from animal supplements present in culture medium. Thus, the content of Neu5Gc needs to be evaluated in glycoproteins produced in animal cells that contain or not a functional CMAH gene. Knowing the initial content of this immunogenic Sia will allow to determine if culture media supplements or feeding strategies commonly used to improve sialylation influence the content of Neu5Gc and at the same time will allow to reduce the risk of causing an antigenic response in humans. Previously it has been shown that murine cell lines have a higher content of Neu5Gc (over 60%) than hamster cells (1-15%) (Chenu, Gregoire *et al.* 2003). In accordance to this, CHO EG2-hFc Mab showed negligible amounts on Neu5Gc-containing glycans (<3%) in comparison to NS0-IgG1 Mab with over 96% of Neu5Gc. These Sia proportions varied depending on the supplement added to cell culture media.

Previous reports have shown that the enzyme responsible for the activation of Sias, CMP-sialic acid synthetase (CMAS), highly depends on divalent cations (e.g. Mg^{2+}, Mn^{2+}, $Ca^{2+,}$ Co^{2+} and Zn^{2+}). Thus, in addition to act as a cofactor for GalT, Mn^{2+} also participates in the activation of Sias by CMAS in the nucleus of mammalian cells. In this chapter, M showed an increase of

about 12% and 100% in Neu5Gc and Neu5Ac respectively which resulted in a change in Neu5Gc:Neu5Ac ratio from 8:2 observed in the control to 7:3. The latter shows that even though a higher increase in Neu5Ac was observed in M containing cultures with respect to Neu5Gc, Neu5Gc remained the main Sias. In contrast, incorporation of M did not influence the Neu5Gc/Ac content in EG2-hFc Mab, with Neu5Ac constituting 97% of the total Sia pool. This is consistent with previous reports where M concentrations (10-340 μM M) did not influence Neu5Gc content in darbepoetin alpha on the cell line used as well as cells' initial Neu5Gc content.

Varki *et al.* also combined Mn^{2+} with ManNAc (5-12 Mm) and/or GlcNAc (0-5 mM) which resulted in a significant reduction of about 50-75% in Neu5Gc content along with a significant increase in the degree of sialylation of up to 10% (Yorke 2013, Ficko 2014). In contrast, Baker *et al.* incorporated 20 mM ManNAc to GS-NS0 cultures and a drop from 52% to 30% in Neu5Gc content was observed with a corresponding increase in Neu5Ac(Baker, Rendall *et al.* 2001). Here, IgG1 Mab also showed a reduction in Neu5Gc content of about 52%-78% in culture media containing Ac_4ManNAc but at much lower concentrations (200-1000 μM). However, a significant decrease of up to 43% in total sialylation was also observed in NS0 cells. Furthermore, there was no change with respect to Neu5Ac content. The latter shows that much lower concentrations of the Ac_4ManNAc can be used to achieve a similar effect regarding Neu5Gc reduction in glycoproteins. With respect to Ac_4GlcNAc supplementation, the 10% (or 0.67mole Sia/mole IgG1 Mab) of sialylated glycans observed in NS0-IgG1 Mab produced in medium without additives was not improved and instead a slight but significant decrease to about 6% (or 0.20 mole Sia/mole IgG1Mab) was observed at 100 μM Ac_4GlcNAc. Still, a significant decrease of about 73% in the

Neu5Gc content was possible when concentrations reached 100 μM in accordance to previous studies (Ficko 2014).

CTP and UTP play critical roles in cellular metabolism acting as common precursors for RNA synthesis and the activation of CMP-Sia and UDP-sugars for protein glycosylation. For this reason, these pyrimidine nucleotides were also evaluated alone or in combination with the Sia precursors analogs Ac_4GlcNAc and Ac_4ManNAc respectively on their effect on Sia proportions. For instance, uridine has been used to improve galactosylation and sialylation of certain glycoproteins (Pels Rijcken, Overdijk *et al.* 1993, Wong, Wati *et al.* 2010, Gramer, Eckblad *et al.* 2011, Liu, Spearman *et al.* 2014). However, little is known about the effect of Urd on Neu5Gc and Neu5Ac content. NS0 experiments showed that concentrations below 2.5 mM did not influence the ratio of Neu5Gc:Neu5Ac previously observed in the control (8:2). However, a significant decrease of about 49% and 32% were observed in the content of Neu5Gc and in the total Sia pool respectively at 5 mM Urd. As a result, Neu5Gc:Neu5Ac ratio changed to 1.4:1. Similarly, a change in the ratio of Neu5Gc:Neu5Ac to 2:1 and 1:1 was observed in M+Urd_1 and M+Urd_2-containing cultures. In contrast to cultures containing Urd alone, combining M+$Urd_{1\text{-}2}$ significantly increased Neu5Ac content by about 66%-131%, as Urd concentration increased from 2.5 mM to 5 mM. Thus, combining Urd and M not only increased the total Sia pool (↑45%), but also increased Neu5Ac content and decreased Neu5Gc in NS0-IgG1 Mab. Finally, cultures containing M+Urd+Gal also showed an improvement in Neu5Gc content with respect to Neu5Ac, with a ratio of 1:1, as a result a significant increase of about 167% in Neu5Ac. The change of Neu5Gc:Neu5Ac ratio to 1:1 probably resulted from the combination of Mn and Urd rather than from Gal supplementation as there was not a significant difference in Neu5Gc and Neu5A content between

M+Gal vs. M+Urd+Gal. The latter is supported by the fact that Neu5Gc content also significantly increased (26% and 46.6%) in Gal- and M+Gal-containing cultures respectively. In EG2-cells, uridine containing cultures (Urd; M+Urd; M+Urd+Gal) also showed a significant decrease in the total Sia pool. However, this decrease was a result from a significant decrease of about 67% in Neu5Ac in comparison to the control. There was no influence over Neu5Gc content in EG2 cells.

Cyt was also evaluated on its effect on Sia proportions in IgG1 Mab. In fact, Cyt has been previously used to replenish intracellular CTP pools which are required for the synthesis of sialoglycoconjugates. However, similarly to Urd containing cultures, a significant decrease of up to 55-65% was observed in Neu5Gc content as Cyt concentration increased from 0.5-2.5 mM. The latter resulted in a change in Neu5Gc:Neu5ac ratio to 1:1 along with a significant decrease in the total pool of sialylated glycans of up to 40% in comparison to the control (8:2). In addition, a significant decrease of about 47 and 51% in Neu5Gc content was observed in cultures containing both, 0.5 mM Cyt+100 μM Ac_4ManNAc and 1 mM Cyt + 100 μM Ac_4ManNAc respectively. However, Neu5Gc was the main Sia in these culture conditions. A Neu5Gc:Neu5ac ratio of 1:1 was observed only when Cyt reached 2.5 mM when combined with 100 μM ManNAc.

As immediate Sia precursors, Ac_4ManNAc and Ac_4GlcNAc alone or in combination with Cyt and uridine respectively were expected to increase sialylation of IgG1 Mab. Instead, a significant decrease in Sia containing glycans was observed as concentrations of these Sia precursors increased. The fact that intracellular concentrations of Cyt nucleotides were not determined does not exclude the possibility that Cyt and Neu5Gc intracellular concentrations were not improved by these supplements. If Cyt nucleotides were indeed increased, an increase in CMP-

Sia would have been expected and subsequently an increase in sialylation. However, the decrease in sialylation suggests that CMP-Sia did not reach the *trans*-Golgi compartment where sialylation takes place. Cacan and Carey *et al.* previously showed that CMP-Sia transport across the Golgi membrane can be inhibited by CMP and UDP sugars (Carey, Sommers *et al.* 1980, Cacan, Cecchelli *et al.* 1984, Maggioni, von Itzstein *et al.* 2013). Another possibility can be that Sia activation in the cytosol was inhibited by high concentrations of CTP (Rodriguez-Aparicio, Luengo *et al.* 1992). Even though vertebrate CMASs are highly specific for CTP, other tri-, di- or mono-phosphate nucleosides can act as competitive inhibitors (e.g ATP, GTP, UTP, TTP, CDP and CMP) (Rodriguez-Aparicio, Luengo *et al.* 1992). In addition, UDP-*N*-acetylglucosamine 2-epimerase enzyme activity can be inhibited by high concentrations of CMP-Sia which can result in blocking further synthesis of Neu5Ac (Pels Rijcken, Hooghwinkel *et al.* 1990, Grammatikos, Valley *et al.* 1998, Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001). In view of the above, an increase in Cyt pools and CMP-Sia does not always translate into an increase in sialylation (Pels Rijcken, Hooghwinkel *et al.* 1990, Pels Rijcken, Overdijk *et al.* 1995, Baker, Rendall *et al.* 2001, Hills, Patel *et al.* 2001). Nevertheless, Neu5Gc content was significantly reduced in NS0-IgG1 Mab produced in media containing Sia precursors.

9.6.Conclusion

- The content of Neu5Gc in the murine cell line NS0-IgG1 Mab was significantly higher (78% of the total Sia pool) than in the hamster cell line CH0-EG2hFc Mab which contained mainly Neu5Ac (97%).

- A significant decrease in the content of Neu5Gc of 12%, 38%, 17% and 42% along with a significant increase in Neu5Ac of 43%, 131%, 58% and 147% were observed in NS0 cells cultured in media containing M, M+Urd, M+Gal, M+Urd+Gal respectively.

- A Neu5Gc:Neu5Ac ratio of 1:1 was observed in uridine containing cultures.

- A significant increase of up to 16% and 78% in total Sia where observed in Gal- and M+Gal – containing cultures. This shows a synergistic effect when Mn^{2+} and Gal are combined.

- None of the galactosylation precursors had an influence on the Neu5Gc content of CHOEG2 cells remaining below limits of detection. However, uridine containing cultures showed a significant reduction (~67%) in the amount of Neu5Ac when compared to the control.

- Ac_4ManNAc did not have any influence on Neu5Ac content. However, a significant reduction of up to 78% in Neu5Gc was observed in cultures containing 1000 μM Ac_4ManNAc, which resulted in a Neu5Gc:Neu5Ac of 1:1. The total amount of Sia slightly decrease in comparison the control, indicating that the Sia precursor was not enough to improve sialylation.

- A significant decrease of up to 65% and 39% was observed in Neu5Gc content and total Sia respectively in IgG1 Mab produced in media containing Cyt (0.5-2.5 mM). When Cyt (2.5 mM) was combined with Ac_4ManNAc (100 μM) a Neu5Gc:Neu5Ac ratio of 1:1 was also observed in NS0 cells.

- Ac_4GlcNAc also induced a significant decrease (73%) in Neu5Gc content in IgG1 Mab at 100 μM. The Neu5Gc:Ac ratio was 4:! in 100 μM Ac_4GlcNAc in comparison to 0-50 μM with 9:1. Supplementation of 25 μM Ac_4GlcNAc alone or in combination with 1-5 mM uridine resulted in a significant decrease in Neu5Gc of up to ~60% and in the total Sia of up to 45% .

Chapter 10

Effect of free Neu5Gc and Neu5Ac supplementation on sialic acid proportions of IgG1 and EG2-hFc Mabs

10.1 Introduction

Mammals (e.g. chimpanzees, gorillas) have Neu5Gc and Neu5Ac as terminal Sias in glycoproteins. In contrast, humans lack Neu5Gc and instead, *N*-glycans, *O*-glycans and gangliosides terminate in Neu5Ac. The lack of Neu5Gc in humans has led to the presence of anti-Neu5Gc antibodies which has compromised the serum half-life of glycoproteins produced by animal and/or mammalian cells due to Neu5Gc epitope's presence. Neu5Gc comprises 95% and 11% of total Sias expressed in mouse and rat liver glycoconjugates respectively (Irie, Koyama *et al.* 1998). In addition, there are currently numerous FDA-approved therapeutics that contain Neu5Gc at different amounts depending on the cell line (e.g. CHO, NS0, Sp2/0, BHK, hybridomas) or animal source (e.g. goat milk; bovine and pig enzymes; equine-, sheep-, rabbit-serum) (Shaw, Schneckenburger *et al.* 1992, Ghaderi, Zhang *et al.* 2012). Even though human cells lack a functional CMAH gene, human cells (e.g. HEK, HT-80) have also shown to metabolically incorporate Neu5Gc when exposed to Neu5Gc-rich exogenous sources. This is no different *in vivo* where human gut epithelial cells are constantly exposed to glycoprotein-bound Neu5Gc from food sources (e.g. read meat, milk products) (Bardor, Nguyen *et al.* 2005). Bardor *et al.* described how

human cells incorporate exogenous Neu5Gc into glycoproteins via non-clathrin-mediated mechanisms, mostly by pynocytosis. The resulting pinocytotic vesicles and endosomes will fuse with lysosomes where glycoprotein-bound Neu5Gc will be cleaved by the lysosomal sialidase and then released to the cytosol by action of the lysosomal Sia transporter. Once in the cytosol, free Sias are salvaged and activated prior to attachment to glycoproteins by sialyltransferases (Bardor, Nguyen *et al.* 2005). Thus, human biosynthetic pathways are not able to differentiate between Neu5Ac and Neu5Gc, which differ by a single oxygen atom (Tangvoranuntakul, Gagneux *et al.* 2003). Similarly, animals with a human-like defect (e.g. *Cmah*$^{-/-}$ mice) are also able to incorporate Neu5Gc in multiple organs after long term feeding with soy chow containing porcine submaxillary mucin (100-250 μg of Neu5Gc /g of chow) (Banda, Gregg *et al.* 2012). Thus, since humans and animals with a dysfunctional CMAH gene can metabolically incorporate Neu5Gc from dietary sources, monitoring culture environment becomes critical to control Neu5Gc content in glycoproteins (Bardor, Nguyen *et al.* 2005). Borys *et al.* observed that sodium butyrate, lowering temperature nearly stationary phase and increasing pCO2 levels reduced Neu5Gc levels to up to 62%, 59% and 46% respectively (Borys, Dalal *et al.* 2010). In addition, exposure of human cells to free Neu5Ac has shown to compete with Neu5Gc, reducing the amount of this immunogenic Sia in therapeutic glycoproteins (Ghaderi, Taylor *et al.* 2010).

In Chapter 8 and 9, it was shown that NS0 cells had Neu5Gc as their main Sia added to IgG1 with a proportion of 3 to 1 with respect to Neu5Ac. In contrast, EG2 cells strongly preferred Neu5Ac over Neu5Gc, showing negligible amounts of Neu5Gc in control cultures. Knowing that Neu5Gc and Neu5Ac ratios can vary according species and tissue type, this Chapter focused on the effect that supplementation of free Sia has on Sias proportions in both NS0-IgG1 and CHO

EG2-hFc Mabs. For instance, incorporation of free Neu5Ac was expected to reduce the amount of Neu5Gc present in NS0-IgG1 Mab, as previously shown in other reports where Neu5Ac acted as a competitive inhibitor of Neu5Gc. Also, free Neu5Gc was added to the media to test if Neu5Gc content could further increase from the original amount found in NS0-IgG1. Finally, a combination of Neu5Gc and Neu5Ac (Neu5Ac+Gc) were also provided to NS0 cells to evaluate the effect on Sias proportions. On the other hand, CHO cells were also exposed to free Neu5Ac aiming to increase sialylation of EG2-hFc Mabs. In addition, free Neu5Gc or a combination of Neu5Gc and Neu5Ac were also supplemented to CHO cells and the change in Sia proportions, if any, was analyzed. Reducing Neu5Gc content while increasing Neu5Ac levels will allow to enhance biotherapeutic products by reducing their immunogenicity and improving their half-life.

10.2 Objectives.

- Reduce the occurrence of murine-specific Neu5Gc motif in IgG1 Mab by feeding of Neu5Ac.
- Evaluate the effect on Sia proportions by feeding Neu5Gc/Ac to NS0 and EG2 cell cultures.

10.3 Experimental set up

Feeding of NS0 and CHO cells with exogenous Sias was evaluated on the sialylation of IgG1 Mab. For this purpose, cells were seeded in 125 mL shake flasks at $2.5x10^5$ cells/mL in 40 mL of Biogro™ media supplemented with 10 μM Neu5Ac; 10 μM Neu5Gc or 10 μM Neu5Gc plus 10 μM Neu5Ac for four days at 37°C, 10% CO_2. Each condition was performed in triplicates and the mean value of two different set of experiments is stated (n=6). Cell density and viability

was monitored daily by trypan blue exclusion assay. After four days, cells were spun down at 1500 rpm for 5 minutes and supernatant was collected, filtered and concentrated 10X before further analysis. Pre-purified Mab (100 μg/mL) was subjected to mild acid hydrolysis and released Sias were incubated in the dark with DMB reagent for 2.5 hours at 50°C. The derivatives were then separated in a C18 column and Sias were identified by fluorescence detection. Sias amount was determined by comparison with known quantities of DMB-derivatized Neu5Gc and Neu5Ac standards and then reported as a ratio of moles Sia per mole of recombinant protein.

10.4 Results

10.4.1 Free Sia supplementation effect on NS0 and CHO cells' growth.

To evaluate the effect of free Sias on the Sia proportions of IgG1 and EG2-hFc Mabs, NS0 and CHO EG2 cells were cultured in the presence of 10 μM of Neu5Ac, 10 μM Neu5Gc or a combination of both. Sias were added at the beginning of a four-day batch culture and their effect on cell growth are shown in **Figure 10.1**. During the first 24 hours, a lag phase took place with a low cell specific cell growth (μ) of ~0.02 h^{-1}in NS0 cells (**Figure 10.1A)**. Then, μ increased to up to 0.04 h^{-1} during exponential phase in all culture conditions. Supplementation of Neu5Gc, Neu5Ac or a combination of both Sias did not affect significantly the maximal cell densities (MCDs) seen on day four with up to 2.88 ±0.24 x 10^6 cells/mL in comparison to the control with 2.82 ±0.23 x 10^6 cells/mL. Furthermore, all cultures showed a viability over >90%. With respect to EG2 cells, there was no evidence of a lag phase during the first 24 hours, with a specific cell growth of ~0.05 h^{-1} in all cultures (**Figure 10.1B)**. Cell growth continued until day three, where cells cultured in the presence of Sias reached their maximal cell densities (MCD), which were

comparable to the control cultures (MCD of 2.97 ± 0.42 x 10^6 cells/mL). EG2 cells' viability also remained over 90% in all cultures all along the culture period.

These results showed that free Neu5Ac and Neu5Gc supplementation did not have an influence on either NS0 and EG2 cells growth nor in cell viability when compared with untreated cultures. Differences in specific cell growth was observed between cell lines, with CHO EG2 cells showing higher growth rates than NS0 cells during the first 48 hours. However, comparable MCD were obtained between both cell lines on day four.

10.4.2 Effect of free Sia supplementation on the *N*-linked glycosylation of NS0-IgG1 and CHO EG2-hFc Mabs.

N-linked glycosylation was analyzed in NS0-IgG1 Mabs harvested on day four from cultures supplemented with free Sias. NS0 cells were cultured in Biogro™ media containing 10 μM Neu5Ac, 10 μM Neu5Gc or 10 μM Neu5Ac plus 10 μM Neu5Gc. Antibodies were harvested and protein A purified prior to glycosylation analysis by HILIC. As previously described, NS0-IgG1 produced in Biogro™ media contains a higher proportion of glycans containing one Gal residue (G1), followed by glycans containing zero and two Gal residues (G0 and G2 respectively) (**Figure 10.2A**). The same distribution of glycans was observed in all culture conditions with minor differences in cultures containing Neu5Ac. For instance, Neu5Ac-fed cultures showed a significant increase of about 20%, 24% and 16% in the amount of G0 glycans with respect to the control (p-Value 0.05), Neu5Gc-fed cultures (p-Value 0.02) and Neu5Ac+Gc-fed cultures (p-Value 0.01) respectively. Furthermore, Neu5Ac containing cultures also had a higher amount of G1 glycans

(G1=51.1% ± 1.56) with respect to all culture conditions, but it was just significantly higher by 6% with respect to the control (G1= 48.3 ± 1.55; p-Value 0.02). Finally, Neu5Ac-fed cultures showed the lowest amount of G2 glycans (18.6% ± 4.62) in comparison to Neu5Gc-fed cultures (25.3%; p-Value 0.05), Neu5Ac+Gc (26.8%; p-Value 0.02) and the control culture (26.4%; p-Value 0.03). Cultures containing Neu5Gc alone or in combination with Neu5Ac (Neu5Ac+Gc) showed a galactosylation index (GI= 0.50 ± 0.05 and 0.52 ± 0.04 respectively) comparable to the control with 0.51±0.07 (**Figure 10.3)**. A slight reduction in GI was observed in Neu5Ac-containing cultures with 0.44 ± 0.04 but was not significant. The relative abundance of alpha-1,3 gal-containing glycans was not significantly influenced by any of the free Sia supplements, varying from 5.76% ± 1.81 to 8.59% ± 1.84 (data not shown). Neu5Gc- and Neu5Gc+Neu5Ac-containing cultures didn't show any change in sialylation (8.97% ± 4.20 and 10.22% ± 2.90 respectively) in NS0-IgG1 Mabs, having comparable amount of sialylated glycans with the control culture (10.6% ± 3.56) (**Figure 10.4**). Neu5Ac cultures showed a lower content of Sias (5.25% ± 2.48) but this reduction was not significant. However, Neu5Gc+Neu5Ac-fed cultures showed a significantly higher sialylation in comparison to cultures containing Neu5Ac alone (p-Value 0.02). This could be a result from containing the double amount of Sia (10μM of each Sia) in comparison to Neu5Ac cultures. However, this effect was not observed when comparing Neu5Gc-fed cultures with Neu5Ac+Gc containing cultures.

With respect to CHO cells, EG2-hFc Mabs produced in media without additives showed 18% ± 3.06, 26% ± 1.97 and 55% ± 5.03 of glycans containing zero, one and two Gal residues respectively. A similar distribution (G0<G1<G2) was observed in Sia supplemented cultures (**Figure 10.5A).**

Figure 10. 1 The effect of free Sia supplementation on NS0 and CHO EG2 cells' growth and viability.

A. NS0 cells and **B.** CHO cells when cultured in Biogro™ media containing free 10 μM Neu5Ac, free 10 μM Neu5Gc or 10 μM Neu5Gc plus 10 μM Neu5Ac. Cells were inoculated at an initial cell density of $2.5x10^5$ cells/mL in 35 mL culture medium and cell growth (solid lines) and viability (dashed lines) were monitored for four days by trypan blue exclusion assay. Control culture did not contain any supplement. Data points represent mean ± SD of triplicates (*n=3*).

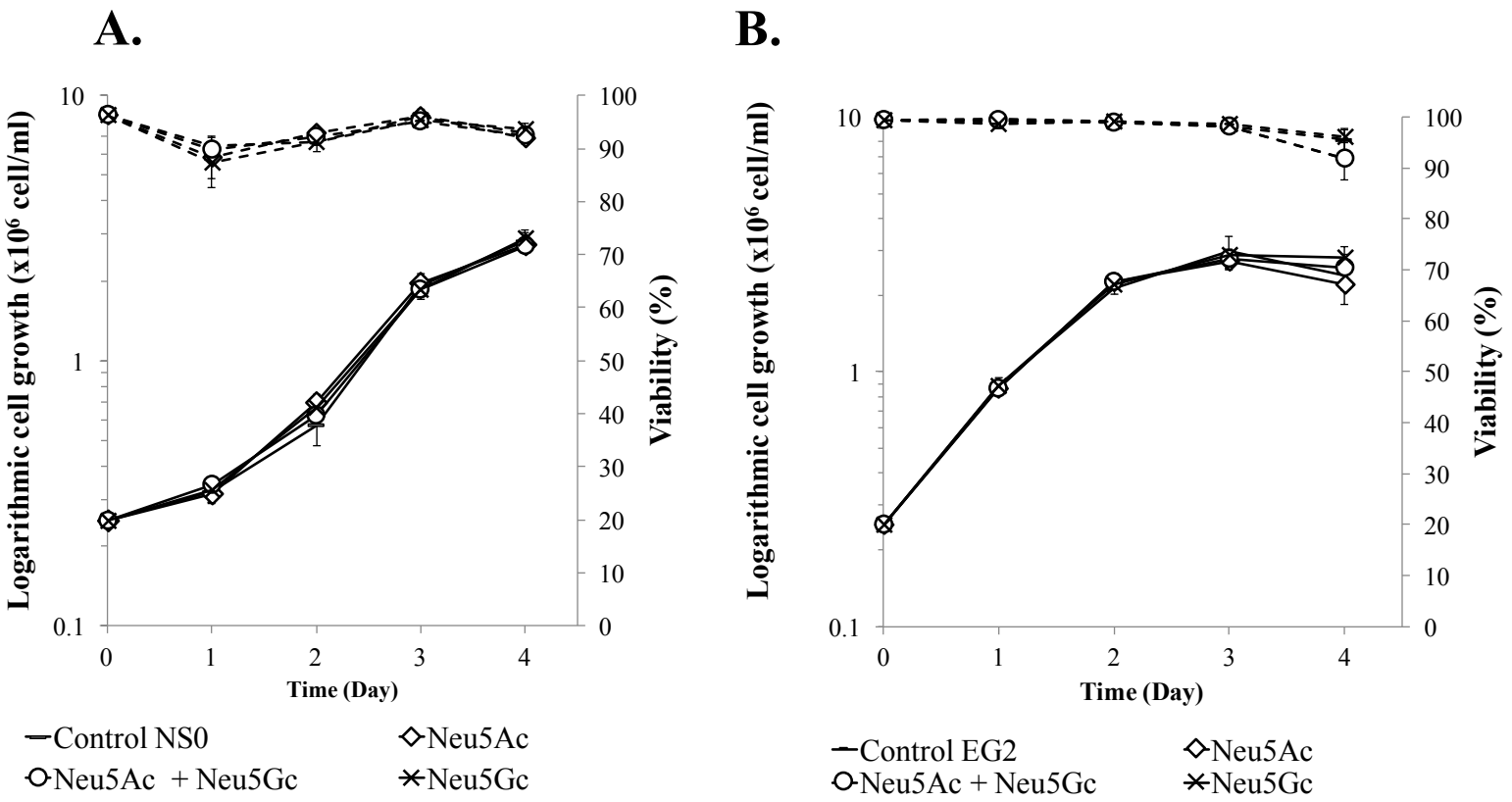
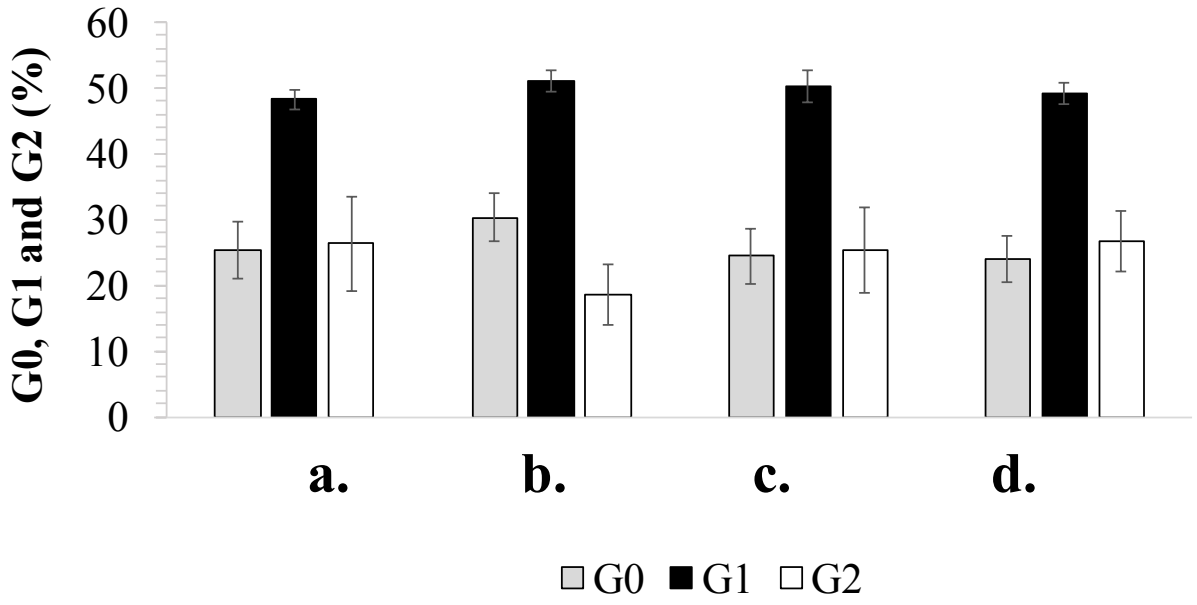


Figure 10. 2 Effect of free Sia supplementation on galactosylation of IgG1 Mab.

The relative abundance of glycans containing zero, one or two Gal (G0 (-■-), G1 (-■-) and G2 (-□-) respectively) residues from NS0-IgG1 Mabs produced in a. Biogro™ media supplemented with: b. 10 μM Neu5Ac, c.10 μM Neu5Gc and d.10 μM Neu5Ac plus 10 μM Neu5Gc (Neu5Ac+Neu5Gc). Mabs were harvested and protein purified prior to HILIC analysis. Control culture did not contain any supplement. Data points represent mean ± SD of triplicates for two independent experiments (*n=6*). Pairs of means ± SD significantly different (p-Value 0.02) with respect to the control).



A slightly decrease in G0 and G1 containing glycans was observed in EG2-hFc Mabs produced in media supplemented with Sias. This decrease was concomitant with an increase in G2 structures in all culture conditions. However, the changes described above were significant just in Neu5Ac+Gc cultures when compared to the control culture. Thus, a significant decrease of 25% (p-Value 0.03) was observed in G0 glycans and a significant increase of 12% (p-Value 0.03) in G2 glycans were observed in Neu5Ac+Gc cultures. No significant differences were observed in between Sia -containing cultures with respect to G0 and G2 glycans. As a result, Neu5Gc- and

Neu5Ac-fed cultures showed a slightly higher GI (0.72 ± 0.03 and 0.73 ± 0.02 respectively) but was not significant when compared to the control (0.68 ± 0.04) (**Figure 10.5B)**. However, cultures containing both Sias (Neu5Ac+Gc) showed an 8% increase in GI (0.74 ± 0.01; p-Value 0.04) in comparison to EG2-hFc Mabs produced in media without additives. Sia-supplemented cultures did not show significant differences among them. Finally, the effect of Neu5Gc and Neu5Ac on the sialylation of EG2-hFc is shown in **Figure 10.5C**. Incorporation of Sias alone or in combination (Neu5Ac+Gc) failed to increase the original amount of Sia found in the control sample (20.9% ± 4.10).

Figure 10. 3 Galactosylation index (GI) of IgG1 Mabs cultured in media supplemented with free Sias.

NS0-IgG1 Mabs produced in **a.** Biogro™ media supplemented with: **b.** 10 μM Neu5Ac, **c.**10 μM Neu5Gc and **d.** 10 μM Neu5Ac plus 10 μM Neu5Gc (Neu5Ac+Neu5Gc). Mabs were harvested and protein purified prior to HILIC analysis. Control culture did not contain any supplement. Data points represent mean ± SD of triplicates for two independent experiments (n=6). Pairs of means ± SD significantly different (p-Value 0.02) with respect to the control)

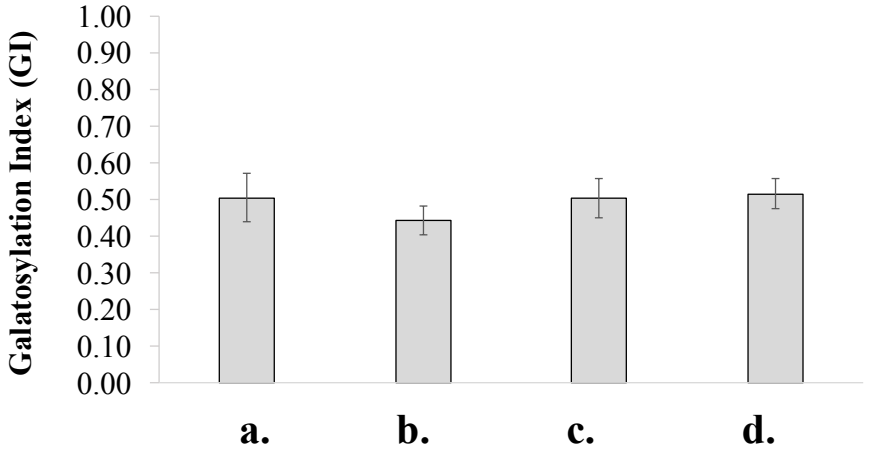
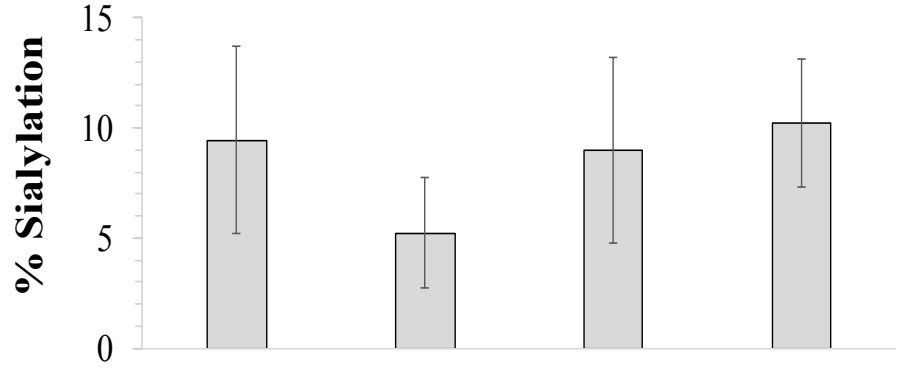


Figure 10. 4 Effect of free Sias supplementation on sialylation of IgG1 Mabs.

Relative abundance of glycans containing Sias (%) from NS0-IgG1 samples cultured in **a.** Biogro™ media supplemented with: **b.** 10 μM Neu5Ac, **c.** 10 μM Neu5Gc and **d**. 10 μM Neu5Ac plus 10 μM Neu5Gc (Neu5Ac+Neu5Gc). Mabs were purified by protein A from cultures at day four, 2-AB-labeled and analyzed by HILIC chromatography. Values correspond to the mean ± SD of triplicates of two independent experiments (n = 6).



These results show that incorporation of free Sias did not improve *N*-linked glycosylation in NS0-IgG1 Mabs in terms of galactosylation and sialylation. However, Neu5Ac cultures showed a slightly lower GI and percentage of sialylation in comparison to the control, which could have resulted from the 20% increase observed in G0 glycans along with the 30% reduction in G2 glycans Cultures containing Neu5Ac+Neu5Gc (10 μM of each Sia) did not had an effect in the *N*-glycosylation of NS0-IgG1 Mabs, even though there was twice as much exogenous Sia than the control and cultures containing either Sia. With respect to EG2 cells, a significant decrease of 25% and a significant increase of 12% was observed in G0 and G2 glycans respectively in Neu5Ac+Gc cultures. No significant differences were observed in between Sia-containing cultures with respect to the control.

10.4.3 Effect of free Sia supplementation on the proportions of Neu5Gc and Neu5Ac in NS0-IgG1 and CHO EG2-hFc Mabs.

Supplementing Biogro™ media with exogenous Neu5Gc and Neu5Ac did not influence the total amount of sialylation originally present in NS0-IgG1 and CHO EG2-hFc Mabs. However, the effect of Sia feeding on sialylation was further explored by considering the proportions of Neu5Ac and Neu5Gc. In control cultures, IgG1 Mabs showed a total amount of Sias of 0.24 ± 0.02 mole Sia/mole IgG1, where 88% corresponded to Neu5Gc. This was equivalent to a ratio of Neu5Gc:Neu5Ac of 7:1 (**Figure 10.6, Table 10.1**). NS0 cells cultured in the presence of 10 μM Neu5Ac alone or in combination with 10 μM Neu5Gc showed a slightly higher, but not significant, amount of total Sia with 0.27 ± 0.02 moles/mole IgG1. Neu5Gc was the predominant Sia (87% and 90% of the total Sia pool) in Neu5Ac- and Neu5Ac+Gc-fed cultures respectively in comparison to the control with 88%. More importantly, the Sia ratio slightly changed to ~6:1 and 9:1 in Neu5Ac- and Neu5Ac+Gc-fed cultures respectively (**Table 10.1**). An increase of 25% in the total amount of Sia (0.30 ± 0.02) was observed in cultures containing 10 μM Neu5Gc, which resulted from an increase of about 18% in Neu5Gc content (p-value 0.01). Thus, the original Neu5Gc to Neu5Ac ratio observed in the control changed slightly to 6:1 in Neu5Gc-fed cultures (**Table 10.1**).

Figure 10. 5 Effect of free Sias on CHO EG2-hFc Mabs' glycosylation.

CHO cells were cultured in shake flasks containing **a.** Biogro™ media supplemented with: **b**. 10 µM Neu5Gc; **c**. 10 µM Neu5Ac; or **d.**10 µM Neu5Gc plus 10 µM Neu5Ac at 37 °C. **A**. The abundance of glycans containing G0 (-■-), G1 (-■-) and G2 (-□-) was used to calculate the **B.** GI of EG2-hFc Mabs after incubation in the presence of free Sias. **C.** Sia content is expressed as a percentage. Data points represent mean ± SD of triplicates for each condition (n=3). *Pair of means ± SD significantly different (p-Value 0.03) with respect to control culture.

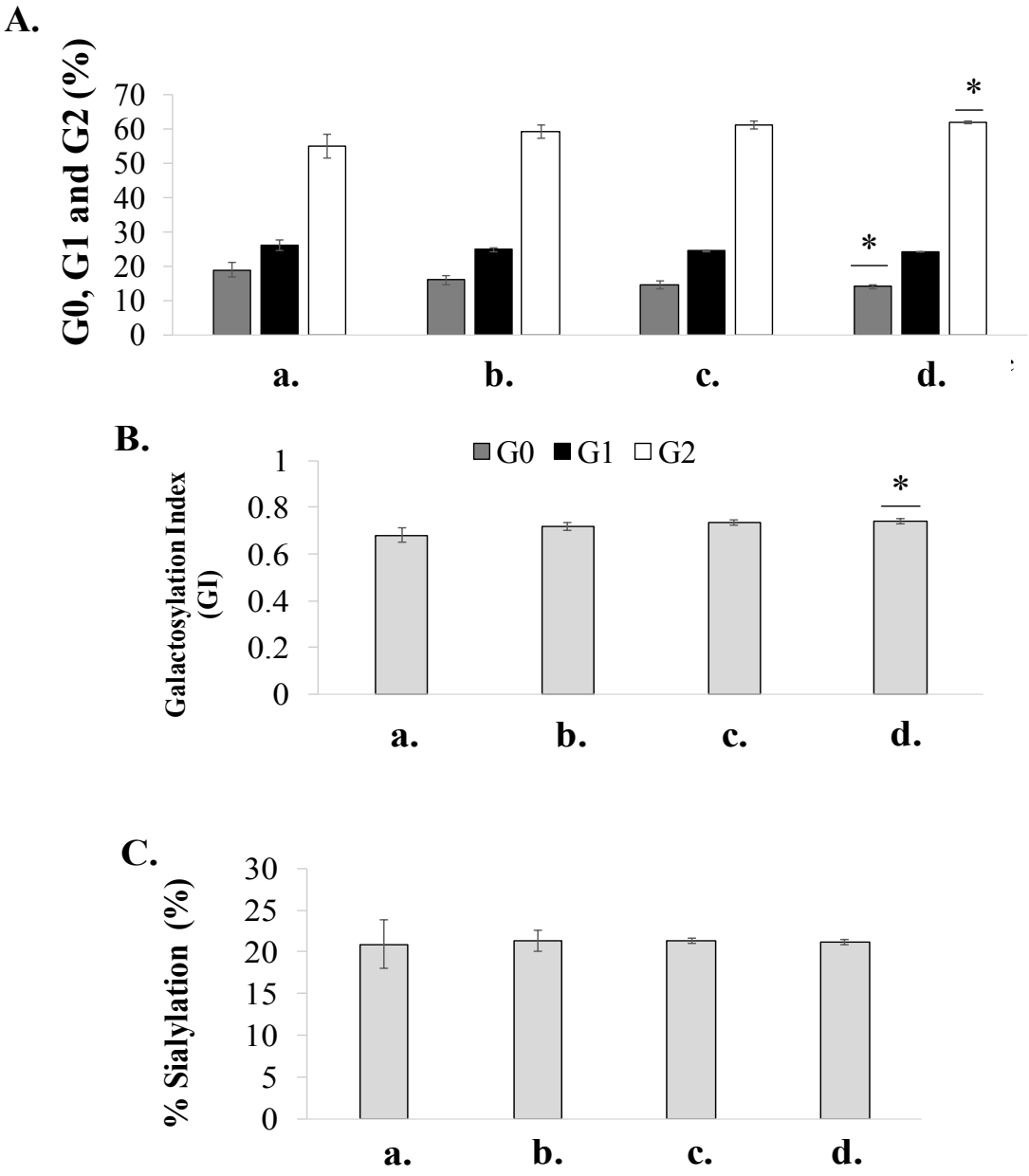
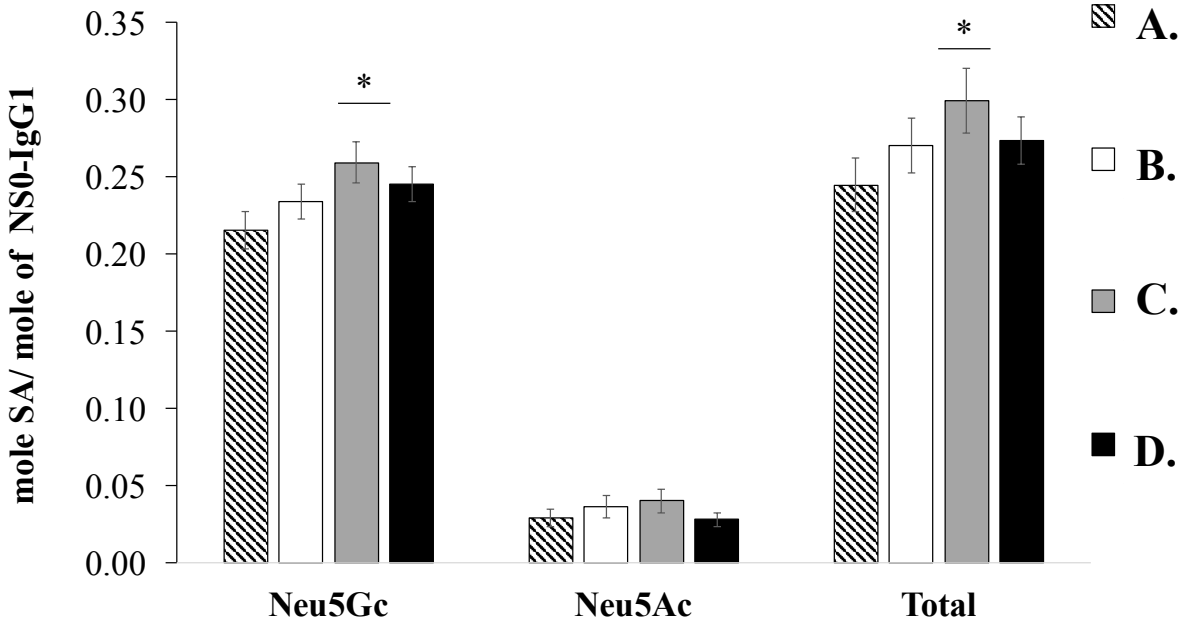


Figure 10. 6 Effect of free Sia supplementation on Sia proportions of IgG1 Mab.

Neu_5Gc and Neu_5Ac content (mole Sia/mole Mab) in NS0-IgG1 Mab produced in A. Biogro™ media (control, (▨-) supplemented with: B. 10 μM Neu5Ac (-□-); C. 10 μM Neu5Gc (-■-); D. 10 μM Neu5Gc and 10 μM Neu5Ac (-■-). Neu5Gc/Neu5Ac content were analyzed quantitatively by reverse-phase HPLC of 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB)-labeled Sias released by mild acid hydrolysis. Values correspond to the mean ± SD of duplicates from two different set of experiments. *Data significantly different with respect to the control sample (p-Value <0.05).

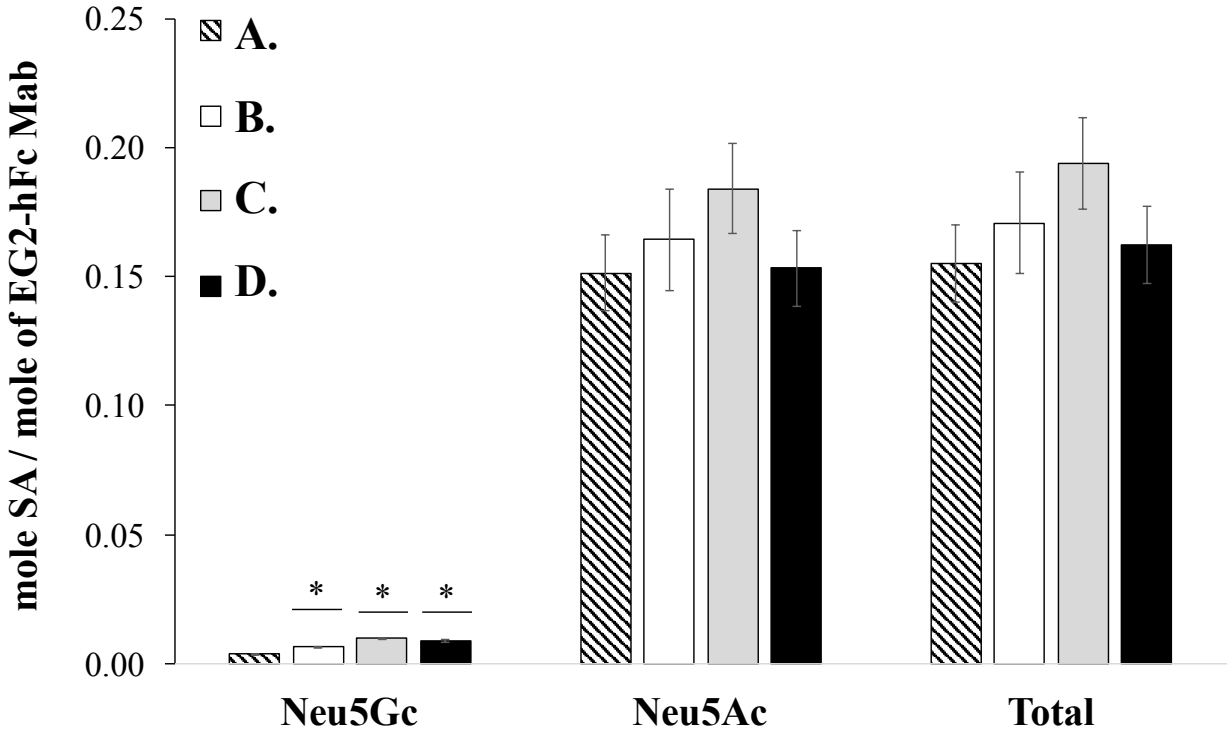


In CHO EG2 cells, control cultures showed a total amount of Sias of 0.16 ± 0.01 with Neu5Ac being the predominant Sia added to EG2-hFc (98%). Cultures containing 10 μM Neu5Ac alone or in combination with 10 μM Neu5Gc showed comparable amounts of total Sia in comparison to the control. However, a slightly increase but significant in Neu5Gc content was observed in Neu5Ac and Neu5Ac+Neu5Gc- fed cultures constituting 4% and 6% of the total Sia

content respectively (p-Value <0.0001). Mabs produced in media containing 10 μM Neu5Gc also showed Neu5Ac as the predominant Sia with 95%. A significant increase in Neu5Gc content was also observed (p-Value 0.0001), constituting 5% of the total Sia. Nevertheless, the ratio observed of Neu5Gc to Neu5Ac was 0:1 in all culture condition (**Table 10.1**).

Figure 10. 7 Neu_5Gc and Neu_5Ac content (mole Sia/mole Mab) in CHO EG2-hFc Mab produced in media supplemented with free Sias.

A. Biogro™ media (control, ▨-) supplemented with: **B.** 10 μM Neu5Ac (-□-); **C.** 10 μM Neu5Gc (-■-); **D.** 10 μM Neu5Gc and 10 μM Neu5Ac (-■-).. Neu5Gc/Neu5Ac content were analyzed quantitatively by reverse-phase HPLC of DMB-labeled Sias released by mild acid hydrolysis. Values correspond to the mean ± SD of duplicates from two different set of experiments. *Data significantly different with respect to the control sample (p-Value <0.0001).



These results show that by supplementing 10 μM Neu5Ac alone or in combination with 10 μM Neu5Gc a slight increase of up to 4.5% in Neu5Gc content was observed with respect to control cultures. In contrast, 10 μM Neu5Gc-containing cultures showed a significant increase of about 18% in Neu5Gc content. The latter corresponded to a change in the Neu5Gc:Neu5Ac ratio observed in the control (7:1) to 9:1 in IgG1 Mab produced in media supplemented with 10 μM Neu5Gc. Furthermore, Neu5Gc-fed cultures was the only culture condition that showed a significant increase in the total amount of Sia with respect to the control. There were not significant differences in the content of Neu5Ac in Sia-supplemented cultures. With respect to EG2 cells, a slightly increase in the amount of Neu5Gc was observed in all cultures containing Neu5Gc, Neu5Ac or a combination of both Sias. Nevertheless, Neu5Gc remained <6% and Neu5Ac continued to be the predominant Sia in EG2-hFc Mabs.

10.5 Discussion

Neu5Gc is widespread throughout the deuterostome lineage of animals, from the echinoderms up to mammals. The latter includes animal cells commonly used to produce recombinant biotherapeutic glycoproteins which can contain high levels of this non-human glycan epitope (Corfield A.P. 1982, Schauer 1995, Davies and Varki 2015). In fact, current FDA-approved biotherapeutic glycoproteins have shown to carry the immunogenic Neu5Gc (Ghaderi, Zhang *et al.* 2012). Another source of undesirable Neu5Gc are mouse fibroblasts commonly used as feeder layers during human embryonic stem cells (hESCs) culture (Ajit Varki 2009). Neu5Gc can compromise drug's efficacy and can induce side effects or immunogenic reactions in patients, since all humans have circulating anti-Neu5Gc antibodies. Thus, minimizing Neu5Gc content

constitutes one of the key quality attributes that pharmaceuticals need to ensure. There are a few reports describing how to decrease or eliminate the Neu5Gc content in glycoproteins. For instance, using disruption of CMAH gene in animals can provide biological material free from Neu5Gc (e.g. serum, red meat, milk, animal cells)(Chenu, Gregoire *et al.* 2003, Ghaderi, Zhang *et al.* 2012). Even though genetic modification can eliminate Neu5Gc expression in animal cells or transgenic animals, unexpected expression has been hypothesized to result via metabolic incorporation of Neu5Gc from dietary sources rich in Neu5Gc (Banda, Gregg *et al.* 2012). Furthermore, it would be more difficult to change well-established cell lines currently used to produce biotherapeutics (e.g. CHO, BHK, murine myeloma) and that have already been approved by the FDA. Thus, it is more feasible to monitor culture conditions that affect Neu5Gc content in glycoproteins than to use genetic engineering. Borys *et al.* observed that sodium butyrate (SB), lowering temperature nearly stationary phase or increasing pCO_2 levels reduced Neu5Gc levels to up to 62%, 59% and 46% respectively (Borys, Dalal *et al.* 2010). As a result, the ratio observed of Neu5Gc:Neu5Ac changed from 1:10 in the control to 1:19 in cultures containing SB. Similarly, a change in Neu5Gc:Neu5Ac ratio was observed as the shift low temperature occurred early- (1:5) vs. late- (1:12) stages of the culture.

Table 10. 1 Summary of the effect of free Sia supplementation on the N-linked glycosylation of A. NS0-IgG1 Mab and B. EG2-hFc Mab.

Mabs were harvested and protein purified prior to HILIC analysis. The galactosylation index and sialylation were determined after four days of culture. Control culture did not contain any supplement. Shaded are corresponds to the amount of Neu5Gc and its ratio with respect to Neu5Ac. Amount of Sias is presented in mole of Sia / mole of Mab. The values correspond to the mean ± SD of duplicates from two different set of experiments. *Data significantly different with respect to the control sample (p-Value <0.05).

A.	**Glycosylation Analysis**		**Sialic Acid Analysis**			
Supplement concentration	**GI**	**% Sialylation**	**Neu5Gc**	**Neu5Ac**	**Total**	**ratio**
Control	0.51 ± 0.07	10.6 ± 3.56	0.22 ± 0.01	0.03 ± 0.01	0.24 ± 0.02	7 : 1
10 uM Neu5Ac	0.44 ± 0.04	5.25 ± 2.48	0.23 ± 0.01	0.04 ± 0.01	0.27 ± 0.02	6 : 1
10 uM Neu5Gc	0.50 ± 0.05	8.97 ± 4.2	0.26 ± 0.01	0.04 ± 0.01	0.30 ± 0.02	6 : 1
10 uM Neu5Ac+ 10 uM Neu5Gc	0.52 ± 0.04	10.2 ± 2.9	0.25 ± 0.01	0.03 ± 0.00	0.27 ± 0.02	9 : 1

B.	**Glycosylation Analysis**		**Sialic Acid Analysis**			
Supplement concentration	**GI**	**% Sialylation**	**Neu5Gc**	**Neu5Ac**	**Total**	**ratio**
Control	0.68 ± 0.03	20.9 ± 2.9	0.00 ± 0.00	0.15 ± 0.01	0.16 ± 0.01	0 : 1
10 uM Neu5Ac	0.72 ± 0.02	21.3 ± 1.23	0.01 ± 0.00	0.16 ± 0.02	0.17 ± 0.02	0 : 1
10 uM Neu5Gc	0.73 ± 0.01	21.4 ± 0.29	0.01 ± 0.00	0.18 ± 0.02	0.19 ± 0.02	0 : 1
10 uM Neu5Ac+ 10 uM Neu5Gc	0.74 ± 0.01	21.2 ± 0.29	0.01 ± 0.00	0.15 ± 0.01	0.16 ± 0.01	0 : 1

Finally, shifting the pCO_2 levels from 20-40 mmHg to 140 mm Hg changed the Neu5Gc:Neu5Ac from 1:6 to 1:11 (Borys, Dalal *et al.* 2010). Thus, an increase in Neu5Ac content at the expense of Neu5Gc occurred under these culture conditions. Knowing that Neu5Ac and Neu5Gc compete for activation by CMP-synthetase prior to sialylation, consecutive exposure of human cells to free Neu5Ac (3 mM-15mM) has shown to reduce Neu5Gc content by metabolic competition (Ghaderi, Taylor *et al.* 2010). Since exogenous Sias can be taken up by animal cells and the fact that Sias constitute the most immediate precursors for sialylation, NS0 and EG2 cells were incubated in media containing free Neu5Gc, Neu5Ac or a combination of Neu5Gc *plus* Neu5Ac to evaluate their effect on the Sia proportions of IgG1 and EG2-hFc Mabs respectively. In EG2 cells, Neu5Ac feeding did not influence EG2-hFc Mab sialylation nor the Neu5Gc:Neu5Ac proportions. With respect to IgG1 Mab, feeding of Neu5Ac was expected to compete with newly synthesized Neu5Gc by NS0 cells and decrease Neu5Gc content based on the aforementioned reports (Ajit Varki 2009). However, NS0-IgG1 Mab produced in media with 10 μM Neu5Ac showed comparable Neu5Gc content with respect to untreated cultures, free Neu5Gc- and Neu5Gc plus Neu5Ac-containing cultures. The lack of effect of Neu5Ac on NS0-IgG1-Neu5Gc content suggests that the Neu5Ac concentrations as well as the time used for exposure were not enough to compete with newly synthesized Neu5Gc. Thus, concentrations > 10 μM Neu5Ac (mM range) could have been more efficient in reducing the content of Neu5Gc in NS0 cells, by increasing the amount of moles of Neu5Ac/ mole of Mab. For example, Varki *et al.* suggested that incorporation concentrations as high as 0.1 mM- 20 mM Neu5Ac in drinking water, food or cell media have been used to reduce Neu5Gc content. However, human 293 T- and mouse cells- containing Neu5Gc required a time of exposure to 5 mM Neu5Ac of 7 days to up to 6 months in order to decrease the content of Neu5Gc respectively (Ajit Varki 2009). Future experiments should focus on exposing

NS0 cells to higher concentrations of free-Neu5Ac as well as extending the time of exposure to free Neu5Ac. The latter may improve the sialylation pool and the content of Neu5Ac vs. Neu5Gc observed in untreated cultures.

With respect to Neu5Gc feeding, Bardor *et al.* showed that a 3 day-exposure to media containing 3 mM Neu5Gc resulted in incorporation of this immunogenic Sia in different subcellular fractions of human fibroblasts, neuroblastoma and Caco-2 cells. Furthermore, ingestion of porcine submaxillary mucin in human volunteers, which contains up to 95% Neu5Gc, resulted in partial incorporation into newly synthesized glyconjugates and partial excretion of Neu5Gc in urine (Tangvoranuntakul, Gagneux *et al.* 2003). In accordance to these reports, incorporation of free NeuGc slightly increase the Neu5Gc content observed in untreated cultures (2.38%) by 2-fold in cultures supplemented with either free Sia, but it remained below <6%. The latter showed that EG2 cells' negligible amounts of Neu5Gc in untreated cultures could be further increased if exposed to exogenous Neu5Gc. Similarly, a slight but significant increase of about 18% and 25% was also observed in Neu5Gc content and in the total Sia pool respectively in NS0 cells when compared to the control. The latter supports the fact that mammalian cells other than human cells are also able to metabolically incorporate Neu5Gc from external sources (Ajit Varki 2009, Varki, Hedlund *et al.* 2013). Thus, it is important to monitor Neu5Gc content in glycoproteins produced by mammalian cells that have been in contact with animal supplements or serum replacements. The experiments used as a reference exposed cells to 0.1 mM – 20 mM Neu5Ac and 3mM Neu5Gc for at least three consecutive days. Even though the culture media volumes used in this publication are not mentioned, free Sias used as supplements were commercially purchased and not

synthesized by the investigators. These concentrations were not possible to reproduce in Biogro™ media due to limitations in the cost of the reagent.

Incorporation of 10 μM Neu5Gc *plus* 10 μM Neu5Ac did not further improve sialylation of untreated cultures, Neu5Gc- or Neu5Ac- containing cultures. Indicating again that higher concentrations are required to induce a more significant change in Sia proportions of EG2 and NS0 cells. However, incorporation of Neu5Ac along with Neu5Gc did not influence Neu5Gc content in the same manner as when Neu5Gc was supplemented alone. The latter may suggest that Neu5Ac was indeed competing with Neu5Gc in NS0 cultures. Thus, exposure to exogenous sources of Neu5Gc needs to be limited. Alternatives include replacing animal serum with human serum or serum from transgenic animals ($CMAH^{-/-}$) and using mammalian cells with a non-functional CMAH gene cultured in serum-free media. In addition, Varki's *et al.* strategy of "flooding the system" with Neu5Ac or Sia precursor (Chapter 9) can also be incorporated to cell culture to decrease or eliminate previously attached Neu5Gc in glycoproteins (Ajit Varki 2009, Ghaderi, Taylor *et al.* 2010, Ghaderi, Zhang *et al.* 2012, Ficko 2014). These strategies will allow to reduce the immunogenicity and clearance rates of recombinant proteins used for human therapies. However, this strategy seems not feasible as the cost of Neu5Ac (~$1,100 per gram) may limit its use during bioprocesses. Nevertheless, the aforementioned studies have provided important insight into how to reduce the amount of Neu5Gc. Thus, altering culture conditions that can improve the amount of Neu5Ac may result more practicable such as altering temperature, incorporating SB, supplementing ManNAc (Chapter 8), or changing the pCO_2 levels.

10.6 Conclusions

- Glycan distribution observed in NS0 untreated cultures (G2<G0<G1) was not affected by free Sia supplementation

- Significant increase of about 20% and 6% in IgG1 Mab-G0 and G1-glycans respectively along with a significant decrease of about 30% in G2 glycans were observed in cultures supplemented with free 10 μM Neu5Ac.

- Free Sias supplementation did not affect the total amount of sialylated glycans in NS0-IgG1 Mab.

- Free Sias did not affect the EG2-hFc Mab glycan distribution observed in control cultures (G0<G1<G2).

- A significant decrease of 25% along with a significant increase off 12% in G0- and G2- glycans respectively was observed in Neu5Ac+Gc- EG2 cultures. The latter did not affect significantly the GI in EG2-hFc Mab.

- Free Sia supplementation did not influence the content of Sias in EG2-hFc Mab.

- In IgG1 Mab, Neu5Gc:Neu5Ac ratio observed in untreated cultures (7:1) slightly changed to ~6:1 and 8:1 in Neu5Ac- and Neu5Ac+Gc-fed cultures respectively.

- Supplementation of 10 μM Neu5Gc showed a significant increase of about 18% in Neu5Gc content without a significant change in the Neu5Gc:Neu5Ac ratio (6.5:1) when compared to the control.

- There were not significant changes in Neu5Ac when free Sias were supplemented to EG2 cultures. A slight increase in Neu5Gc content was observed, but Neu5Gc remained below 6% in free-Sia supplemented EG2 cultures.

Chapter 11

Differences in sialic acid content of monoclonal antibodies produced by NS0 and CHO cells cultured in serum-supplemented media

11.1 Introduction

Serum has been widely used as a supplement for *in vitro* cell culture because it is rich in transport proteins, cytokines, hormones, co-factors, essential minerals, trace elements and growth factors, all of which promote animal cell proliferation (Gstraunthaler 2003). Bovine, equine and fetal calf serum (FCS) are among the most commonly used sera at a concentration of 10% (v/v) (Butler 2004). Fetal Bovine Serum (FBS) manufacture is a non-sterile process where blood is collected from several thousand fetuses to produce a single 1500 L batch of serum (Geigert 2013). In addition, FBS also provides a rich source of salvaged sugars for glycosylation. However, the undefined and variable composition of serum can introduce major inconsistencies between batches. Furthermore, serum can be a potential source of adverse factors (e.g. endotoxins) as well as a source of microbial contamination (e.g. fungi, bacteria, viruses or prions) (Brunner, Frank *et al.* 2010). Most importantly, FBS is a source of contamination with proteins that contain Neu5Gc which can be incorporated by cultured cells (**Chapter 10**).

The ratio of Neu5Gc:Neu5Ac varies in glycans depending on a strain, tissue, or cell within a species (Schauer 1995). In addition, the percentage of Neu5Gc will depend on the developmental stage, with adults having higher concentrations of Neu5Gc than fetus (Corfield A.P. 1982). For

instance, FCS contains 58-90% Neu5Ac with the remainder as Neu5Gc in comparison to adult animals where there is a higher ratio of Neu5Gc:Neu5Ac of 7:3. In contrast, equine serum contains a ratio of Neu5Ac:Neu5Gc of 85:15 (Corfield A.P. 1982). As previously mentioned in Chapter 9 and 10, human cells produce exclusively Sias of the Neu5Ac-type, due to an inactivation of CMP-*N*-acetylneuraminic hydroxylase (CMAH) gene responsible for the conversion of CMP-Neu5Ac to Neu5Gc (Tangvoranuntakul, Gagneux *et al.* 2003). For this reason, human serum is mainly constituted by 80% Neu5Ac, 20% 9-*O*-lactoylated (Neu5Ac9Lt) and traces of 9-*O*-acetylated (Neu5,9Ac2) (Corfield A.P. 1982).

In humans, the lack of an alternate pathway for Neu5Gc synthesis suggests that food rich in Neu5Gc (e.g. red meats, milk products) is responsible for the presence of Neu5Gc in normal tissues, tumors and fetal tissues (Bardor, Nguyen *et al.* 2005). Similarly, studies have shown that culturing human cells in media containing fetal bovine proteins present in serum, led to low levels of membrane-bound Neu5Gc (Bardor, Nguyen *et al.* 2005, Ajit Varki 2009). It was determined that the exogenous Neu5Gc was taken up by pinocytosis and then metabolically incorporated by human biosynthetic pathways which cannot distinguish between Neu5Ac and Neu5Gc, which differ by only one oxygen atom. (Furukawa K 1988 , Tangvoranuntakul, Gagneux *et al.* 2003, Bardor, Nguyen *et al.* 2005, Ajit Varki 2009). Indeed, after re-suspension of human cell lines in serum-free media and complete loss of Neu5Gc in glycoproteins it was suggested that serum was the source of Neu5Gc contamination in human cells (Muchmore EA 1998). Another example of Neu5Gc contamination is the case of stem cells which depend upon feeder layers of mouse embryonic fibroblasts which are also maintained in fetal calf serum (FCS) (Martin, Muotri *et al.* 2005).

11.2 Objective

- Investigate FBS's influence on sialylation, particularly on the content of the immunogenic Sia residue Neu5Gc in antibodies produced by two different mammalian cell lines, NS0 and EG2, known to have different initial Neu5Gc content.

11.3 Experimental set up

The effect of serum supplementation on the sialylation of IgG1-NS0 and CHO EG2-hFc Mabs was evaluated in a four-day batch culture. Cells were inoculated at $2.5x10^5$ cells/mL in 125 mL shake flasks (n=3) containing 75 mL of Biogro™ culture media supplemented 10% (v/v) of Ultra Low IgG Fetal bovine serum (UL-IgG FBS) (Thermo Fisher Scientific Inc. Catalog #16250078). UL-IgG FBS was chosen because of its lower IgG levels (<5 μg/mL) to avoid possibly contamination by bovine IgG which could compromise the extraction and purification of our secreted antibodies. The average level of IgG in normal FCS is approximately 70-300 μg/mL(RMBIO). During the experiment, shake flasks were placed in a shaker platform at 120 rpm and incubated at 37°C with 10% CO_2. Daily samples (500 μl) were taken to monitor cell growth and viability by trypan blue exclusion method; and productivity by ELISA. Mabs were harvested on day four where an extra sample containing $1x10^7$ cells was taken for nucleotide and nucleotide sugar analysis. Harvested antibodies were protein A purified prior to glycan and Sia analysis.

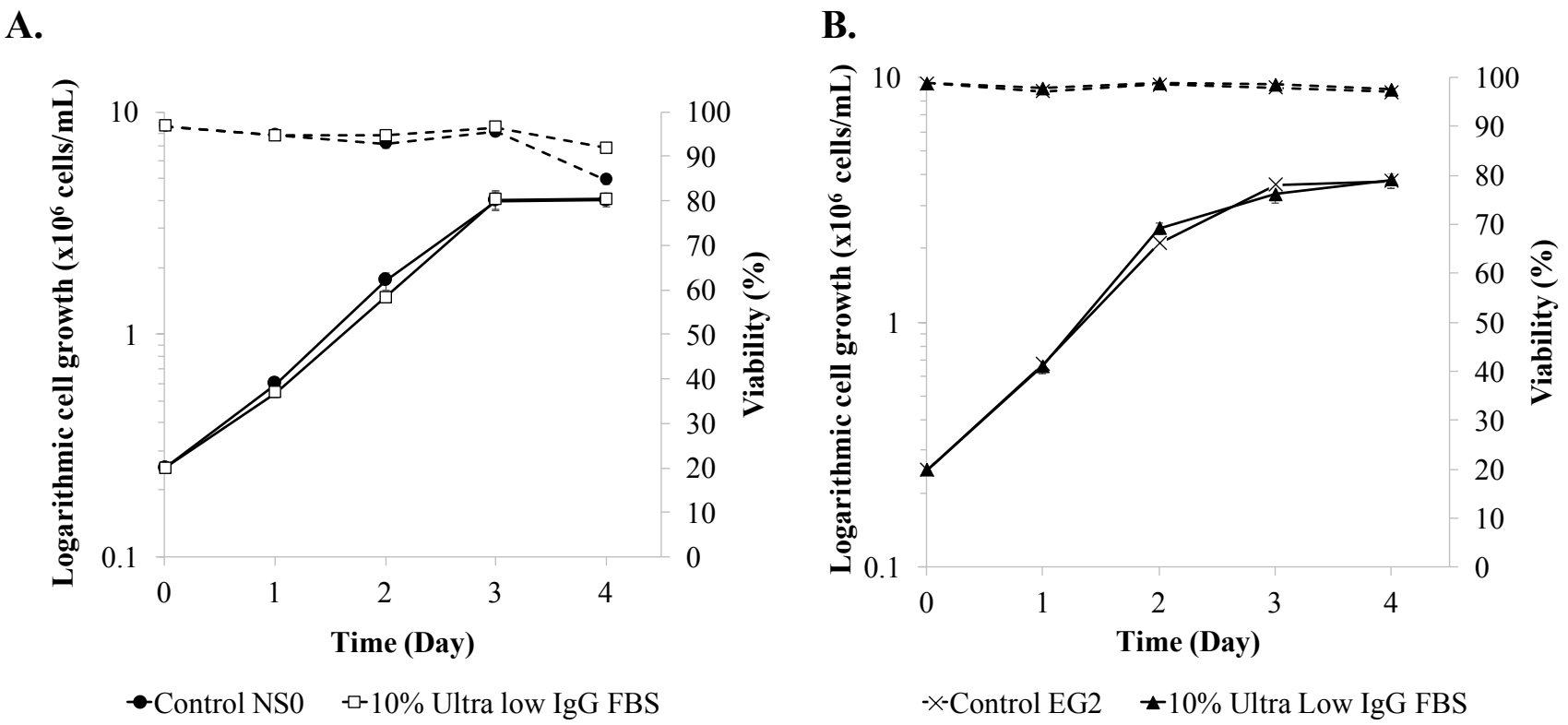
11.4 Results

11.4.1. Effect of Ultra-Low IgG FBS in NS0 and EG2 cells growth and viability

Figure 11.1A shows NS0 cells' growth and viability when cultured in the presence of 10% of Ultra low IgG FBS. Comparable cell densities were observed between serum-fed cultures and control culture along the culture period, reflected by their similar specific growth rates (0.04 h^{-1}). Stationary phase was reached on day three and maximal cell densities were reached on day four with 4.01 ± 0.27 x10^6 cells/mL and 4.05± 0.24 x10^6 cells/mL in control cultures and FBS-fed cultures respectively. Viable cell densities were above 94% during the first three days in control cultures, declining to 84.7% ± 1.32 on day four. Serum-fed cultures showed viabilities over 91% along the culture period. On the other hand, CHO cells' growth and viability after culturing for four days in the presence of 10% UL-IgG FBS is shown in **Figure 11.1B**. There is a lack of lag phase during the first hours of cell culture, with an exponential phase lasting until day three in control and FBS-fed cultures. Furthermore, both culture conditions reached their MCDs on day four with 3.76 ± 0.23 x10^6 cells/mL 3.81 ± 0.19 x10^6 cells/mL, showing not significant difference. No significant differences were observed between control and FBS-fed cultures, with viabilities over 97% along the culture period. These results show that NS0 and EG2 cultures cell growth and viability were not affected when cultured in media containing 10% Ultra-Low IgG FBS.

Figure 11. 1 Effect of serum supplementation on NS0 and CHO EG2 cells' growth and viability.

A. NS0 cells' and **B.** CHO EG2 cells' growth during a four-day batch culture. NS0 cells (-□-) and EG2 cells (-▲-)were seeded at $2.5x10^5$ cells/mL into 40 mL of media in 125 mL shake flasks with 10% Ultra Low IgG FBS. NS0 cells (-●-) and EG2 cells (-×-) control cultures had no additives. Cell density (solid lines) and viability (dashed lines) were monitored in each culture at daily intervals over the culture period by trypan blue exclusion assay. Data points represent mean ± SD of triplicates for each condition (n=3).



11.4.2. Effect of Ultra-Low IgG FBS on the *N*-linked glycosylation profile of NS0-IgG1 and CHO EG2-hFc Mabs.

Figure 11.2A shows the effect of 10% Ultra-Low IgG FBS on the glycosylation of NS0 cells. Cells cultured media without additives showed 21.6% ± 2.50, 39.6% ± 1.81 and 38.8% ± 3.66 of glycans containing zero, one and two Gal residues (G0, G1 and G2) respectively. A similar glycan pool distribution was observed in media containing 10% Ultra-Low IgG FBS with not significant difference in the amount of G0 and G2 glycoforms. However, G1 glycans content was slightly higher in FBS-supplemented cultures with a 14.7% increase with respect to the control (p-Value 0.0004). The relative abundance of Gal residues was used to calculate the galactosylation index (GI) of IgG1 Mabs shown in **Figure 11.2B**. Control cultures showed a GI of 0.59 ± 0.03 comparable to the GI seen in FBS-fed cultures with 0.58 ± 0.03. Relative abundance of alpha-1,3 gal residues in IgG1 Mabs was not affected by FBS supplementation, showing 12.1% ± 2.1 and 13.2% ± 1.3 in control and FBS-fed cultures (data not shown). A comparison in the percentage of sialylation of NS0-IgG1 Mabs produced in control and FBS-supplemented cultures is shown in Figure 10.3. Sialylated structures constituted 19.3% ± 2.4 of the total glycan pool in control cultures. A significant decrease of 22% in total sialylation was observed in FBS-supplemented cultures (p-Value 0.01) (**Figure 11.3)**.

In CHO EG2-hFc Mabs, *N*-glycosylation was characterized by glycans containing mainly biantennary glycans constituted with two Gal residues (G2) followed by glycans with one and zero Gal residues (G1 and G2 respectively) (**Figure 11.4A)**. Thus, in control cultures, the proportions were as follows: G2= 51.9 ± 1.93; G1= 29.5% ±0.82 and G0= 15.4% ± 1.11. In FBS-supplemented

cultures, glycans containing zero Gal residues (G0) significantly decreased by 37%, whereas G1 glycoforms increased by 29% (p-Value 0.01) when compared to cultures without serum. A small decrease in the content of G2 containing glycans was also observed in FBS-supplemented cultures, but this reduction was not significant when compared to the control. Even though it was possible to observe differences in the relative abundance of Gal containing glycans, galactosylation index in control cultures did not significantly differ from FBS-supplemented cultures **(Figure 11.4B)**. Furthermore, sialylation was also slightly lower in Mabs produced in culture media containing serum (14.2% ± 2.94); however, this reduction was not significant compared to the control culture (19.7% ±0.77) (**Figure 11.4C)**. These results show that there were not significant changes in galactosylation of IgG1-Mab produced by NS0 cells cultured in media containing 10% Ultra-Low IgG FBS when compared to untreated cultures. However, a slight but significant decrease in sialylation was detected in serum supplemented cultures. Similarly, EG2-hFc Mab's glycosylation showed subtle changes in G2-containing glycans, which in turn resulted in a slight decrease in sialylation. However, these changes were not significant in comparison to control cultures.

Figure 11. 2 Effect of Ultra-Low IgG FBS on the N-linked glycosylation of NS0-IgG1 Mabs.

NS0 cells were cultured in shake flasks media with 10% Ultra-Low IgG FBS (□). Control (■) did not contain serum. Mabs were harvested on day four and protein purified prior to HPLC analysis. **A.** Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2 respectively) were used to calculate the **B.** galactosylation index (GI) of Mabs. Data points represent mean ± SD of triplicates for each condition (n=3) *Values are significantly different from the control (p-Value 0.0004).

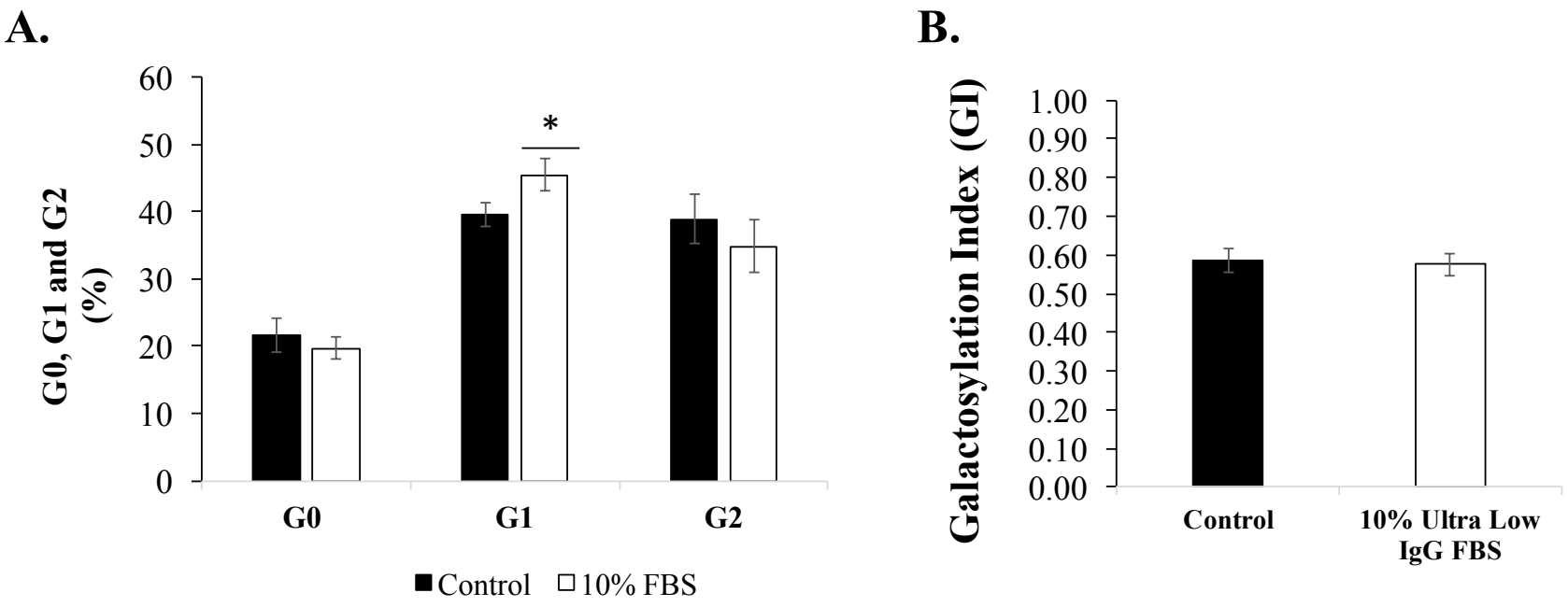
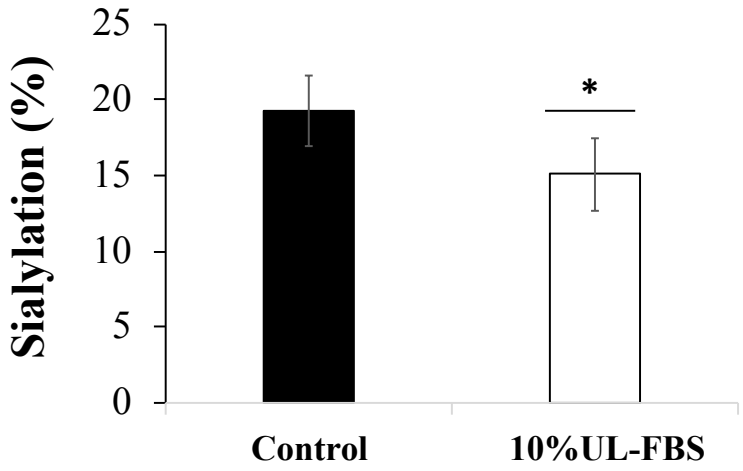


Figure 11. 3 Effect of Ultra-Low IgG FBS on sialylation of NS0-IgG1 Mabs.

NS0 cells were cultured in shake flasks media with 10 % Ultra-Low IgG FBS (□). Control (■) did not contain any serum. Mabs were harvested on day four and protein purified prior to HPLC analysis. Data points represent mean ± SD of triplicates for each condition (n=3) *Values are significantly different from the control (p-Value <0.05).

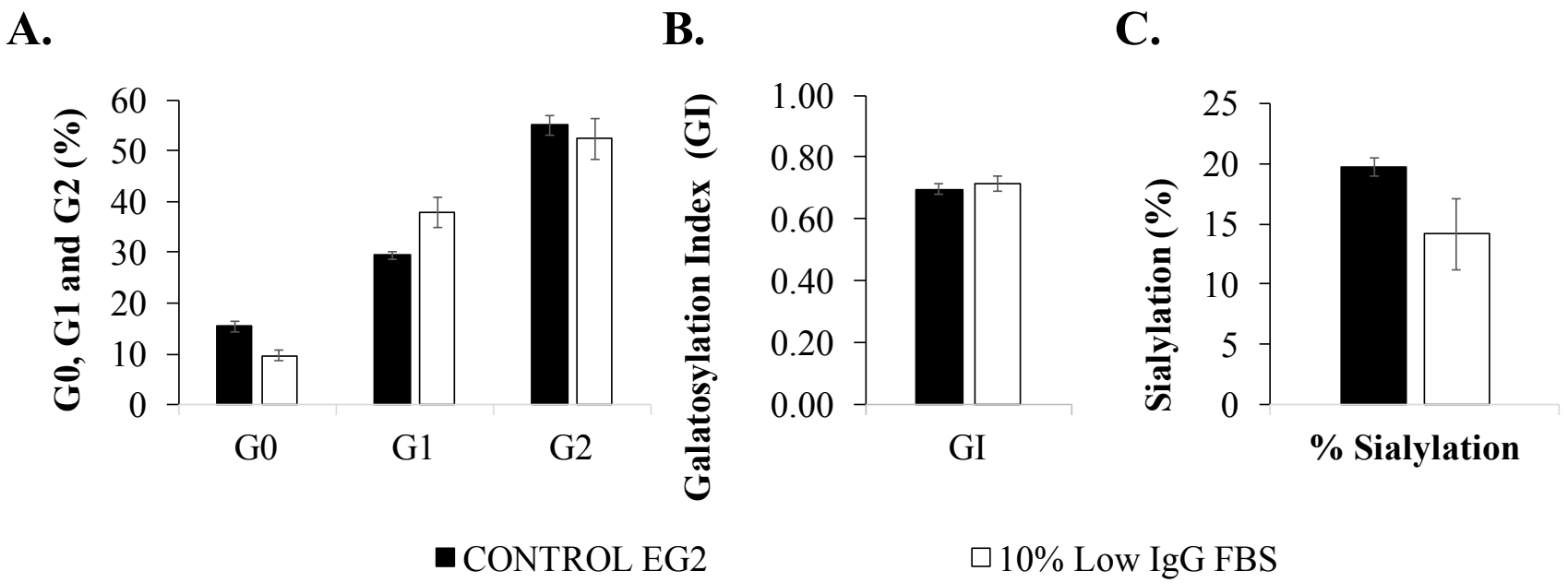


11.4.3. Effect of Ultra-Low IgG FBS on the proportion of Sias of NS0-IgG1 and CHO EG2-hFc Mabs

The effect of serum in sialylation of NS0-IgG1 and CHO EG2-hFc Mabs was further explored by considering the content of Neu5Gc and Neu5Ac. In addition, a sample from Ultra Low IgG FBS was taken to determine the original amount of Sia present in the serum used as a supplement. Figure shows the content of Sia from NS0 cells produced in control cultures and in media supplemented with 10% Ultra-Low IgG FBS along with pure FBS without media. As previously shown, NS0 cells produce IgG1 Mabs where the predominant Sia is Neu5Gc. Control cultures showed a Neu5Gc to Neu5Ac ratio of 7:1, with 0.29 mole Neu5Gc/mole IgG1 and 0.04 mole Neu5Ac/mole IgG1 **(Figure 11.5)**.

Figure 11. 4 Effect of Ultra-Low IgG FBS on the N-linked glycosylation of CHO EG2-hFc Mab.

NS0 cells were cultured in shake flasks media with 10% Ultra-Low IgG FBS (□). Control (■) did not contain any serum. Mabs were harvested on day four and protein purified prior to HPLC analysis. **A.** Relative abundance of glycans containing zero, one or two Gal residues (G0, G1 and G2 respectively) were used to calculate the **B.** galactosylation index (GI) of Mabs. **C.** Percentage of sialylation (%). Data points represent mean ± SD of triplicates for each condition (n=3).



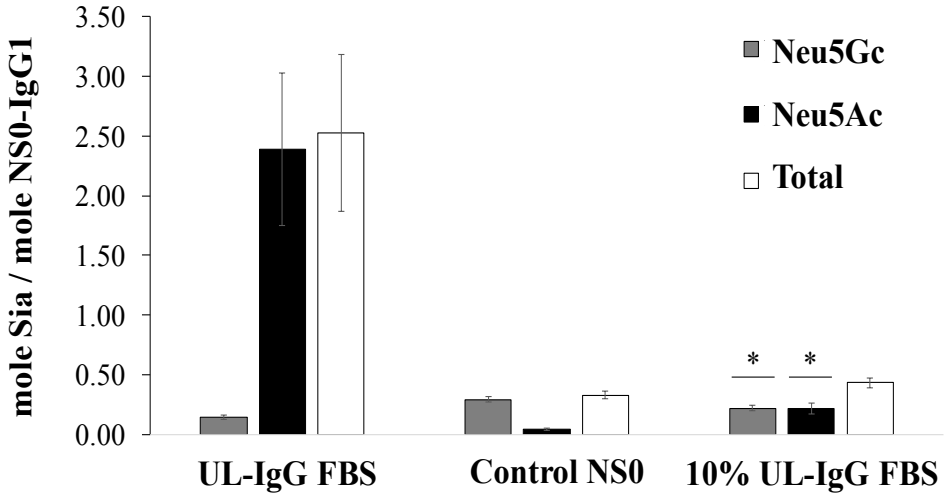
Mabs produced in media containing 10% UL-IgG FBS showed a reduction in Neu5Gc content of about 24% with respect to the control (p-Value <0.05) along with a 5-fold increase in Neu5Ac content with respect to the control. This change in Sia content changed the proportions of Sia to 1:1 in Mabs produced in media with FBS. As a result, the total amount of Sia was increased by 1.3-fold in IgG1 made in FBS-supplemented media with respect to the control. The changes observed in serum supplemented cultures can be further explained when considering the original amount of Sia found in pure Ultra Low IgG FBS. The total amount of Sia in the serum sample was about 2.53±0.66 moles mole Sia /mole of Mab Interestingly, a higher content of Neu5Ac was observed in the serum sample, constituting ~94.5% of the total amount of Sia. The small proportion of Neu5Gc in the original serum could explain how Neu5Gc content did not increase in NS0-IgG1 Mabs. In contrast, Neu5Ac was slightly increased in Mabs produced in serum containing media, indicating that serum could have been the source of this increase. However, the total amount of Sias was not significantly influenced by serum, with Mabs containing 0.43±0.04 moles Sia/mole of IgG1 Mab in FBS-fed cultures when compared to untreated cultures **(Figure 11.5).** CHO EG2 cells were also cultured in the presence of 10% Ultra-Low IgG FBS to study the effect of serum on the amount of total Sia found in EG2-hFc Mabs. Negligible amounts of Neu5Gc were found in both culture conditions, with Neu5Ac constituting 96% and 94% of the total amount of Sia in control and FBS-supplemented cultures respectively. These results indicate that serum failed to influence the proportions of Neu5Gc and Neu5Ac in EG2-hFc Mabs (**Figure 11.6**).

These results show that supplementation of 10% Ultra-Low IgG FBS influenced Sia distribution in NS0 cells. The original ratio of Neu5Gc:Neu5Ac in untreated cultures (7:1) changed to 1:1 because of a significant decrease of about 24% in Neu5Gc content along with a 5.5-fold

increase in Neu5Ac content. The latter resulted in an increase in the total Sia pool of about 30%. However, this increase in total Sia content was not significant when compared to the control.

Figure 11. 5 Effect of Ultra low IgG FBS on Sia proportions of NS0-IgG1 Mab.

Neu_5Gc (■) and Neu_5Ac (■) content and total amount of Sias (□) are presented as mole Sia/mole Mab) in NS0-IgG1 Mab produced in Biogro™ media supplemented with/without 10% Ultra Low IgG FBS after a four-day batch culture. Neu5Gc/Neu5Ac content were analyzed quantitatively by reverse-phase HPLC of 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB)-labeled Sias released by mild acid hydrolysis. Values correspond to the mean ± SD of triplicates (n = 3). Pairs of means ± SD significantly different (p-Value <0.05*) with respect to the control culture.



11.4.4. Effect of Ultra-Low IgG FBS on the intracellular concentrations of nucleotide and nucleotide sugars of NS0 and EG2 cells.

Sialylation was reduced when cells were cultured in presence of 10% Ultra-Low IgG FBS. To further understand what caused this decrease in Sia content, the intracellular nucleotide and nucleotide sugar content was evaluated in NS0 cells. For this purpose, NS0 cells were cultured

in media containing 10% Ultra-Low IgG FBS in a working volume of 35 mL of media in rotary 125 mL shake flasks. $1x10^7$ cells were harvested on day four and rapidly quenched using 60% methanol plus AMBIC to stop cells' metabolism. Then, the metabolites were extracted using solid-phase extraction cartridges, recovered and separated by ion-pair reversed phase high-performance liquid chromatography (RP-HPLC).

Figure 11. 6 Effect of Ultra low IgG FBS on Sia proportions of EG2-hFc Mab.

Neu_5Gc (■) and Neu_5Ac (■) content and total amount of Sia (□) are presented as mole Sia/mole Mab) in CHO EG2-hFc Mab produced in Biogro™ media supplemented with/without 10% Ultra Low IgG FBS after a four-day batch culture. Neu5Gc/Neu5Ac content were analyzed quantitatively by reverse-phase HPLC of 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB)-labeled Sias released by mild acid hydrolysis. Values correspond to the mean ± SD of triplicates (n = 3).

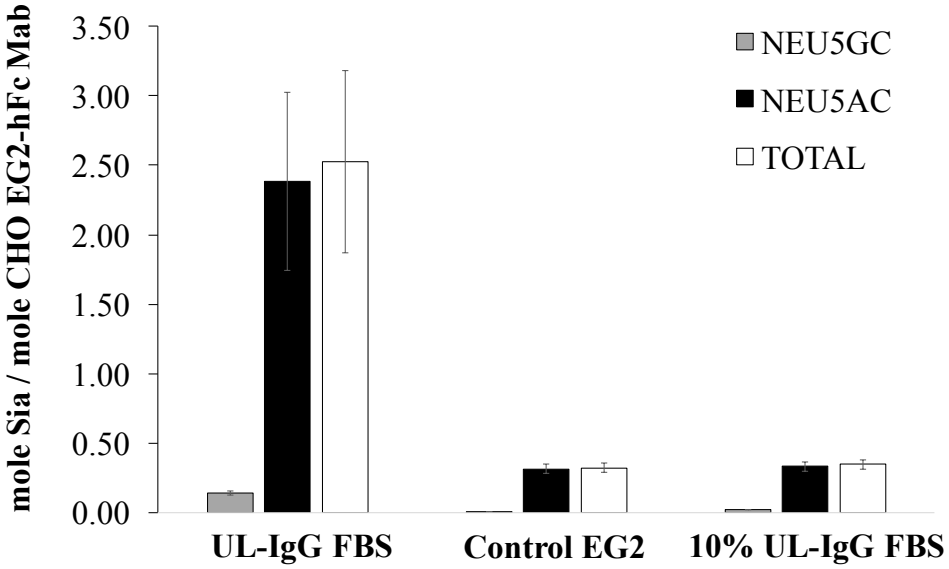


Figure 11.7 shows the concentrations of nucleotides from NS0 cells cultured in the presence of 10% of Ultra-Low IgG FBS. Control culture and FBS-supplemented cultures showed a higher number of mono-phosphate nucleotides with respect to di- and tri-phosphate nucleotides (**Figure 11.7A-D)**. For instance, the Ade pool was constituted by 91.6%, 7.4 % and 0.9% of AMP, ADP, and ATP nucleotides respectively in control cultures. In FBS-supplemented cultures, an increase in the AMP proportion to 95.3% along with a reduction in ADP and ATP to 4.4% and 0.3% caused the total Ade pool to be lower in comparison to the control sample. However, the differences observed were not significant in comparison to the control (**Figure 11.7A**). It is important to mention that the metabolic state of the cells was not properly stopped at the moment of quenching. This is reflected by the higher levels of monophosphates in media with and without serum. Cells were still growing and higher values of ATP would have been expected. Since we are examining the content of sugar nucleotides, the total nucleotide pool was analyzed instead. The uridine pool content found on untreated control cultures was constituted by 80.8%, 18.5% and 0.5% of UMP, UDP and UTP nucleotides respectively. Comparable levels of uridine nucleotides were observed in cells cultured in media containing FBS, indicating that serum did not influence the uridine pool in NS0 cells. With respect to the Gua pool in NS0 cells, control cultures showed an 83.7%, 13.7% and 2.5 % of GMP, GDP and GTP nucleotides. No significant difference was detected in serum fed cultures when comparing with control cells (**Figure 11.6C).** Cyt pool showed the lowest concentrations in comparison to other nucleotides present in NS0 cells grown in control cultures and/or FBS-supplemented media. Furthermore, no significant differences were noticed between untreated cultures and FBS-containing cultures showing close to 78%, 21.6% and 1.2 % of CMP, CDP and CMP-Neu5Ac respectively in control cultures. There were not significant differences in the UDP-Hex pool, with a ratio of 1:2 of UDP-Gal to UDP-Glc in both FBS-

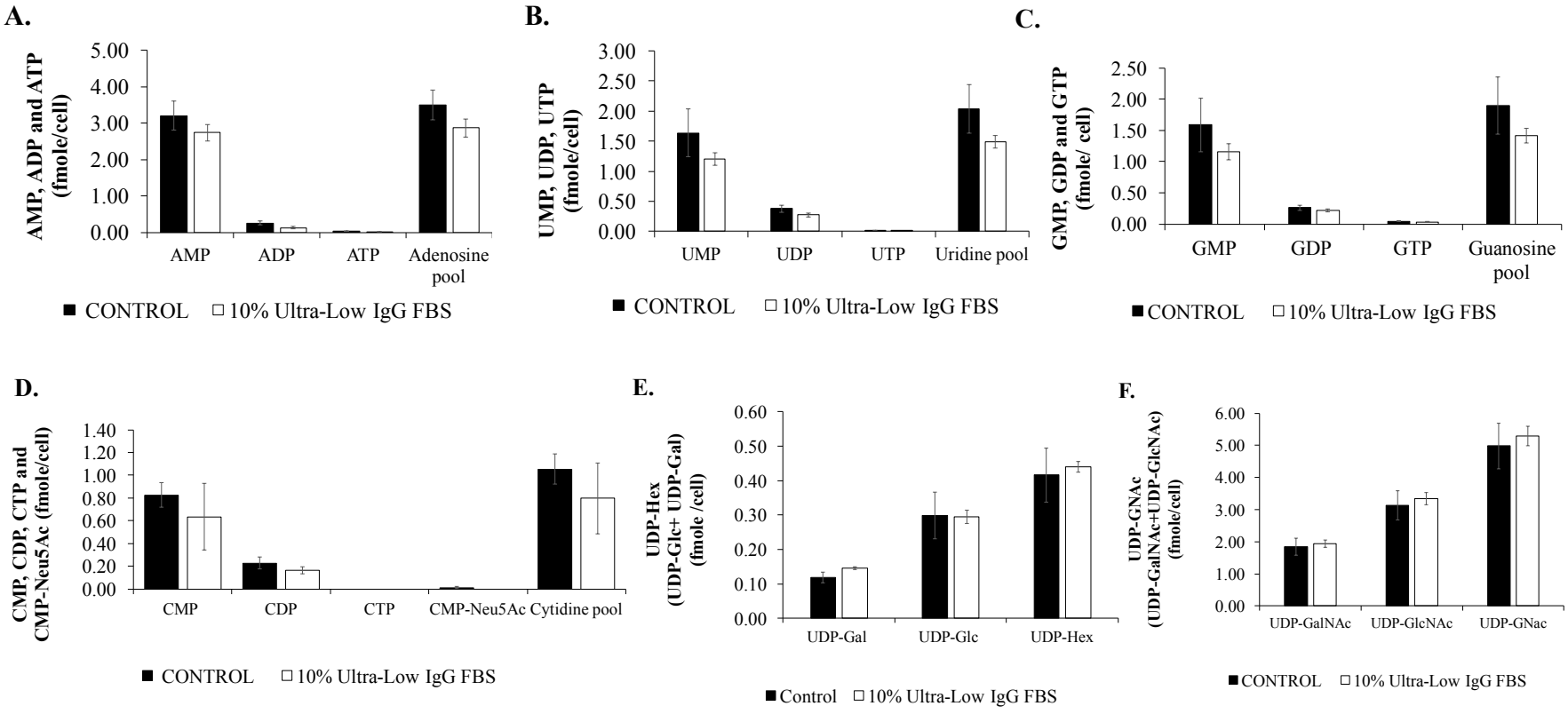
supplemented cultures and control (**Figure 11.6E**). Also, FBS-supplemented cultures did not show any influence in the UDP-GNAc pool when compared to the control. UDP-GlcNAc constituted about 63% of the total UDP-GNAc pool in both control cultures and FBS-fed culture. In resume, NS0 cells' nucleotide analysis did not give information to explain the decrease in sialylation observed in FBS supplemented cultures. For instance, the Cyt pool constituted by Cyt nucleotides and CMP-Neu5Ac were slightly lower but not significantly with respect to the control.

11.5 Discussion

Serum has been used as rich nutrient mixture to aid in the cell growth, proliferation, maturation and differentiation of a variety of cell types during biological manufacturing processes. Serum provides growth factors, hormones, binding and transport proteins, attachment and spreading factors, amino acids, vitamins trace elements, fatty acids and lipids (Brunner, Frank *et al.* 2010). However, since animal serum implies several disadvantages in cell culture, recombinant cell lines have continuously been adapted to serum-free media formulations because of ethical, economic and regulatory reasons (Lefloch, Tessier *et al.* 2006). For instance, serum can contain infectious agents (e.g. virus, prions) that can be introduced into cell culture processes. In addition, higher costs of operation can arise because of additional required purification steps to remove serum-sourced proteins present as impurities in a biologic. Furthermore, big concerns arise because of variability in performance between lots, reliability of supply and immunogenicity (Lefloch, Tessier *et al.* 2006, Brunner, Frank *et al.* 2010, Yokoyama 2011) . Indeed, among other animal-derived culture components, serum has been found to be an important source of *N*-glycolylneuraminic (Neu5Gc) contamination.

Figure 11. 7 Effect of 10% Ultra low IgG (□) in NS0 cells' intracellular metabolites.

Control culture did not contain serum (■). Nucleotides: **A.** AMP, ADP and ATP; **B.** UMP, UDP and UTP **C.** GMP, GDP and GTP and **D.** CMP, CDP, CTP and CMP-Neu5Ac. Nucleotide amounts are presented as pools of Ade, Urd, Cyt and Gua nucleotides respectively in fmole/cell. NS0 cells were seeded in shake flasks containing Biogro™ media supplemented with 10% Ultra-Low IgG FBS and harvested on day 4. Nucleotide sugars were extracted and resolved by anion-exchange chromatography. Values correspond to the mean ± SD of triplicates (n = 3). CTP content are not represented as they co-eluted with UDPGalNAc



Thus, in this chapter the effect that serum had on Neu5Gc content was evaluated in two monoclonal antibodies. Since fetal bovine serum (FBS) IgG levels fell between 50-300 μg/mL, an Ultra-Low IgG FBS (<5μg/mL) was used as an alternative to avoid co-purification and analysis of bovine IgG (RMBIO).

Several reports have studied the effect that serum can have on the *N*-glycosylation of monoclonal antibodies when compared to serum-free media (SFM) or chemically-defined media (CDM). However, serum's effect on glycosylation seems to be dependent on the cell line used as well as on the protein being expressed. For example, SFM-containing hybridoma cultures showed antibodies with higher percentage of non-galactosylated glycans (58%) in comparison to DMEM-FBS (28%) and CDM (32%) (Serrato, Hernandez *et al.* 2007). In contrast, Patel *et al.* observed that sialylation of IgG1 produced by a murine hybridoma was decreased when produced in media containing serum (Patel, Parekh *et al.* 1992). Similarly, BHK21 cells produced IL-Mu6 with two times lower sialylation when cultured in 2% FCS-containing media (Gawlitzek, Valley *et al.* 1995). In the experiments outlined in this chapter, serum affected NS0-IgG Mab's glycosylation more significantly than EG2-hFc Mabs. IgG1 Mab showed an increase in G1 glycans (14.7%) along with a significant decrease in sialylation (22%) when compared to untreated NS0 cultures. There were not significant differences in the content of nucleotide and nucleotide sugars in NS0 cells that could explain the changes observed in Sia proportions. A possible explanation for the decreased sialylation is the change of environment to which NS0 cells were exposed in serum-containing media (e.g. rich mixture of nutrients, hormones and other molecules)(Goochee and Monica 1990). The latter can affect oligosaccharide biosynthesis as well as enzymatic degradation which consequently induces variability. In fact, a lower terminal sialylation in serum-produced

antibodies has been previously associated with the presence of high activities of sialidase in serum (Gawlitzek, Valley *et al.* 1995, Restelli and Butler 2002, Lefloch, Tessier *et al.* 2006). However, the effect of sialidase on NS0-IgG1 and EG2-hFc Mab remains to be investigated to determine if protein de-sialylation occurred at the beginning or at late phases of the batch culture due to sialidase release.

Serum's influence on sialylation was further evaluated by analyzing the content of Neu5Gc in both Mabs. Knowing that serum has been considered as a main Neu5Gc-source of contamination in cell culture processes, an increase in Neu5Gc content was expected in the two monoclonal antibodies tested in this chapter. However, EG2-hFc antibodies, with originally 96% of Neu5Ac sialylated glycans, were not affected by serum-supplemented media. In contrast, NS0-IgG1 Mab's Neu5Gc:Neu5Ac proportion in untreated cultures (7:1) was significantly changed in cultures containing serum to 1:1. The latter came from a significant increase of about 24% in Neu5Gc content and a 5-fold increase in Neu5Ac content. Since there were not significant changes in the total Sia pool, the changes in Sia proportions may have resulted either from changes in the expression levels and activity on CMAH in the cytosol or changes in any of the co-factors involved in Neu5Gc-production reaction (e.g. NADH levels) (Borys, Dalal *et al.* 2010). On the other hand, sialyltransferases may have also had more accessibility to Neu5Ac-derived from UL-FBS than newly synthesized Neu5Gc. Indeed, pure Ultra Low IgG FBS (UL-IgG FBS) mainly showed Neu5Ac (94.5%) and negligible amounts of Neu5Gc. This suggests that UL-IgG FBS was a rich source of Neu5Ac, which could be the reason of the Neu5Ac-increase observed in NS0-IgG1 as opposed to influencing Neu5Gc content. Previous reports have suggested that flooding cells environment with Neu5Ac can result in a significant decrease if not elimination of Neu5Gc in

glycoproteins (Ajit Varki 2009). Thus, it can be suggested that the main source of Neu5Gc in commercial-grade FBS, the bovine IgG (50-300 μg/mL), was removed during UL-IgG FBS manufacturing (<5μg/mL). Thus UL-IgG FBS was not able to increase Neu5Gc content in NS0 cells as shown in other reports with commercial-grade FBS. Even though UL-FBS could be used as an alternative to reduce Neu5Gc content in glycoproteins, there is the disadvantage of variability between lots of serum. The latter has been one of the reasons why industry has moved to the use of more defined media (e.g. protein-free media, chemically defined media).

11.6 Conclusion

- A significant reduction of about 22% in sialylation was observed in NS0-IgG1 Mab produced in media containing 10%-Ultra-Low IgG FBS when compared to untreated cultures.
- Nucleotide sugar analysis depletion was not responsible for the changes observed, as the values for cells cultured in serum showed comparable intracellular concentrations of metabolites with respect to the control,
- A significant decrease of 24% in Neu5Gc content along with a 5.5-fold increase in Neu5Ac content was observed in NS0-IgG1 Mab supplemented in serum.
- The original ratio of Neu5Gc to Neu5Ac of 7:1 was reduced to 1:1 in cultures supplemented with 10% Ultra-Low IgG FBS.
- Pure Ultra Low IgG FBS was mainly constituted by 96% of Neu5Ac and may have been the source of Neu5Ac in NS0 cells cultures.
- Subtle but not significant reduction in G2-glycans and subsequently in sialylation were observed in EG2-hFc Mabs.

Chapter 12

Iron supplementation effect on the amount of Neu5Gc on IgG1 Mab.

12.1 Introduction

Mammalian cells require iron for cell growth, DNA synthesis, regulation of gene expression, oxygen transport and cellular metabolism (Wang and Pantopoulos 2011, Arigony, de Oliveira *et al.* 2013). The most well-known uptake mechanism of iron is through the process of transferrin receptor-mediated endocytosis (Nagira, Hara *et al.* 1995, Rolfs and Hediger 1999, Bai, Wu *et al.* 2011). In cell culture media, transferrin is supplied in the form of serum-derived human transferrin (hTf) and bovine transferrin (bTf). In addition to ferrous and ferric ions present in undefined supplements such as serum, iron can also be incorporated as components of salts and trace elements in serum-free media. In physiological solutions, iron can undergo cyclic oxidation, existing in either of two stable states: ferrous (Fe^{2+}) or ferric state (Fe^{3+}) (Winterbourn 1995).

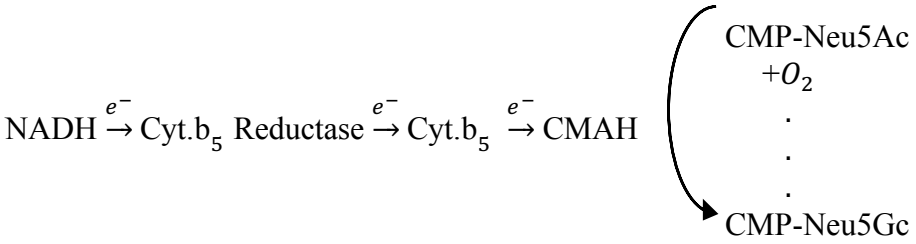
In culture media, iron can interact with amino acids, nucleotides, physiological chelators and proteins. The resulting complexes will determine if iron will be available to support cell life or if it will become toxic to cells. For example, ferric iron can be reduced to ferrous iron by strong reducing agents present in the medium (e.g. ascorbate or Vitamin C). On the other hand, ferric iron not bound to any chelator or carrier molecule can form ferric hydroxide complexes which are known to be insoluble(Okam 1999). Both, reduction to ferrous iron and the loss of ferric iron due

to precipitations are undesirable in cell culture. In addition, to deliver high-density cell cultures with enough iron to satisfy cell growth and productivity, elevated concentrations of iron salts are required. However, iron can result toxic to cells at high concentrations due to the formation of hydroxyl radicals (•OH) which are highly reactive. As a result, iron salts can be removed or replaced by a chelated iron with the aim of dampening iron's reactivity by stabilizing its oxidative status and assisting in its transportation to cells, specially in serum-free medium where the protective qualities of serum are absent (Olaf Kruger 2009, Sigma-Aldrich 2018).

Iron has also been found to be important for the resulting protein glycoform profile. For instance, the oligosaccharyltransferase' (OST) activity depends on divalent metal cations such as Fe^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+}, increasing the site-occupancy in recombinant glycoproteins (Hendrickson and Imperiali 1995, Gawlitzek, Estacio *et al.* 2009). Iron supplementation has also shown to improve fully glycosylated proteins (F6A2G1, F6A2G2) vs. non-glycosylated glycoforms (F6A1G0, F6A2G0)(Clincke, Guedon *et al.* 2011, Wentz, Hemmavanh *et al.* 2017). Furthermore, iron is also involved in the conversion of CMP-Neu5Ac into its hydroxylated form, CMP-Neu5Gc, reaction that is catalyzed by CMP-Neu5Ac hydroxylase (CMAH) (Schlenzka, Shaw *et al.* 1993, Varki and Schauer 2009, Ficko 2014). In fact, exogenously added Fe^{2+} and Fe^{3+} have shown to stimulate CMAH enzyme's activity (Shaw, Schneckenburger *et al.* 1992).

Malykh *et al.* found that CMAH expression varied between species and tissues and that the CMAH activity correlated with the extent of glycoconjugate sialylation with Neu5Gc **(Malykh, Shaw *et al.* 1998).** For CMP-Neu5Gc synthesis, CMAH uses oxygen, a hydrogen donor

($NADPH_2$ or ascorbate), the electron transport chain of cytochrome *b5* and *b5* reductase and iron (Fe^{2+}) as follows:



(Schauer 1995)

Knowing that iron is an important cofactor for glycosylation enzymes, the foregoing experiments tested the effect of increasing iron concentrations on the *N*-linked glycosylation of IgG1 Mab, particularly on galactosylation, sialylation and Neu5Gc content. NS0 cells previously showed to have an elevated Neu5Gc level in IgG1 Mab with respect to Neu5Ac. For this reason, NS0 cells were chosen over EG2 cells, which in turn prefer to add Neu5Ac with negligible amounts of Neu5Gc **(Chapter 8-10)**.

12.2 Objectives.

Knowing that cofactors are essential for some glycosylation enzymes, the following experiments aimed to:

- Investigate ferric citrate and ferrous sulfate's effect on glycosylation of IgG1 Mab produced by NS0 cells, particularly galactosylation and sialylation.
- Test ferric citrate and ferrous sulfate's effect on the amount of Neu5Gc
- Investigate the effect of ferric citrate plus the chelating agent tropolone on the *N*-glycosylation profile as well as on the amount of Neu5Gc of IgG1-Mab.

12.3Experimental set up

Originally, the in-house developed Biogro™ media contains both ferric citrate (FC) and ferrous sulfate (FS) in its basal formulation (0.0135 mM $FeSO_{4.7}H_2O$; 0.057 μM Ferric citrate). To this basal formulation, 0.05 mM, 0.1 mM, 0.5 mM and/or 1 mM FC or 0.05 mM, 0.1 mM and 0.5 mM FS were added before inoculation. These range of concentrations were based on previous reports were iron supplements were used to modulate glycosylation on recombinant antibodies (Ficko 2014, Wentz, Hemmavanh *et al.* 2017). NS0 cells were seeded at $2.5x10^5$ cells/mL in 80 mL of media containing either FC or FS.

A second set of experiments involved the incorporation of FC along a chelating agent to increase the probability of iron being taken up by the cells. For this purpose, NS0 cells were also seeded at $2.5x10^5$ cells/mL in Biogro™ media containing FC (0.1 μM, 0.5 μM, 1 μM, 5 μM and 10 μM) in combination with the chelating agent tropolone (2 hydroxy-2,4,6-cycloheptatrien-1-one). The concentration of tropolone was five times the concentration of FC (0.5 μM, 2.5 μM, 5 μM, 25 μM and 50 μM). Tropolone was chosen over other chelating agents based on Field *et al.* report (Field 1995). In both set of experiments, shake flasks were placed in a rotating platform at 120 rpm and incubated for four days at 37°C with 10% CO_2. Daily sampling (900 μL) allowed to monitor cell growth and viability by trypan blue exclusion method and to analyze the metabolism and Mab production. On day four, cells were spun down and supernatant was collected, filtered and concentrated for further analysis.

12.4 Results

12.4.1 Cell culture performance in iron-supplemented NS0 cultures.

The proprietary media Biogro™ CHO is constituted by basal levels of 0.014 mM FS and 0.057 mM FC and served as a control culture. Basal low-iron concentrations were further enriched by adding either FS (0.05, 0.1, 0.5 mM) or FC (0.05, 0.1, 0.5 and 1 mM). **Figure 12.1** and **12.2** show the effect that FC and FS had on NS0 cells' growth and viability respectively during a four-day batch culture. There was no evidence of a lag-phase during the first 24 hours, showing a similar growth rate (0.02 h^{-1}) in media containing either FC or FS with respect to the control. No significant differences where observed during exponential phase between cultures, with a specific growth rate of about 0.04 h^{-1}. Cells reached stationary phase and their maximal cell densities (MCDs) on day four with up to $3.89x10^6$ cells/mL. FC and FS did not influence MCDs as well as NS0 cells' viability (>93%) when compared to the control.

A separate set of experiments consisted on examining the effect of FC along with the chelating agent 2-hydroxy-2,4,6-cycloheptatrien-1- one, also known as tropolone, on NS0 cells' growth and viability (**Figure 12.3**). The latter was to clarify if incorporation of a chelating agent allowed for a better uptake of iron by the cells. Thus, tropolone was added to culture media in an excess molar concentration to FC at a molar ratio of around 5 to 1 as previously suggested by Field *et al*(Field 1995). As shown in **Figure 12.3**, incorporation of FC along with tropolone affected differently NS0 cells' growth than when FC was added alone (**Figure 12.1** and **12.2**). For instance, cultures containing <1 μM of FC plus 5X tropolone showed comparable growth rates along the culture period (μ= 0.01-0.04 h^{-1}) and MCDs with respect to control cultures with $3.68 \pm 0.29 x10^6$

cells/mL on day four. In contrast, cultures with ≥1 μM FC plus tropolone did not show any growth since day one (μ = -0.002 h^{-1}) with a cell density of < 0.30 x 10^6 cells/mL. The latter was concomitant with a significant decrease of up to 20%-29% in viability since day one in comparison to the control.

Figure 12. 1 Effect of FC on growth and viability of NS0 cells.

Cells were inoculated (2.5x10^5 cells/mL) into 40 mL of media in 125 mL shake flasks with 0.05, 0.1, 0.5 and 1 mM FC. Control culture contain basal concentrations of FC and FS. Data points represent mean ± SD of duplicates for each condition of two independent experiments (n=4). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay using a computer-based image analyzer.

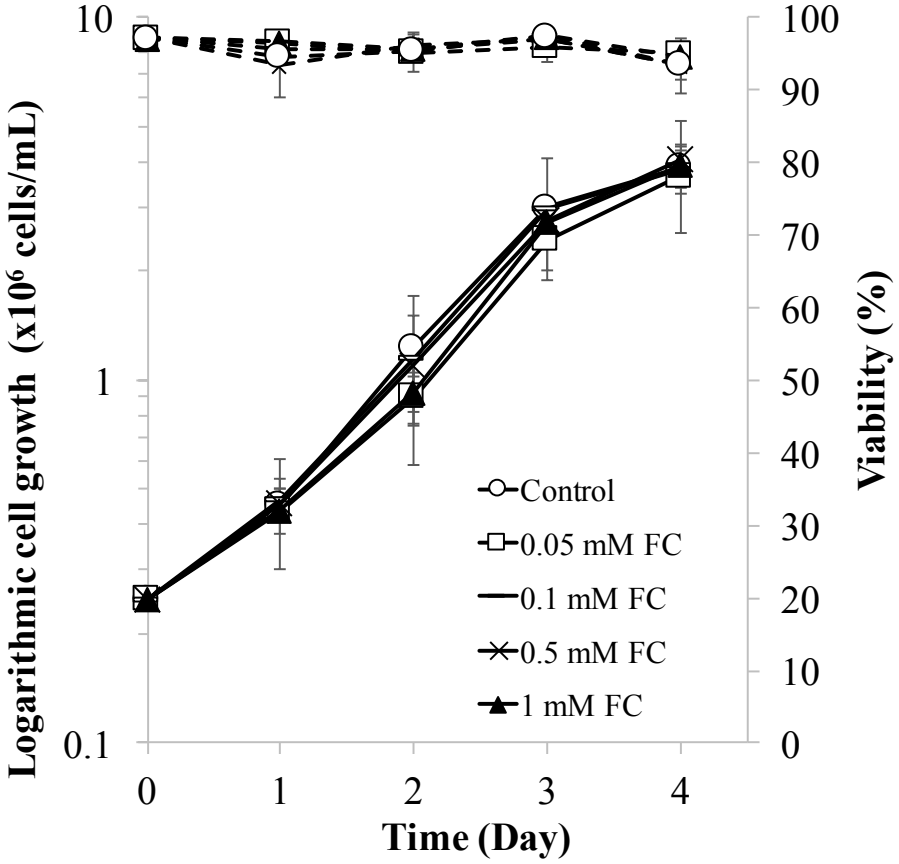
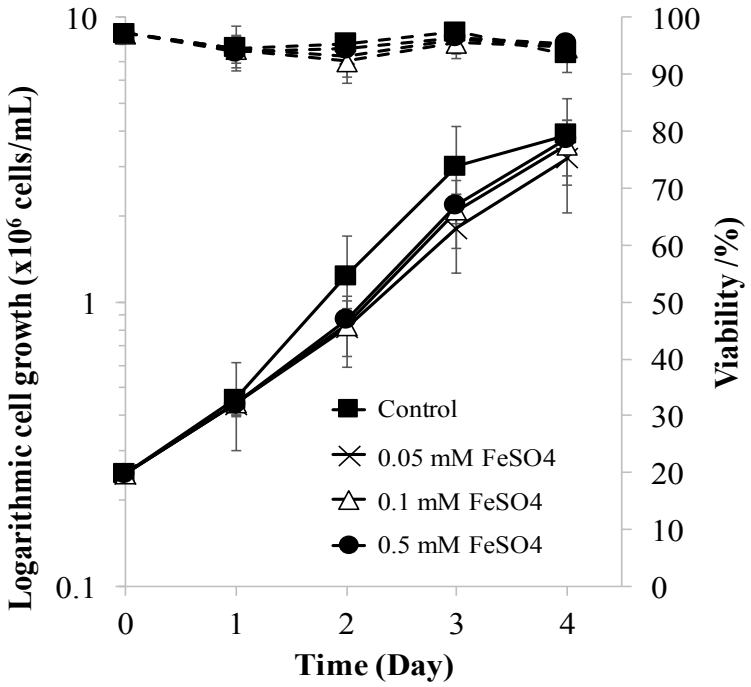


Figure 12. 2 Effect of FS on growth and viability of NS0 cells.

Cells were inoculated ($2.5x10^5$ cells/mL) into 40 mL of media in 125 mL shake flasks with 0.05, 0.1, 0.5 mM FS. Control culture contain basal concentrations of FC and FS. Data points represent mean ± SD of duplicates for each condition of two independent experiments (n=4). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay using a computer-based image analyzer.

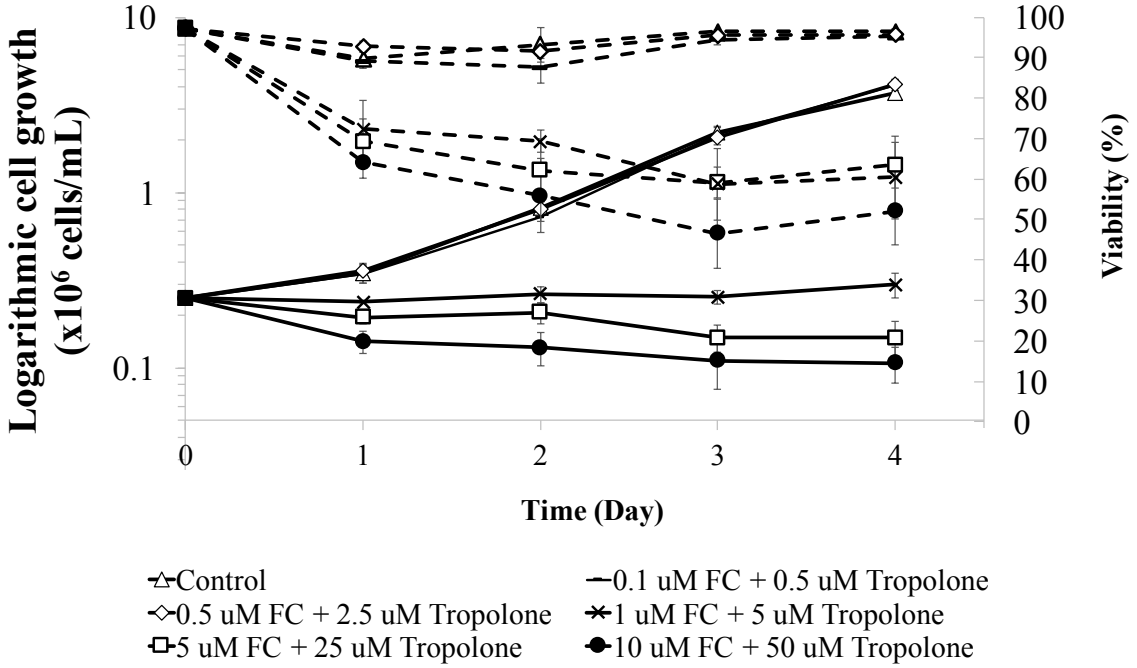


These results show that both iron supplements, FS and FC, did not affect NS0 cells' growth nor their viability when supplied without a chelating agent during a four-day batch culture. In contrast, FC incorporation along with tropolone induced important changes in growth rates, MCDs and viabilities when concentrations were ≥1 μM FC. At these concentrations, FC plus tropolone inhibited cell growth and affected cell viability since day one. On day four, NS0 cultures containing ≥1 μM FC plus 5X tropolone showed a significant decrease of up to 92-97% and 34%-46% in MCDs and viabilities respectively when compared to untreated cultures. Thus, it is possible that when FC was added without the presence of a chelating agent, it was not accessible to cells

for its uptake. In contrast, cells did not tolerate the toxicity of iron at concentrations higher than 1 μM when incorporated with the chelating agent. The fact that much lower concentrations of FC were used with tropolone also supports the idea that the chelating agent indeed help the uptake of iron by NS0 cells. Tropolone's effect on cell growth was also evaluated independently from FC (data not shown). Increasing concentrations of tropolone inhibited cell growth, indicating that tropolone and iron had a negative effect on cells' growth.

Figure 12. 3 Effect of FC and tropolone on the cell growth and viability of NS0 cells.

Cells were inoculated ($2.5x10^5$ cells/mL) into 40 mL of media in 125 mL shake flasks supplemented with 0.05, 0.1, 0.5 and 1 mM FC plus tropolone. The concentration of the chelating agent was 5-fold of the supplemented amount of FC. Control culture contain basal concentrations of FC. Data points represent mean ± SD of duplicates for each condition (n=2). Viable cell densities (solid lines) and viability (dashed lines) were determined at daily intervals over four days by a trypan blue exclusion assay using a computer-based image analyzer.



12.4.2 Influence of FC and FS on *N*-linked glycosylation of IgG1 produced by NS0 cells.

The effect of FC on Fc *N*-glycosylation of IgG1 was examined in NS0 cell cultures. IgG1 Mab produced in media without additives showed 17.5%, 48.6% and 33.9% of glycans containing G0, G2 and G1 glycans respectively. A similar distribution was observed in Mab produced in media containing 0.05-1 mM FC. Also, neither the galactosylation index (GI= 0.58 ± 0.03**)** or terminal sialylation (12.3% ± 3.58) observed in untreated cultures were affected by the incorporation of FC **(Table 12.1A)**. In the same manner as FC, FS did not influence the initial glycan distribution found in IgG1 produced in control cultures (G0<G2<G1). Similarly, there was not influence of FS over the amount of Gal -and Sia-containing glycans when compared to untreated cultures (**Table 12.1B**). These results show that there were not significant differences in IgG1 Mab glycosylation when FC or FS were supplemented to culture media.

Table 12.1C shows the effect of FC on glycosylation of IgG1 Mab when combined with tropolone. A significant decrease of about ~5% to up to 60% in galactosylation index was observed as the concentration of FC plus tropolone increased from 0.1 μM to 10 μM FC plus 5X- tropolone (p-Value <0.0001). The latter resulted from a significant increase of 10.9% to up to 233% in G0-containing glycans as FC concentration increased from 0.1 μM FC to 10 μM FC respectively (p-Value ≤0.001). In addition, G1- containing glycans decreased from up to 5.52% to up to 57.4% in cultures with 1-10 μM FC plus 5X-tropolone respectively (p-Value ≤0.001). With respect to G2-containing glycans, a significant decrease of up to 10.8% to up to 67.6% was observed in Mab produced in media with 1-10 μM FC plus 5-50 μM tropolone respectively (p-Value ≤0.001). As

expected, with a decrease in Gal -containing glycans, a decrease in the attachment of Sia residues to IgG1 Mab was observed in FC plus tropolone containing cultures. A slight but not significant decrease of 21.6, 5.43%, 29.3% and 24.6% on sialylation was observed in IgG1 Mab produced in cultures containing 0.1 μM, 0.5 μM, 1μM and 5 μM plus 5X-tropolone respectively when compared to the control. This decrease became significant (76.6%) once FC reached 10 μM FC plus 50 μM tropolone.

These results show that supplementation of either FC or FS alone did not impact the glycosylation profile of IgG1 Mab produced by NS0 cells. However, a significant difference was observed when FC was incorporated with 5-times the concentration of a chelating agent "tropolone". FC plus tropolone supplementation had a negative impact on the *N*-glycosylation pattern of IgG1 Mab with a significant increase of G0-containing glycans of about 233% along with a significant decrease in G1- and G2- containing glycans of up to 57% and 67.6% respectively. In addition, a decrease in sialylation of 5.43% to up to 76.6% was observed in cultures containing FC (0.1 μM- 10 μM) along with tropolone.

12.4.3 Effect of FS and FC supplementation on the Sias proportions of IgG1 Mab.

CMP-Neu5Ac can be transferred or further converted to CMP-Neu5Gc prior to transfer to newly synthesized glycoconjugates at the trans-Golgi compartment. As mentioned above, this reaction takes place in the cytoplasm and requires the action of the iron-dependent CMP-hydroxylase. Here, Neu5Gc and Neu5Ac proportions in NS0-IgG1 Mab were examined in the presence of increasing concentrations of either FC or FS (**Figure 12.7A** and **12.7B** respectively).

Control cultures showed a Neu5Gc to Neu5Ac ratio of 8 to 2, which represents 0.36 ± 0.07 moles of Sia per mole of IgG1. With respect to cultures supplemented with 0.05, 0.1, 0.5 and 1 mM FC, a similar ratio of Neu5Gc:Neu5Ac of 8:2 was observed showing no influence of FC over Neu5Gc content (**Table 12.1A**). In addition, the total amount of Sia was not significantly influenced by FC when compared to the control, showing up to 0.46 ± 0.05 mole of Sia per mole of Mab. **Figure 12.7B** shows the effect on increasing concentrations of FS on the proportions of Sia in NS0-IgG1. Cultures containing 0.05, 0.1 and 0.5 mM FS also showed no significant differences in the amount of Neu5Gc with 0.29 ± 0.09, 0.30 ± 0.17 and 0.31 ± 0.06 moles Neu5Gc per mole of IgG1 respectively with respect to the control (0.29 ± 0.07) (**Table 12.1B**). In addition, the amount of Neu5Ac was not significantly affected in FS -supplemented cultures with respect to the control with 0.07 ± 0.03 mole of Neu5Ac per mole of Mab. The latter corresponded to a ratio of Neu5Gc to Neu5Ac of 8:2 like the one observed in the control (**Table 12.1B**).

The results showed that FC and FS did not influence the amount of Sia found on NS0-IgG1 Mab produced in Biogro™ media without additives. FC and FS were expected to influence the proportions of Neu5Gc with respect to Neu5Ac during CMP-Neu5Ac hydroxylase's conversion of CMP-Neu5Ac to CMP-Neu5Gc. However, in the absence of any change in Sia proportions, it was suspected that iron was not been taken up by NS0 cells. As a result, a chelating agent was incorporated along FC expecting to facilitate the uptake of iron by NS0 cells as well as increasing the amount of Neu5Gc in IgG1 Mab. Incorporation of 0.1 μM plus 0.5 μM tropolone to up to 0.5 μM FC plus 2.5 μM tropolone did not influence the amount of Neu5Gc nor the amount of Neu5Ac previously observed in the control with 0.21 ± 0.001 and 0.05 ± 0.001 respectively (**Figure 12.8; Table 12.1C**). Neu5Gc content significantly decreased by 82.6%, 94.2% and 97.12% in cultures

containing 1 μM FC plus 5 μM tropolone, 5 μM FC plus 25 μM tropolone and 10 μM FC plus 50 μM tropolone respectively (p-Value <0.0001). In addition, a significant decrease of about 45.3%, 80.4% and 87.4% in Neu5Ac content was also observed in 1 μM FC plus 5 μM tropolone, 5 μM FC plus 25 μM tropolone and 10 μM FC plus 50 μM tropolone-containing cultures respectively (p-Value <0.0001). These results show that Sia content was comparable between cultures containing 0-0.5 μM FC plus 2.5 μM tropolone with 0.24-0.29 moles Sia/ mole IgG1 Mab. In contrast, cultures supplemented with 1-10 μM FC plus 5-50 μM tropolone showed a significant decrease in Neu5Ac content of 45.3% to up to 87.4% respectively. These changes in Sia proportions are presented in **Table 12.1.** The original Neu5Gc:Neu5Ac ratio observed in control cultures of 8:2 significantly changed to 7:3, 6:4, 6:4 and 5:5 in cultures containing 0.5 μM, 1 μM, 5 μM and 10 μM Fc plus 5X tropolone. Thus, increasing the concentration of the chelated iron FC induce a reduction in Neu5Gc content while increasing Neu5Ac content. However, the total amount of Sia was significantly reduced by up to 75.8-95.3% at >0.5 μM FC plus 2.5 μM tropolone.

These results show that incorporation of FC and tropolone had a significantly different effect over Sia proportions of IgG1 Mab than when FC was supplied alone. A positive outcome was the significant decrease of up to 83-97% in the amount of immunogenic Sia Neu5Gc as the concentration increased from 1μM – 10 μM FC plus 5X tropolone. However, this decrease in NeuGc content was not concomitant with an increase in Neu5Ac. Instead, a significant reduction (45%-87%) in Neu5Ac content was also observed >0.5 μM FC plus 5X-tropolone. Thus, the original Neu5Gc:Neu5Ac ratio of 8:2 observed in untreated cultures changed to 7:3, 6:4, 6:4 and 5:5 in cultures containing: 0.5μM FC, 1 μM FC, 5 μM FC and 10 μM FC plus 5X tropolone

respectively. Altogether, a significant decrease in the total amount of Sias of up to 76%-95% was observed when FC at >0.5 μM was combined with 5X tropolone.

12.5 Discussion

The glycosylation profile of recombinant proteins has been previously modulated by the incorporation of metal ions in various salt forms (e.g. $MgCl_2$, $MnSO_4$, Fe_2SO_4). Metal ions act as cofactors during enzyme catalysis. For instance, iron and manganese have shown to be necessary for oligosaccharyltransferase (OST) maximal activity, improving glycosylation in a concentration manner (10-100 μM) by increasing the site-occupancy of t-PA produced by CHO cells (Gawlitzek, Estacio *et al.* 2009). Furthermore, Wentz *et al.* showed that iron salts (e.g. 0.1 mM, 0.5mM, 1 mM and 2 mM ferric nitrate; 0.1 mM and 1 mM FC; or 1mM FS) could reduce by up to 25% the amount of non-galactosylated glycans (e.g. F6A2G0, F6A1G0) and increase by 7X and 50X-fold the amount of F6A2G1 and F6A2G2-glycans respectively in Mabs produced by CHO cells (Wentz, Hemmavanh *et al.* 2017). However, these iron's effects on glycosylation were not consistent with the results presented in this chapter. Further enrichment of Biogro™ media with FC (0.05 mM, 0.1mM, 0.5 mM and 1 mM) or FS (0.05 mM, 0.1 mM and 0.5 mM) did not have any influence over galactosylation nor sialylation of IgG1 Mab with respect to untreated cultures. Even though iron salts have previously shown to stimulate the CMAH enzyme's activity in mouse liver homogenates, few reports have shown the effect of iron salts on the relative amounts of CMP-Neu5Gc and Neu5Ac (Shaw, Schneckenburger *et al.* 1992, Rosenberg 1995, Montreuil, Vliegenthart *et al.* 1997).

Table 12. 1. Iron supplementation effect on N-linked glycosylation of NS0-IgG1 Mab.

Galactosylation index (GI), % sialylated glycans and proportions of Neu5Gc vs. Neu5Ac. Culture media was supplemented with **A**. FC (0.05, 0.1, 0.5 and 1 mM, **B.** FS (0.05, 0.1and 0.5 mM) and **C.** FC plus 5X-tropolone (0.1, 0.5, 1, 5, 10 μM FC).

A.

Ferric citrate (mM)	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Ratio
			Mole Neu5Gc/mole Mab	Mole Neu5Ac/mole Mab	Total mole Sia/mole Mab	Neu5Gc : Neu5Ac
0	0.58 ± 0.03	12.3 ± 3.58	0.29 ± 0.06	0.07 ± 0.05	0.36 ± 0.07	8 : 2
0.05	0.59 ± 0.03	12.5 ± 4.63	0.30 ± 0.05	0.10 ± 0.06	0.40 ± 0.05	8 : 2
0.1	0.54 ± 0.08	10.4 ± 7.86	0.35 ± 0.08	0.11 ± 0.07	0.46 ± 0.05	8 : 2
0.5	0.59 ± 0.04	14.4 ± 3.19	0.28 ± 0.08	0.10 ± 0.06	0.38 ± 0.04	7 : 3
1	0.59 ± 0.05	11.9 ± 5.95	0.28 ± 0.10	0.07 ± 0.04	0.36 ± 0.08	8 : 2

B.

Ferrous sulfate (mM)	GI	% Sialylation	Neu5Gc	Neu5Ac	Total	Ratio
			Mole Neu5Gc/mole Mab	Mole Neu5Ac/mole Mab	Total mole Sia/mole Mab	Neu5Gc : Neu5Ac
0	0.58 ± 0.01	12.3 ± 1.79	0.29 ± 0.06	0.07 ± 0.05	0.36 ± 0.07	8 : 2
0.05	0.53 ± 0.05	10.5 ± 3.29	0.29 ± 0.09	0.07 ± 0.03	0.36 ± 0.09	8 : 2
0.1	0.59 ± 0.03	11.8 ± 2.36	0.30 ± 0.14	0.07 ± 0.04	0.37 ± 0.17	8 : 2
0.5	0.59 ± 0.03	12.6 ± 3.00	0.31 ± 0.07	0.05 ± 0.02	0.36 ± 0.06	9 : 1

C.

Supplement	GI	% SIALYLATION	Neu5Gc	Neu5Ac	Total	Ratio
			Mole Neu5Gc/mole Mab	Mole Neu5Ac/mole Mab	Total mole Sia/mole Mab	Neu5Gc : Neu5Ac
Control	0.58 ± 0.02	18.2 ± 3.00	0.21 ± 0.01	0.05 ± 0.01	0.26 ± 0.02	8 : 2
0.1 μM FC + 0.5 μM Tropolone	0.55 ± 0.07	14.2 ± 4.14	0.19 ± 0.01	0.04 ± 0.02	0.24 ± 0.03	8 : 2
0.5 μM FC + 2.5 μM Tropolone	0.59 ± 0.00	17.2 ± 0.53	0.21 ± 0.02	0.08 ± 0.01	0.29 ± 0.01	7 : 3
1 μM FC + 5 μM Tropolone	0.46 ± 0.09	12.8 ± 5.79	0.04 ± 0.01	0.03 ± 0.00	0.06 ± 0.00	6 : 4
5 μM FC + 25 μM Tropolone	0.32 ± 0.04	13.7 ± 2.39	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	6 : 4
10 μM FC + 50 μM Tropolone	0.23 ± 0.05	4.25 ± 0.49	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	5 : 5

However, it has been suggested that CMAH enzyme's activity along with CMP-Neu5Gc's concentrations in the cytosol are key regulators of sialylation with Neu5Gc (Montreuil, Vliegenthart *et al.* 1997). In this chapter, Ficko *et al.* report was taken as a reference for iron concentrations, where iron concentrations of 0.05-0.1 mM were tested on their effect over Neu5Gc content. In NS0 cultures, iron supplementation did not influence the amount of Neu5Gc, showing a Neu5Gc:Neu5Ac ratio of 8:2 similar to untreated cultures. The latter suggests that FS (0.05 - 0.5 mM) and FC (0.05 mM-1 mM) failed to influence CMAH enzyme's activity. In contrast, Ficko's results showed that a reduction in media's iron concentration from 0.2 to 0.07 mM induced a slight decrease of up to 2% in the content of Neu5Gc. However, these experiments tested iron along with ManNAc and GlcNAc which have shown to decrease Neu5Gc content in glycoproteins (**See section C, Chapter 7 and 8**).

Thus, in despite the high iron concentrations used in these experiments, the lack of iron salts' influence over glycosylation and Neu5Gc content as well as their minimal impact in cell growth and viability suggested that the iron salts were probably not able to effectively enter NS0 cells. Thus, the next set of experiments looked if iron transport was a limiting factor by incorporating 2-hydroxy-2,4,6-cycloheptatrien-1 one (tropolone) along with FC. As a chelating agent, tropolone was expected to bind to iron, stabilise their oxidative status and contribute in their transport into the cells (Field 1995). In fact, tropolone has been previously used in cell culture in replacement of transferrin. Tropolone has been chosen over other chelating agents (e.g. transferrin, ferritin and organic acids) because has shown to be non-toxic, cheap, readily available and of synthetic and not animal origin, which prevents from any unwanted contamination (Field 1995). As a result, the chelating agent allowed the use of much lower concentrations of FC (μM vs mM)

than when FC was supplemented alone to NS0 cells. FC and tropolone significantly reduced NS0 cells' growth and viability at ≥1 μM FC plus 5X-tropolone since day one. The latter indicated that NS0 cells did not tolerate the toxicity of iron at concentrations higher than 1 μM when incorporated with the chelating agent. In addition, an unexpected significant decrease (5.52% - 57.4%; 10.8% - 67.6%) in G1- and G2- containing glycans respectively was observed in cultures with 1-10 μM FC plus 5X-tropolone. Furthermore, an unexpected decrease in galactosylation (5% to up to 60%) along with a decrease on sialylation (21.6 to up to 76.6%) were also observed in IgG1 Mab produced in cultures containing much lower concentrations of FC (0.1-10μM plus 5X-tropolone) when compared to the control.

With respect to iron and tropolone supplementation's effect over Neu5Gc content, a significant decrease in the amount of Neu5Gc was observed in FC plus 5X-tropolone-containing cultures. A decrease in the amount of Neu5Gc has previously been related to limited cell growth (Ficko 2014). In NS0 cell cultures, the highest cell growth inhibition was observed when tropolone reached over 5 μM, with up to 50% decrease in cell densities since day one in comparison to untreated cultures (data not shown). Thus, the more prominent decrease in Neu5Gc content observed at 1μM-10 μM FC plus 5 μM-50 μM tropolone could be related to tropolone's high concentrations. However, IgG1Mab showed a decrease in Neu5Gc content even at 0.5-1 μM where NS0 cells' growth and viability was equivalent to untreated cultures. Thus, the decrease in Neu5Gc content might be due to a limited CMAH activity induced by chelated iron. For instance, the original Neu5Gc:Neu5Ac ratio observed in control cultures of 8:2 significantly changed to 7:3, 6:4, 6:4 and 5:5 in cultures containing 0.5 μM, 1 μM, 5 μM and 10 μM Fc plus 5X tropolone respectively. These results are consistent with previous reports where the catalytic properties of

CMAH in fractionated mouse liver were inhibited by up to 25-100% because of an increase in ionic strength caused by metal ligands in the presence of chelating agents (e.g. EDTA, ferrozine, tiron, *o*-phenanthroline) (Shaw and Schauer 1989, Shaw, Schneckenburger *et al.* 1992). This inhibitory effect over CMAH enzyme was suggested to be a result of chelation of endogenous iron.

In addition to a significant reduction in Neu5Gc content, a significant decrease of up to 87.4% was also observed in the amount of Neu5Ac in FC plus tropolone containing cultures when compared to untreated cultures. These decrease in Neu5Ac content along with the decrease in Neu5Gc were reflected in a significant reduction of total sialylation of up to 10-95% in FC plus tropolone-containing cultures. The latter could indicate that other factor might have influenced the decrease in sialylation besides the chelated iron's effect on CMAH enzyme's activity involving the synthesis of CMP-Neu5Ac. Finally, the possibility of extracellular degradation by glycosidases cannot be discarded as a factor of decrease in Sia in cultures with higher FC plus tropolone concentrations.

12.6 Conclusions.

- FS (0.05, 0.1, 0.5 mM) and FC (0.05, 0.1, 0.5 and 1 mM) did not have a significant effect on cell growth and viability of NS0 cells.
- There was not influence of FS and FC in the *N*-linked glycosylation of IgG1 Mab, particularly on sialylation with a Neu5Gc: Neu5Ac ratio of 8:2 similar to untreated cultures

- Supplementation of FC and tropolone (1 μM-10 μM FC plus 5 μM-50 μM tropolone) decreased significantly NS0 cells' growth (up to 59%) and viability (up to 29%) since day one, indicating that at these concentrations iron was toxic to cells.

- A significant increase in non-galactosylated glycans (10.9%-233%) along with a significant decrease in G1- and G2- containing glycans (5.52%-57.4%; 10.8%-67.6%) of up to 10.9%- was observed in IgG1 Mab produced in media containing 1-10 μM FC plus 5X tropolone.

- A significant decrease (5-60%) in galactosylation index was observed as the concentration of FC increased from 0.1 μM to 10 μM plus 5X tropolone.

- Sialylation decreased by up to 76.6% in Mab produced in media containing 10 μM FC plus 5X-tropolone.

- Neu5Gc content significantly decreased by 82.6%, 94.2% and 97.12% in cultures containing 1 μM FC plus 5 μM tropolone, 5 μM FC plus 25 μM tropolone and 10 μM FC plus 50 μM tropolone respectively.

- A significant decrease of about 45.3%, 80.4% and 87.4% in Neu5Ac content was also observed in 1 μM FC plus 5 μM tropolone, 5 μM FC plus 25 μM tropolone and 10 μM FC plus 50 μM tropolone-containing cultures respectively.

- A significant decrease in Neu5Gc:Neu5Ac ratio of 8:2 observed in control cultures was significantly change 7.3; 6:4; and 5:5 in the presence of ≥ 0.5-10 μM FC plus 5X tropolone.

- Decrease in growth and viability can be attributed to NS0cells'intolerance to high concentrations of FC and tropolone induced toxicity.

- The decrease in galactosylation, sialylation and Neu5Gc content were attributed to different factor such as chelating agent sequestration of endogenous iron, iron toxicity, reduction of CMAH enzyme's activity or possible presence of extracellular glycosylases.

Chapter 13

Conclusion

Manufacturing bioprocesses have focused on extending longevity of cells in culture while promoting the yield of recombinant proteins. For instance, feeding strategies are more closely monitored to ensure safety and reproducibility of the bioprocess by ensuring a proper glycosylation profile. In Chapter 3 and 4, the effect of glucose depletion and increase in cell inoculum on the metabolism, productivity and particularly *N*-glycosylation was investigated on the chimeric human-llama monoclonal antibody (EG2-hFc) secreted by CHO EG2 cells. Unlike regular IgGs, EG2-hFc Mabs constitute single-domain antibodies which are smaller in size (80 kDa) allowing for better permeability in tissues (Zhang, Liu *et al.* 2009). A comparison of EG2-hFc to a regular IgG (anti-IL-8 IgG) secreted by DP12 cells showed a similar decrease of the macro- and microheterogeneity under glucose depletion. The data indicated that under glucose deprivation, low levels of intracellular nucleotides and nucleotide sugars reduced the availability of the immediate precursors of glycosylation. Hence, it is important to avoid nutrient depletion as it can compromise the glycosylation process of recombinant proteins.

Knowing that glycosylation is dependent on nucleotide sugars availability, the glycosylation pathways of NS0 and EG2 cells were manipulated by supplementation of galactosylation precursors (Chapter 5). Both cell lines showed the highest GI in media

supplemented with M and Gal, showing a synergistic effect over UDP-Gal intracellular concentrations. In addition, the results emphasize that cell lines and recombinant proteins need to be evaluated individually. First, the increment in galactosylated glycans induced by M and Urd allowed to further improvement of sialylation of NS0-IgG1 but not of EG2-hFc Mabs. The latter was not a result of a decrease in CMP-Sia pool, as the concentrations of this Sia precursor were comparable to untreated cultures. Thus, there are more factors influencing sialylation of monoclonal antibodies than just improving the availability of Gal terminal residues. Second, an increase in UDP-Gal intracellular concentrations translated also into a significant increase of alpha-Gal residues in NS0-IgG1 (Chapter 6). The alpha-Gal epitope has previously been found at higher concentrations in murine- than in hamster-cell lines. However, there is limited understanding of the effect that glycosylation precursors have on the relative amount of alpha-Gal residues. Thus, the results presented here can contribute to a better understanding of the influence of feeding strategies on immunogenic residues such as the alpha-Gal epitope. In addition, it was shown that exoglycosidase digestion can be used to define the contribution of galactosylation precursor supplementation on alpha-Gal residues vs. beta Gal residues.

In Chapter 7 and 8, a substrate-based approach was used to intercept the Sia pathway to increase the intracellular concentration of Sia precursors such as UDP-GlcNAc and ManNAc. In contrast to previous studies, peracetylated hexosamines were used to help diffusion across the plasma membrane. This allowed the usage of much lower concentrations (10-1000 μM). Even though a subtle increase in GI was observed in IgG1 Mabs, an unexpected decrease in sialylation was observed in 0-50 μM peracetylated glucosamine (Ac_4GlcNAc) cultures. These results were consistent with previous reports were higher intracellular concentrations of UDP-GlcNAc

(induced by GlcN+Urd feeding) were assumed to be responsible for inhibiting transport of CMP-Sia to the Golgi, resulting in low sialylation. Thus, peracetylated mannosamine (Ac_4ManNAc) was used to overpass the feedback inhibition of GNE by CMP-Sia. However, ≥200 μM Ac_4ManNAc had a negative impact on galactosylation and subsequently on sialylation of IgG1 Mabs. Little is known about the effect of peracetylated hexosamines in Sia proportions of monoclonal antibodies. Interestingly, peracetylated hexosamines changed Neu5Gc:Neu5Ac ratio to 4:1 and 1:1 at >50 μM Ac_4GlcNAc and >200 μM Ac_4ManNAc respectively. Further research must be done to explain why peracetylated hexosamines can induce a decrease in Neu5Gc. In addition, it is suggested that Sia precursor feeding strategies should be combined with M+Gal supplementation to increase terminal Gal residues required prior to sialylation.

An improvement in sialylation of recombinant proteins has been related to improved serum half-life *in vivo,* resistance to proteases, thermal stability, and anti-inflammatory properties. However, it is desired that the relative abundance of the immunogenic Sia Neu5Gc stays at a negligible amounts relative to Neu5Ac. That was the case of CHO EG2 cells, with a Neu5Gc:Neu5Ac ratio of 0:1. In contrast, NS0 cells showed preference towards Neu5Gc, with a Neu5Gc:Neu5Ac ratio of 8:2. In Chapter 9, a positive outcome was observed in NS0 cultures supplemented with galactosylation precursors. This was a decrease in the amount of the immunogenic Neu5Gc while increasing the total degree of sialylation. Particularly uridine containing cultures, which showed a Neu5Gc:Neu5Ac ratio of 1:1. Previous studies have shown that Neu5Ac can compete with newly synthesized Neu5Gc when present at higher concentrations. In Chapter 10, free Neu5Ac supplementation slightly changed the Neu5Gc:Neu5Ac ratio to 6:1. However, the reduction of Neu5Gc was not enough to increase the amount of the desired Sia

Neu5Ac over Neu5Gc. Here we used micromolar range concentrations, different from previous studies were millimolar concentrations were successful in the reduction of Neu5Gc to negligible amounts.

One of the key supplements widely used in mammalian cell culture is serum. Serum's rich composition provides cells with proteins, growth factors, essential minerals, trace elements and cytokines, all of which are required for animal cell growth and proliferation. However, serum also has been identified as a rich source of salvaged sugars for glycosylation. The later includes the immunogenic Neu5Gc Sia which has been incorporated to glycoproteins by cultured cells. This includes human cells which cannot produce Neu5Gc as well as cells were the CMAH gene has been knocked-out. In contrast to previous results, a fetal bovine serum containing <5 μM IgG (Ultra-Low IgG FBS) was used to evaluate NS0-IgG1 and EG2-hFc Mab Sia proportions. Surprisingly, a significant decrease of up to 24% in Neu5Gc along with a 5-fold increase in Neu5Ac-glycans was observed in 10% Ultra-Low IgG FBS-supplemented cultures. The latter resulted in a change in Neu5Gc:Neu5Ac to 1:1. Analysis of serum bottle content confirmed that Ultra-Low IgG FBS was the main source of Neu5Ac, showing 94.5% Neu5Ac. Thus, results shown in Chapter 11 showed that Ultra-low IgG FBS can be used as a strategy to reduce Neu5Gc by metabolic competition. Accordingly, Ultra-Low IgG FBS failed to influence the proportions of Sia s in EG2-hFc Mab, which original have mainly Neu5Ac-containing glycans.

Finally in Chapter 12, iron supplementation has previously shown to improve fully glycosylated proteins. In particular, iron is required for the conversion of CMP-Neu5Ac to CMP-Neu5Gc by CMP-Neu5Ac hydroxylase (CMAH). Chelated iron had a negative impact on IgG1

Mab's *N*-glycosylation with a significant decrease in galactosylated glycans of up to 67.6% along with a significant decrease in sialylation of up to 76.6%. Surprisingly, a significant reduction in Neu5Gc-containing glycans was observed as the concentration of chelating iron increased from 0.5 μM to 1μM. The latter meant a Neu5Gc:Neu5Ac ratio change to 1:1 in comparison to untreated cultures with 8:2. However, it is important to mention that cultures which contained lower Neu5Gc-glycans showed a reduction in cell growth, probably due to iron's toxicity as concentration increased in the media. Thus, further experimentation needs to focus on examining if iron was indeed responsible of the observed reduction on Neu5Gc.

The results presented in this thesis show that glycosylation patterns were strongly dependent on the cell producers' machinery, protein itself, nutrient availability and glycosylation precursors. Thus, galactosylation was improved by increasing the intracellular concentrations of UDP-Gal and uridine pool by supplementation of M, Urd and Gal. However, improving sialylation was more complicated than just providing with Sia precursors to mammalian cells. Future work should include feeding experiments with the same Sia precursors but along with M+Gal. The latter will guarantee an increase in galactosylation, which is required for sialylation to occur. This thesis provided with valuable information in regard to the effect that Sia precursors (e.g. peracetylated hexosamines, Cyt, uridine and iron) have on not only glycosylation of monoclonal antibodies but also on their effect over the proportion of Neu5Gc vs. Neu5Ac, which has not been profoundly characterized yet.

Chapter 14

References.

Abès, R. and Teillaud, J.-L. (2010). "Impact of Glycosylation on Effector Functions of Therapeutic IgG." Pharmaceuticals 3(1): 146-157.

Ahn, W. S. and Antoniewicz, M. R. (2012). "Towards dynamic metabolic flux analysis in CHO cell cultures." Biotechnology Journal 7(1): 61-74.

Aires da Silva, F., Corte-Real, S. & Goncalves, (2008). "Recombinant Antibodies as Therapeutic Agents. Pathways for Modeling New Biodrugs." J. BioDrugs: 301-314.

Ajit Varki, S. D., Rachel Taylor (2009).Elimination of a contamining non-human sialic acid by metabolic competition. University of California. 20110195921.

Almaraz, R. T.*et al.* (2012). "Metabolic flux increases glycoprotein sialylation: implications for cell adhesion and cancer metastasis." Mol Cell Proteomics 11(7): M112 017558.

Altamirano, C.*et al.* (2006). "Considerations on the lactate consumption by CHO cells in the presence of galactose." Journal of Biotechnology 125(4): 547-556.

Altamirano, C.*et al.* (2000). "Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine." Biotechnol Prog 16(1): 69-75.

Amon, R.*et al.* (2014). "Glycans in immune recognition and response." Carbohydr Res 389: 115-122.

Andersen, D. C. and Goochee, C. F. (1994). "The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins." Curr Opin Biotechnol 5(5): 546-549.

Arigony, A. L.*et al.* (2013). "The influence of micronutrients in cell culture: a reflection on viability and genomic stability." Biomed Res Int 2013: 597282.

Arnold, J. N.*et al.* (2007). "The impact of glycosylation on the biological function and structure of human immunoglobulins." Annu Rev Immunol 25: 21-50.

Bai, Y.*et al.* (2011). "Role of iron and sodium citrate in animal protein-free CHO cell culture medium on cell growth and monoclonal antibody production." Biotechnol Prog 27(1): 209-219.

Baker, K. N.*et al.* (2001). "Metabolic control of recombinant protein N-glycan processing in NS0 and CHO cells." Biotechnol Bioeng 73(3): 188-202.

Banda, K.*et al.* (2012). "Metabolism of vertebrate amino sugars with N-glycolyl groups: mechanisms underlying gastrointestinal incorporation of the non-human sialic acid xeno-autoantigen N-glycolylneuraminic acid." J Biol Chem 287(34): 28852-28864.
Bardor, M.*et al.* (2005). "Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells." J Biol Chem 280(6): 4228-4237.
Barnabe, N. and Butler, M. (2000). "The effect of glucose and glutamine on the intracellular nucleotide pool and oxygen uptake rate of a murine hybridoma." Cytotechnology 34(1-2): 47-57.
Bayer, N.*et al.* (2013). "Artificial and Natural Sialic Acid Precursors Influence the Angiogenic Capacity of Human Umbilical Vein Endothelial Cells." Molecules 18(3): 2571.
Bekesi, J. G. and Winzler, R. J. (1969). "The effect of D-glucosamine on the adenine and uridine nucleotides of sarcoma 180 ascites tumor cells." J Biol Chem 244(20): 5663-5668.
Bell, A.*et al.* (2010). "Differential tumor-targeting abilities of three single-domain antibody formats." Cancer Lett 289(1): 81-90.
Berger M., K. M., Blanchard V. (2011). Protein Glycosylation and Its Impact on Biotechnology Genomics and Systems Biology of Mammalian Cell Culture. Advances in Biochemical Engineering Biotechnology. Hu W., Z. A. Berlin, Heidelberg, Springer. 127.
Blondeel, E. J. M. and Aucoin, M. G. (2018). "Supplementing glycosylation: A review of applying nucleotide-sugar precursors to growth medium to affect therapeutic recombinant protein glycoform distributions." Biotechnol Adv 36(5): 1505-1523.
Bodnar, E. D. and Perreault, H. (2013). "Qualitative and Quantitative Assessment on the Use of Magnetic Nanoparticles for Glycopeptide Enrichment." Analytical Chemistry 85(22): 10895-10903.
Bork, K.*et al.* (2009). "Increasing the sialylation of therapeutic glycoproteins: the potential of the sialic acid biosynthetic pathway." J Pharm Sci 98(10): 3499-3508.
Borys, M. C.*et al.* (2010). "Effects of culture conditions on N-glycolylneuraminic acid (Neu5Gc) content of a recombinant fusion protein produced in CHO cells." Biotechnol Bioeng 105(6): 1048-1057.
Borys, M. C.*et al.* (1993). "Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells." Biotechnology (N Y) 11(6): 720-724.

Bosques, C. J.*et al.* (2010). "Chinese hamster ovary cells can produce galactose-alpha-1,3-galactose antigens on proteins." Nat Biotechnol 28(11): 1153-1156.
Boyd, P. N.*et al.* (1995). "The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H." Mol Immunol 32(17-18): 1311-1318.
Brekke, O. H. and Sandlie, I. (2003). "Therapeutic antibodies for human diseases at the dawn of the twenty-first century." Nature Reviews Drug Discovery 2(1): 52-62.
Brunner, D.*et al.* (2010). "Serum-free cell culture: the serum-free media interactive online database." ALTEX 27(1): 53-62.
Burgener, A.*et al.* (2006). "Intracellular ATP and total adenylate concentrations are critical predictors of reovirus productivity from Vero cells." Biotechnol Bioeng 94(4): 667-679.
Butler, M. (2004). Animal cell culture and technology, Taylor and Francis Publishers.
Butler, M. (2005). "Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals." Appl Microbiol Biotechnol 68(3): 283-291.
Butler, M. (2007). Cell culture and upstream processing. London, Taylor & Francis.
Butler, M. (2009). Mammalian Cell Lines and Glycosylation: A Case Study. Post-translational Modification of Protein Biopharmaceuticals, Wiley-VCH Verlag GmbH & Co. KGaA**:** 51-77.
Butler, M. and Spearman, M. (2014). "The choice of mammalian cell host and possibilities for glycosylation engineering." Curr Opin Biotechnol 30: 107-112.
Byrne, K. (2014). High density CHO cell cultures: Improved productivity and product quality, Dublin City University. PhD Thesis.
Cabrera, G.*et al.* (2005). "Influence of culture conditions on the N-glycosylation of a monoclonal antibody specific for recombinant hepatitis B surface antigen." Biotechnol Appl Biochem 41(Pt 1): 67-76.
Cacan, R.*et al.* (1984). "Intralumenal pool and transport of CMP-N-acetylneuraminic acid, GDP-fucose and UDP-galactose. Study with plasma-membrane-permeabilized mouse thymocytes." Biochem J 224(1): 277-284.
Cacan, R.*et al.* (1987). "Catabolic pathway of oligosaccharide-diphospho-dolichol." European Journal of Biochemistry 166(2): 469-474.
Cao, H. and Chen, X. (2012). "General consideration on sialic acid chemistry." Methods Mol Biol 808: 31-56.

Capasso, J. M. and Hirschberg, C. B. (1984). "Effect of nucleotides on translocation of sugar nucleotides and adenosine 3'-phosphate 5'-phosphosulfate into Golgi apparatus vesicles." Biochim Biophys Acta 777(1): 133-139.

Carey, D. J.*et al.* (1980). "CMP-N-acetylneuraminic acid: isolation from and penetration into mouse liver microsomes." Cell 19(3): 597-605.

Castilho, L. d. R. (2008). Animal cell technology : from biopharmaceuticals to gene therapy. New York ; Abingdon [England], Taylor & Francis Group.

Chapman, A. E. and Calhoun, J. C. t. (1988). "Effects of glucose starvation and puromycin treatment on lipid-linked oligosaccharide precursors and biosynthetic enzymes in Chinese hamster ovary cells in vivo and in vitro." Arch Biochem Biophys 260(1): 320-333.

Chapman, A. G.*et al.* (1971). "Adenylate energy charge in Escherichia coli during growth and starvation." J Bacteriol 108(3): 1072-1086.

Chee Furng Wong, D.*et al.* (2005). "Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures." Biotechnology and Bioengineering 89(2): 164-177.

Chen, L. and Liang, J. F. (2012). "Metabolic monosaccharides altered cell responses to anticancer drugs." Eur J Pharm Biopharm 81(2): 339-345.

Chen, S. C.*et al.* (2016). "Mechanism and inhibition of human UDP-GlcNAc 2-epimerase, the key enzyme in sialic acid biosynthesis." Sci Rep 6: 23274.

Chenu, S.*et al.* (2003). "Reduction of CMP-N-acetylneuraminic acid hydroxylase activity in engineered Chinese hamster ovary cells using an antisense-RNA strategy." Biochim Biophys Acta 1622(2): 133-144.

Chou, H. H.*et al.* (2002). "Inactivation of CMP-N-acetylneuraminic acid hydroxylase occurred prior to brain expansion during human evolution." Proc Natl Acad Sci U S A 99(18): 11736-11741.

Clark, K. J.*et al.* (2005). "Gene-expression profiles for five key glycosylation genes for galactose-fed CHO cells expressing recombinant IL-4/13 cytokine trap." Biotechnol Bioeng 90(5): 568-577.

Clincke, M. F.*et al.* (2011). "Effect of iron sources on the glycosylation macroheterogeneity of human recombinant IFN-gamma produced by CHO cells during batch processes." BMC Proc 5 Suppl 8: P114.

Commins, S. P.*et al.* (2016). "Delayed anaphylaxis to alpha-gal, an oligosaccharide in mammalian meat." Allergol Int 65(1): 16-20.
Commins, S. P. and Platts-Mills, T. A. (2009). "Anaphylaxis syndromes related to a new mammalian cross-reactive carbohydrate determinant." J Allergy Clin Immunol 124(4): 652-657.
Commins, S. P. and Platts-Mills, T. A. (2013). "Delayed anaphylaxis to red meat in patients with IgE specific for galactose alpha-1,3-galactose (alpha-gal)." Curr Allergy Asthma Rep 13(1): 72-77.
Corfield A.P., S. R. (1982). Occurrence of Sialic Acids. Sialic Acids. Cell Biology Monographs. R., S., Springer, Vienna. 10.
Craig Hammond, A. H. (1995). "Quality control in the secretory pathway." Current Opinion in Cell Biology 7(4): 623-529.
Crowell, C. K.*et al.* (2007). "Amino acid and manganese supplementation modulates the glycosylation state of erythropoietin in a CHO culture system." Biotechnol Bioeng 96(3): 538-549.
Cruz, H. J.*et al.* (1999). "Metabolic shifts by nutrient manipulation in continuous cultures of BHK cells." Biotechnol Bioeng 66(2): 104-113.
Cura, A. J. and Carruthers, A. (2012). "Role of monosaccharide transport proteins in carbohydrate assimilation, distribution, metabolism, and homeostasis." Compr Physiol 2(2): 863-914.
Curling, E. M.*et al.* (1990). "Recombinant human interferon-gamma. Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture." Biochem J 272(2): 333-337.
Dan, N. and Lehrman, M. A. (1997). "Oligomerization of hamster UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase, an enzyme with multiple transmembrane spans." J Biol Chem 272(22): 14214-14219.
Davies, L. R. L. and Varki, A. (2015). "Why Is N-Glycolylneuraminic Acid Rare in the Vertebrate Brain?" Topics in current chemistry 366: 31-54.
Delves, P. J. and Roitt, I. M. (1998). Encyclopedia of Immunology, Academic Press.
Diaz, S. L.*et al.* (2009). "Sensitive and specific detection of the non-human sialic Acid N-glycolylneuraminic acid in human tissues and biotherapeutic products." PLoS One 4(1): e4241.

Doi K., H. M., Ebata I., Yamaguchi S., Ohta Y., Terada S. (2010). "Effect of ManNAc and its Derivatives on Glycosylation to Proteins Produced by Mammalian Cell Culture. ." Cells and Culture. ESACT Proceedings. Springer, Dordrecht 4.

Doyle, C. and Butler, M. (1990). "The effect of pH on the toxicity of ammonia to a murine hybridoma." Journal of Biotechnology 15(1-2): 91-100.

Du, J.*et al.* (2009). "Metabolic glycoengineering: sialic acid and beyond." Glycobiology 19(12): 1382-1401.

Dumont, J.*et al.* (2016). "Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives." Crit Rev Biotechnol 36(6): 1110-1122.

Durocher, Y. and Butler, M. (2009). "Expression systems for therapeutic glycoprotein production." Curr Opin Biotechnol 20(6): 700-707.

El Mai, N.*et al.* (2013). "Engineering a human-like glycosylation to produce therapeutic glycoproteins based on 6-linked sialylation in CHO cells." Methods Mol Biol 988: 19-29.

Ezzelarab, M. and Cooper, D. K. (2005). "Reducing Gal expression on the pig organ - a retrospective review." Xenotransplantation 12(4): 278-285.

Ficko, T. T. (2014).Production of glycoproteins with low N-glycolylneuraminic acid (Neu5Gc) content. LEK Pharmaceuticals D.D. 9562082.

Field, R. P. (1995).Animal cell culture. Lonza Group, AG. 6593140.

Fitzpatrick, L.*et al.* (1993). "Glucose and glutamine metabolism of a murine B-lymphocyte hybridoma grown in batch culture." Appl Biochem Biotechnol 43(2): 93-116.

Flickinger, M. C. (2013). Upstream Industrial Biotechnology, 2 Volume Set, Wiley.

Freeze, H. H. and Elbein, A. D. (2009). Glycosylation Precursors. Essentials of Glycobiology. Varki, A. *et al.* Cold Spring Harbor (NY).

Furukawa K, Y. H., Oettgen HF, Old LJ, Lloyd KO. (1988). "Analysis of the expression of N-glycolylneuraminic acid-containing gangliosides in cells and tissues using two human monoclonal antibodies." J Biol Chem 263(34): 18507-18512.

Gadgil, M. (2017). "Cell culture processes for biopharmaceutical manufacturing." Current Science 112(7): 1478-1488.

Gala, F. A. and Morrison, S. L. (2002). "The role of constant region carbohydrate in the assembly and secretion of human IgD and IgA1." J Biol Chem 277(32): 29005-29011.

Galili, U. (2001). "The alpha-Gal epitope (Galalpha1-3Galbeta1-4GlcNAc-R) in xenotransplantation." Biochimie 83(7): 557-563.
Galili, U.*et al.* (1984). "A unique natural human IgG antibody with anti-alpha-galactosyl specificity." J Exp Med 160(5): 1519-1531.
Galili, U.*et al.* (1988). "Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells." J Biol Chem 263(33): 17755-17762.
Gambhir, A.*et al.* (1999). "Alteration of cellular metabolism by consecutive fed-batch cultures of mammalian cells." J Biosci Bioeng 87(6): 805-810.
Gawlitzek, M.*et al.* (2009). "Identification of cell culture conditions to control N-glycosylation site-occupancy of recombinant glycoproteins expressed in CHO cells." Biotechnol Bioeng 103(6): 1164-1175.
Gawlitzek, M.*et al.* (2000). "Ammonium alters N-glycan structures of recombinant TNFR-IgG: degradative versus biosynthetic mechanisms." Biotechnol Bioeng 68(6): 637-646.
Gawlitzek, M.*et al.* (1995). "Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions." Journal of Biotechnology 42(2): 117-131.
Gawlitzek, M.*et al.* (1998). "Ammonium ion and glucosamine dependent increases of oligosaccharide complexity in recombinant glycoproteins secreted from cultivated BHK-21 cells." Biotechnol Bioeng 57(5): 518-528.
Geigert, J. (2013). Challenge of Adventitious Agent Control. The challenge of CMC regulatory compliance for biopharmaceuticals and other biologics, New York: Springer**:** 59-104.
Gershman, H. and Robbins, P. W. (1981). "Transitory effects of glucose starvation on the synthesis of dolichol-linked oligosaccharides in mammalian cells." J Biol Chem 256(15): 7774-7780.
Ghaderi, D.*et al.* (2010). "Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins." Nat Biotechnol 28(8): 863-867.
Ghaderi, D.*et al.* (2012). "Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation." Biotechnology & genetic engineering reviews 28: 147-175.

Glacken, M. W.*et al.* (1986). "Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells." Biotechnol Bioeng 28(9): 1376-1389.
Gomord, V.*et al.* (2005). "Biopharmaceutical production in plants: problems, solutions and opportunities." Trends Biotechnol 23(11): 559-565.
Goochee, C. F. and Monica, T. (1990). "Environmental effects on protein glycosylation." Biotechnology (N Y) 8(5): 421-427.
Grainger, R. K. and James, D. C. (2013). "CHO cell line specific prediction and control of recombinant monoclonal antibody N-glycosylation." Biotechnol Bioeng 110(11): 2970-2983.
Gramer, M. J.*et al.* (2011). "Modulation of antibody galactosylation through feeding of uridine, manganese chloride, and galactose." Biotechnol Bioeng 108(7): 1591-1602.
Gramer, M. J. and Goochee, C. F. (1993). "Glycosidase activities in Chinese hamster ovary cell lysate and cell culture supernatant." Biotechnol Prog 9(4): 366-373.
Gramer, M. J.*et al.* (1995). "Removal of sialic acid from a glycoprotein in CHO cell culture supernatant by action of an extracellular CHO cell sialidase." Biotechnology (N Y) 13(7): 692-698.
Grammatikos, S. I.*et al.* (1999). "Monitoring of intracellular ribonucleotide pools is a powerful tool in the development and characterization of mammalian cell culture processes." Biotechnol Bioeng 64(3): 357-367.
Grammatikos, S. I.*et al.* (1998). "Intracellular UDP-N-acetylhexosamine pool affects N-glycan complexity: a mechanism of ammonium action on protein glycosylation." Biotechnol Prog 14(3): 410-419.
Gstraunthaler, G. (2003). "Alternatives to the use of fetal bovine serum: serum-free cell culture." ALTEX 20(4): 275:281.
Gu, X. and Wang, D. I. (1998). "Improvement of interferon-gamma sialylation in Chinese hamster ovary cell culture by feeding of N-acetylmannosamine." Biotechnol Bioeng 58(6): 642-648.
Harris, R. J.*et al.* (2010). Analytical Characterization of Monoclonal Antibodies: Linking Structure to Function. Current Trends in Monoclonal Antibody Development and Manufacturing. Shire, S. J. *et al.* New York, NY, Springer New York**:** 193-205.

Hayter, P. M.*et al.* (1991). "Chinese hamster ovary cell growth and interferon production kinetics in stirred batch culture." Appl Microbiol Biotechnol 34(5): 559-564.
Hayter, P. M.*et al.* (1992). "Glucose-limited chemostat culture of Chinese hamster ovary cells producing recombinant human interferon-gamma." Biotechnol Bioeng 39(3): 327-335.
Hayter, P. M.*et al.* (1993). "The effect of the dilution rate on CHO cell physiology and recombinant interferon-gamma production in glucose-limited chemostat culture." Biotechnol Bioeng 42(9): 1077-1085.
Helenius, A. (1994). "How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum." Mol Biol Cell 5(3): 253-265.
Hendrickson, T. L. and Imperiali, B. (1995). "Metal ion dependence of oligosaccharyl transferase: implications for catalysis." Biochemistry 34(29): 9444-9450.
Higa, H. H. and Paulson, J. C. (1985). "Sialylation of glycoprotein oligosaccharides with N-acetyl-, N-glycolyl-, and N-O-diacetylneuraminic acids." J Biol Chem 260(15): 8838-8849.
Higashi, H.*et al.* (1985). "Characterization of N-glycolylneuraminic acid-containing gangliosides as tumor-associated Hanganutziu-Deicher antigen in human colon cancer." Cancer Res 45(8): 3796-3802.
Higel, F.*et al.* (2016). "N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins." Eur J Pharm Biopharm 100: 94-100.
Hills, A. E.*et al.* (2001). "Metabolic control of recombinant monoclonal antibody N-glycosylation in GS-NS0 cells." Biotechnol Bioeng 75(2): 239-251.
Hodge, G. (2005). "Media Development for Mammalian Cell Culture." BioPharm International 18(5).
Hodoniczky, J.*et al.* (2005). "Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro." Biotechnol Prog 21(6): 1644-1652.
Hossler, P. (2011). "Protein Glycosylation Control in Mammalian Cell Culture: Past Precedents and Contemporary Prospects."
Hossler, P. (2011). Protein Glycosylation Control in Mammalian Cell Culture: Past Precedents and Contemporary Prospects. . Genomics and Systems Biology of Mammalian Cell Culture. Hu W., Z. A. Berlin, Heidelberg, Springer. 127.

Hossler, P.*et al.* (2009). "Optimal and consistent protein glycosylation in mammalian cell culture." Glycobiology 19(9): 936-949.
Hristodorov, D., Fischer, R. & Linden, L. (2013). "With or Without Sugar? (A)glycosylation of Therapeutic Antibodies." Molecular Biotechnology 54(3): 1056-1068.
Huai, G.*et al.* (2016). "Characteristics of alpha-Gal epitope, anti-Gal antibody, alpha1,3 galactosyltransferase and its clinical exploitation (Review)." Int J Mol Med 37(1): 11-20.
Huber, R.*et al.* (1976). "Crystallographic structure studies of an IgG molecule and an Fc fragment." Nature 264(5585): 415-420.
Huhn, C.*et al.* (2009). "IgG glycosylation analysis." Proteomics 9(4): 882-913.
Irie, A.*et al.* (1998). "The molecular basis for the absence of N-glycolylneuraminic acid in humans." J Biol Chem 273(25): 15866-15871.
Jedrzejewski PM, D. V. I., Polizzi KM, Kontoravdi C. (2013). "Applying quality by design to glycoprotein therapeutics: experimental and computational efforts of process control. ." Pharmaceutical Bioprocessing.(1): 51-69.
Jefferis, R. (2002). Glycosylation of Recombinant IgG Antibodies and Its Relevance for Therapeutic Applications. Cell Engineering: Glycosylation. Al-Rubeai, M. Dordrecht, Springer Netherlands**:** 93-107.
Jefferis, R. (2009). Antibody glycosylation Post-translational Modification of Protein Biopharmaceuticals. Walsh, G., Wiley-Blackwell**:** 79-108.
Jefferis, R. (2009). "Glycosylation as a strategy to improve antibody-based therapeutics." Nat Rev Drug Discov 8(3): 226-234.
Jenkins, N.*et al.* (1994). "Effect of lipid supplements on the production and glycosylation of recombinant interferon-gamma expressed in CHO cells." Cytotechnology 15(1-3): 209-215.
Jones, M. B.*et al.* (2010). "Analysis and metabolic engineering of lipid-linked oligosaccharides in glycosylation-deficient CHO cells." Biochem Biophys Res Commun 395(1): 36-41.
Kaluza, G. (1975). "Effect of impaired glycosylation on the biosynthesis of Semliki forest virus glycoproteins." J Virol 16(3): 602-612.
Kaneko, Y.*et al.* (2010). "Changes in the quality of antibodies produced by Chinese hamster ovary cells during the death phase of cell culture." J Biosci Bioeng 109(3): 281-287.

Kim, E. J.*et al.* (2004). "Characterization of the metabolic flux and apoptotic effects of O-hydroxyl- and N-acyl-modified N-acetylmannosamine analogs in Jurkat cells." J Biol Chem 279(18): 18342-18352.
Kochanowski, N.*et al.* (2006). "Intracellular nucleotide and nucleotide sugar contents of cultured CHO cells determined by a fast, sensitive, and high-resolution ion-pair RP-HPLC." Analytical biochemistry 348(2): 243-251.
Kornfeld, S.*et al.* (1964). "The Feedback Control of Sugar Nucleotide Biosynthesis in Liver." Proc Natl Acad Sci U S A 52: 371-379.
Krapp, S.*et al.* (2003). "Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity." J Mol Biol 325(5): 979-989.
Krokhin, O.*et al.* (2004). "Site-specific N-glycosylation analysis: matrix-assisted laser desorption/ionization quadrupole-quadrupole time-of-flight tandem mass spectral signatures for recognition and identification of glycopeptides." Rapid Commun Mass Spectrom 18(18): 2020-2030.
Krug, E.*et al.* (1984). "D-glucosamine-induced changes in nucleotide metabolism and growth of colon-carcinoma cells in culture." Biochem J 217(3): 701-708.
Kunkel, J. P.*et al.* (1998). "Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody." Journal of Biotechnology 62(1): 55-71.
Kuriakose, A.*et al.* (2016). "Immunogenicity of Biotherapeutics: Causes and Association with Posttranslational Modifications." J Immunol Res 2016: 1298473.
Lai, T.*et al.* (2013). "Advances in Mammalian cell line development technologies for recombinant protein production." Pharmaceuticals (Basel) 6(5): 579-603.
Lalonde, M. E. and Durocher, Y. (2017). "Therapeutic glycoprotein production in mammalian cells." Journal of Biotechnology 251: 128-140.
Lammerts van Bueren, J. J.*et al.* (2011). "Anti-galactose-alpha-1,3-galactose IgE from allergic patients does not bind alpha-galactosylated glycans on intact therapeutic antibody Fc domains." Nat Biotechnol 29(7): 574-576.
Lattová, E. and Perreault, H. (2009). Method for Investigation of Oligosaccharides Using Phenylhydrazine Derivatization. Glycomics. Packer, N. and Karlsson, N., Humana Press. 534**:** 65-77.

Lattova, E.*et al.* (2004). "Matrix-assisted laser desorption/ionization tandem mass spectrometry and post-source decay fragmentation study of phenylhydrazones of N-linked oligosaccharides from ovalbumin." Journal of the American Society for Mass Spectrometry 15(5): 725-735.
Lefloch, F.*et al.* (2006). "Related effects of cell adaptation to serum-free conditions on murine EPO production and glycosylation by CHO cells." Cytotechnology 52(1): 39-53.
Leszek A., K. a. D. D. (2015). "Sugar activation for production of nucleotide sugars as substrates for glycosyltransferases in plants." J. Appl. Glycosci.
Li, F.*et al.* (2010). "Cell culture processes for monoclonal antibody production." MAbs 2(5): 466-479.
Lin, N.*et al.* (2015).Cells deficient in CMP-N-acetylneuraminic acid hydroxylase and/or glycoprotein alpha-1,3-galactosyltransferase. Sigma Aldrich Co LLC. 20150152463.
Liu, B.*et al.* (2014). "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." Journal of Biotechnology 170: 17-27.
Liu, Y.*et al.* (2003). "Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line." J Cell Mol Med 7(1): 49-56.
Loboda, A. V.*et al.* (2000). "A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance." Rapid Commun Mass Spectrom 14(12): 1047-1057.
Luchansky, S. J.*et al.* (2004). "Expanding the diversity of unnatural cell-surface sialic acids." Chembiochem 5(3): 371-374.
Lund, J.*et al.* (1996). "Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fc gamma receptor I and influence the synthesis of its oligosaccharide chains." J Immunol 157(11): 4963-4969.
Macher, B. A. and Galili, U. (2008). "The Galalpha1,3Galbeta1,4GlcNAc-R (alpha-Gal) epitope: a carbohydrate of unique evolution and clinical relevance." Biochim Biophys Acta 1780(2): 75-88.
Maggioni, A.*et al.* (2013). "Characterisation of CMP-sialic acid transporter substrate recognition." Chembiochem 14(15): 1936-1942.

Majid, F. A.*et al.* (2007). "Glycosylation of an immunoglobulin produced from a murine hybridoma cell line: the effect of culture mode and the anti-apoptotic gene, bcl-2." Biotechnol Bioeng 97(1): 156-169.
Malykh, Y. N.*et al.* (1998). "The role of CMP-N-acetylneuraminic acid hydroxylase in determining the level of N-glycolylneuraminic acid in porcine tissues." Glycoconj J 15(9): 885-893.
Markely, L. R. A. (2011). High-Throughput Quantification of Glycoprotein Sialylation, University of Wisconsin-Madison. Doctor of Philosophy in Chemical Engineering.
Martin, M. J.*et al.* (2005). "Human embryonic stem cells express an immunogenic nonhuman sialic acid." Nature medicine 11(2): 228-232.
Martinez-Duncker, I. and Tiralongo, J. (2013). Sialobiology : Structure, Biosynthesis and Function: Sialic Acid Glycoconjugates in Health and Disease. Sharjah, U.A.E., Bentham Science Publishers.
Miller, W. M.*et al.* (1988). "Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture." Bioprocess Engineering 3(3): 113-122.
Mimura, Y.*et al.* (2000). "The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms." Mol Immunol 37(12-13): 697-706.
Montreuil, J.*et al.* (1997). Chemistry, Biochemistry and Biology of Sialic Acids. Glycoproteins II. J. Montreuil, J. F. G. V., H. Schachter, Elsevier. 29 of New Comprehensive Biochemistry**:** 243-402.
Morihisa Fujita, X.-D. G. (2015). Glycan-Mediated Protein Transport from the Endoplasmic Reticulum. Sugar Chains. Decoding the Functions of Glycans. Tadashi Suzuki, K. O., Naoyuki Taniguchi. Japan, Springer**:** 21-34.
Morito, T.*et al.* (1982). "Hanganutziu-Deicher antibodies in infectious mononucleosis and other diseases." J Immunol 129(6): 2524-2528.
Muchmore EA, D. S., Varki A. (1998). "A structural difference between the cell surfaces of humans and the great apes." Am J Phys Anthropol. 107((2): 187-198.
Muchmore EA, M. M., Varki A, Diaz S. (1989). "Biosynthesis of N-glycolyneuraminic acid. The primary site of hydroxylation of N-acetylneuraminic acid is the cytosolic sugar nucleotide pool." J Biol Chem. 34(264): 10216-10223.

Mulukutla, B. C.*et al.* (2012). "On metabolic shift to lactate consumption in fed-batch culture of mammalian cells." Metab Eng 14(2): 138-149.

Mulukutla, B. C.*et al.* (2010). "Glucose metabolism in mammalian cell culture: new insights for tweaking vintage pathways." Trends Biotechnol 28(9): 476-484.

Nagira, K.*et al.* (1995). "Development of a protein-free medium with iron salts replacing transferrin for a human-human hybridoma." Biosci Biotechnol Biochem 59(4): 743-745.

Naito-Matsui, Y.*et al.* (2017). "Physiological Exploration of the Long Term Evolutionary Selection against Expression of N-Glycolylneuraminic Acid in the Brain." J Biol Chem 292(7): 2557-2570.

Nakajima, K.*et al.* (2010). "Simultaneous determination of nucleotide sugars with ion-pair reversed-phase HPLC." Glycobiology 20(7): 865-871.

Ng, Y. C.*et al.* (1996). "Optimization of physical parameters for cell attachment and growth on macroporous microcarriers." Biotechnol Bioeng 50(6): 627-635.

Nimmerjahn, F. and Ravetch, J. V. (2007). "Fc-receptors as regulators of immunity." Adv Immunol 96: 179-204.

Nobuko Hosokawa, T. S. (2015). N-Glycans and Quality Control of Proteins
. Sugar Chains. Decoding the Functions of Glycans. Tadashi Suzuki, K. O., Naoyuki Taniguchi. Japan, Springer**:** 1-20.

Norrby, K. (1974). "Cell inoculum size and rate of subsequent exponential multiplication in human cell lines." Virchows Arch B Cell Pathol 16(1): 63-69.

Nyberg, F.*et al.* (1998). "A European validation study of smoking and environmental tobacco smoke exposure in nonsmoking lung cancer cases and controls." Cancer causes & control : CCC 9(2): 173-182.

Nyberg, G. B. (1998). Glycosylation site occupancy heterogeneity in Chinese hamster ovary cell culture. Massachussetts, Massachusetts Institute of Technology. Doctor of Phylosophy.

Nyberg, G. B.*et al.* (1999). "Metabolic effects on recombinant interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells." Biotechnol Bioeng 62(3): 336-347.

Okam, M. (1999, October 8, 1999). "Chelators for Iron overload." Information Center for Sickle Cell and Thalassemic Disorders. from http://sickle.bwh.harvard.edu/chelators.html.

Olaf Kruger, K. O., Lars Kober (2009).Mammalian culture media with polyamine and iron. Cellca GmbH. 8637312.

Ozturk, S. and Hu, W. S. (2005). Cell Culture Technology for Pharmaceutical and Cell-Based Therapies, CRC Press.
Ozturk, S. S. and Palsson, B. O. (1990). "Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production." Journal of Biotechnology 16(3-4): 259-278.
Patel, T. P.*et al.* (1992). "Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody." Biochem J 285 (Pt 3): 839-845.
Pederson, N. V.*et al.* (1992). "UDP-N-acetylhexosamine modulation by glucosamine and uridine in NCI N-417 variant small cell lung cancer cells: 31P nuclear magnetic resonance results." Cancer Res 52(13): 3782-3786.
Pels Rijcken, W. R.*et al.* (1990). "Pyrimidine metabolism and sugar nucleotide synthesis in rat liver." Biochem J 266(3): 777-783.
Pels Rijcken, W. R.*et al.* (1993). "Pyrimidine nucleotide metabolism in rat hepatocytes: evidence for compartmentation of nucleotide pools." Biochem J 293 (Pt 1): 207-213.
Pels Rijcken, W. R.*et al.* (1995). "The effect of increasing nucleotide-sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes." Biochem J 305 (Pt 3): 865-870.
Peng, J.*et al.* (2018). "Chemical Structure and Composition of Major Glycans Covalently Linked to Therapeutic Monoclonal Antibodies by Middle-Down Nuclear Magnetic Resonance." Anal Chem.
Pham, T.*et al.* (2009). "Evidence for a novel human-specific xeno-auto-antibody response against vascular endothelium." Blood 114(25): 5225-5235.
Powell, J. T. and Brew, K. (1976). "Metal ion activation of galactosyltransferase." J Biol Chem 251(12): 3645-3652.
Quast, I.*et al.* (2015). "Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity." J Clin Invest 125(11): 4160-4170.
Rabina, J.*et al.* (2001). "Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography." Glycoconj J 18(10): 799-805.
Raju, T. S. (2003). "Glycosylation variations with expression systems and their impact on biological activity of therapeutic immunoglobulins." Bioprocess Int. 1: 10.
Raju, T. S. (2008). "Terminal sugars of Fc glycans influence antibody effector functions of IgGs." Curr Opin Immunol 20(4): 471-478.

Raju, T. S.*et al.* (2000). "Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics." Glycobiology 10(5): 477-486.

Ramakrishnan, B. and Qasba, P. K. (2014). UDP-Gal: BetaGlcNAc Beta 1,4-Galactosyltransferase, Polypeptide 1 (B4GALT1). Handbook of Glycosyltransferases and Related Genes. Taniguchi, N. *et al.* Tokyo, Springer Japan**:** 51-62.

Raman, R.*et al.* (2005). "Glycomics: an integrated systems approach to structure-function relationships of glycans." Nat Methods 2(11): 817-824.

Rearick, J. I.*et al.* (1981). "Glucose starvation alters lipid-linked oligosaccharide biosynthesis in Chinese hamster ovary cells." J Biol Chem 256(12): 6255-6261.

Regine Eibl, D. E., Ralf Portner, Gerardo Catapano, Peter Czermak (2009). Cell and Tissue Reaction Engineering Heidelberg, Germany, Springer.

Restelli, V. and Butler, M. (2002). The Effect of Cell Culture Parameters on Protein Glycosylation. Cell Engineering: Glycosylation. Al-Rubeai, M. Dordrecht, Springer Netherlands**:** 61-92.

Rijcken, W. (1995). "the effect of increasing nucleotide sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes." Biochem. J.: 865-870.

RMBIO. "IgG Depleted Fetal Bovine Serum (FBS)." from https://www.rmbio.com/igg-fbs-low-igg-low-endotoxin-igg-depleted-fbs.

Rodriguez-Aparicio, L. B.*et al.* (1992). "Purification and characterization of the nuclear cytidine 5'-monophosphate N-acetylneuraminic acid synthetase from rat liver." J Biol Chem 267(13): 9257-9263.

Roepstorff, P. and Fohlman, J. (1984). "Proposal for a common nomenclature for sequence ions in mass spectra of peptides." Biomed Mass Spectrom 11(11): 601.

Rolfs, A. and Hediger, M. A. (1999). "Metal ion transporters in mammals: structure, function and pathological implications." J Physiol 518(Pt 1): 1-12.

Rosenberg, A. (1995). Biology of the sialic acids. New York ; London, Plenum Press.

Royle, L.*et al.* (2006). "Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions." Methods Mol Biol 347: 125-143.

Ryll, T. (2006).Galactosylation of recombinant glycoproteins. Gennentech, INC. . 20070111284.

Ryll, T. and Wagner, R. (1992). "Intracellular ribonucleotide pools as a tool for monitoring the physiological state of in vitro cultivated mammalian cells during production processes." Biotechnol Bioeng 40(8): 934-946.

Salama, A.*et al.* (2017). "Neu5Gc and alpha1-3 GAL Xenoantigen Knockout Does Not Affect Glycemia Homeostasis and Insulin Secretion in Pigs." Diabetes 66(4): 987-993.

Samraj, A. N.*et al.* (2014). "Involvement of a non-human sialic Acid in human cancer." Front Oncol 4: 33.

Sarkar, A. K.*et al.* (1995). "Disaccharide uptake and priming in animal cells: inhibition of sialyl Lewis X by acetylated Gal beta 1-->4GlcNAc beta-O-naphthalenemethanol." Proc Natl Acad Sci U S A 92(8): 3323-3327.

Savage, A. (1997). Glycosylation: a post-translational modification. Mammalian Cell Biotechnology in Protein Production. Hauser, H. r. W., Roland, DE GRUYTER**:** 233-276.

Savinova, I. N.*et al.* (2015). "The efficiency of fatty acids, N-acetyl-D-mannosamine, and N-acetylneuraminic acid for a change in the sialylation profile of recombinant darbepoetin alfa in CHO cell culture." Applied Biochemistry and Microbiology 51(8): 827-833.

Scallon, B. J.*et al.* (2007). "Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality." Mol Immunol 44(7): 1524-1534.

Schauer, R. (1995). Biochemistry and Role of Sialic acids. Biology of the sialic acids. Rosenberg, A., Springer, Boston, MA.

Schauer, R. (2000). "Achievements and challenges of sialic acid research." Glycoconj J 17(7-9): 485-499.

Schauer, R.*et al.* (2009). "Low incidence of N-glycolylneuraminic acid in birds and reptiles and its absence in the platypus." Carbohydr Res 344(12): 1494-1500.

Schenk, B.*et al.* (2001). "The ins(ide) and outs(ide) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum." Glycobiology 11(5): 61R-70R.

Schilling, B. M.*et al.* (2008).Product quality enhancement in mammalian cell culture processes for protein production. Bristol-Myers Squibb Co. 20080070280.

Schlenzka, W.*et al.* (1993). "Catalytic properties of the CMP-N-acetylneuraminic acid hydroxylase from the starfish Asterias rubens: comparison with the mammalian enzyme." Biochim Biophys Acta 1161(2-3): 131-138.

Schroeder, H. W., Jr. and Cavacini, L. (2010). "Structure and function of immunoglobulins." J Allergy Clin Immunol 125(2 Suppl 2): S41-52.
Sefton, B. M. (1977). "Immediate glycosylation of Sindbis virus membrane proteins." Cell 10(4): 659-668.
Sellick, C. A.*et al.* (2009). "Effective quenching processes for physiologically valid metabolite profiling of suspension cultured Mammalian cells." Anal Chem 81(1): 174-183.
Seppala, R.*et al.* (1991). "Sialic acid metabolism in sialuria fibroblasts." J Biol Chem 266(12): 7456-7461.
Serrato, J. A.*et al.* (2007). "Differences in the glycosylation profile of a monoclonal antibody produced by hybridomas cultured in serum-supplemented, serum-free or chemically defined media." Biotechnol Appl Biochem 47(Pt 2): 113-124.
Sharma, A.*et al.* (1996). "Reduction in the level of Gal(alpha1,3)Gal in transgenic mice and pigs by the expression of an alpha(1,2)fucosyltransferase." Proc Natl Acad Sci U S A 93(14): 7190-7195.
Shaw, L. and Schauer, R. (1989). "Detection of CMP-N-acetylneuraminic acid hydroxylase activity in fractionated mouse liver." Biochem J 263(2): 355-363.
Shaw, L.*et al.* (1992). "Mouse liver cytidine-5'-monophosphate-N-acetylneuraminic acid hydroxylase. Catalytic function and regulation." Eur J Biochem 206(1): 269-277.
Shields, R. L.*et al.* (2002). "Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity." J Biol Chem 277(30): 26733-26740.
Sigma-Aldrich (2018). "Ferric and Ferrous Iron in Cell Culture." Retrieved January 2nd, 2018.
Sokoloski JA1, L. C., Handschumacher RE, Nigam A, Sartorelli AC. (1991). "Effects of uridine on the growth and differentiation of HL-60 leukemia cells." Leuk Res. 15(11): 1051-1058.
Sola, R. J. and Griebenow, K. (2010). "Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy." BioDrugs 24(1): 9-21.
Spiro, R. G. (2000). "Glucose residues as key determinants in the biosynthesis and quality control of glycoproteins with N-linked oligosaccharides." The Journal of biological chemistry 275(46): 35657-35660.
Stark, N. J. and Heath, E. C. (1979). "Glucose-dependent glycosylation of secretory glycoprotein in mouse myeloma cells." Arch Biochem Biophys 192(2): 599-609.

Steinke, J. W.*et al.* (2015). "The alpha-gal story: lessons learned from connecting the dots." J Allergy Clin Immunol 135(3): 589-596; quiz 597.
Subramanian, G. (2012). Biopharmaceutical Production Technology. Somerset, GERMANY, John Wiley & Sons, Incorporated.
Sutton, B. J. and Phillips, D. C. (1983). "The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G." Biochem Soc Trans 11 Pt 2: 130-132.
Swiech, K.*et al.* (2012). "Human cells: new platform for recombinant therapeutic protein production." Protein Expr Purif 84(1): 147-153.
Tanemura, M.*et al.* (1998). "Reduction of the major swine xenoantigen, the alpha-galactosyl epitope by transfection of the alpha2,3-sialyltransferase gene." J Biol Chem 273(26): 16421-16425.
Tangvoranuntakul, P.*et al.* (2003). "Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid." Proc Natl Acad Sci U S A 100(21): 12045-12050.
Tayi, V. S. B., Michael. (2015). "Isolation and quantification of N-glycans from immunoglobulin G antibodies for quantitative glycosylation analysis. ." Journal of Biological Methods 2(3): 19.
Taylor, M. E. and Drickamer, K. (2006). Introduction to glycobiology. Oxford, Oxford University.
Turco, S. J. (1980). "Modification of oligosaccharide-lipid synthesis and protein glycosylation in glucose-deprived cells." Arch Biochem Biophys 205(2): 330-339.
Uldry, M.*et al.* (2002). "GLUT2 is a high affinity glucosamine transporter." FEBS Letters 524(1-3): 199-203.
Ullrey, D. B. and Kalckar, H. M. (1979). "Methods for specific characterization of trace amounts of uridine nucleotides in animal cell cultures." Analytical biochemistry 95(1): 245-249.
Umana, P.*et al.* (1999). "Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity." Nat Biotechnol 17(2): 176-180.
UZH (2016). "Nucleotide-activated sugars." Retrieved Feb 2018.
van Bueren, J. J. L.*et al.* (2011). "Anti-galactose-α-1,3-galactose IgE from allergic patients does not bind α-galactosylated glycans on intact therapeutic antibody Fc domains." Nature Biotechnology 29: 574.

Van Venrooij, W. J. W.*et al.* (1972). "Effects of deprival of glucose or individual amino acids on polyribosome distribution and rate of protein synthesis in cultured mammalian cells." Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis 259(1): 127-137.

Varki, A. (2001). "N-glycolylneuraminic acid deficiency in humans." Biochimie 83(7): 615-622.

Varki, A. (2010). "Colloquium paper: uniquely human evolution of sialic acid genetics and biology." Proc Natl Acad Sci U S A 107 Suppl 2: 8939-8946.

Varki A, C. R., Esko J, et al. (1999). Degradation and Turnover of Glycans. Essentials of Glycobiology. Ajit Varki, R. C., Jeffrey Esko, Hudson Freeze, Gerald Hart, Jamey Marth. United States of America, Cold Spring Harbor Laboratory Press.

Varki A, C. R., Esko J, et al. (1999). Monosaccharide Metabolism. Essentials of Glycobiology. Ajit Varki, R. C., Jeffrey Esko, Hudson Freeze, Gerald Hart, Jamey Marth. United States of America, Cold Spring Harbor Laboratory Press.

Varki A, C. R., Esko J, et al. (1999). N-Glycans. Essentials of Glycobiology. , Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Varki A, C. R., Esko J, et al. (2009). Essentials of Glycobiology, Cold Spring Harbor (NY).

Varki, A.*et al.* (2013).Elimination of N-Glycolylneuraminic Acid From Animal Products For Human Use. The Regents of the University of California. 8828652.

Varki, A. and Schauer, R. (2009). Sialic Acids. Essentials of Glycobiology. Varki, A. *et al.* Cold Spring Harbor (NY).

Verheijen, F. W.*et al.* (1999). "A new gene, encoding an anion transporter, is mutated in sialic acid storage diseases." Nat Genet 23(4): 462-465.

Villacres, C.*et al.* (2015). "Low glucose depletes glycan precursors, reduces site occupancy and galactosylation of a monoclonal antibody in CHO cell culture." Biotechnol J 10(7): 1051-1066.

Vriezen, N.*et al.* (1997). "Effects of glutamine supply on growth and metabolism of mammalian cells in chemostat culture." Biotechnol Bioeng 54(3): 272-286.

Wagner, R. (1997). Biological Aspects of Animal Cells. Mammalian cell biotechnology in protein production. Hauser, H. r. and Wagner, R. Berlin, Walter de Gruyter.

Wagner, R. (2011). Metabolic Control of Animal Cell Processes. Mammalian Cell Biotechnology in Protein Production. Munchen, DEU, Walter de Gruyter**:** 193–232.

Walsh, G. (2003). Biopharmaceuticals : Biochemistry and Biotechnology. Chichester, West Sussex, England, Wiley-Blackwell.

Walsh, G. (2014). "Biopharmaceutical benchmarks 2014." Nat Biotechnol 32(10): 992-1000.
Walsh, G. and Jefferis, R. (2006). "Post-translational modifications in the context of therapeutic proteins." Nat Biotechnol 24(10): 1241-1252.
Wang, J. and Pantopoulos, K. (2011). "Regulation of cellular iron metabolism." Biochem J 434(3): 365-381.
Wentz, A. E.*et al.* (2017).Use of metal ions for modulation of protein glycosylation profiles of recombinant proteins. Abbvie Inc. 20170145465.
Werner, R. G.*et al.* (2007). "Glycosylation of therapeutic proteins in different production systems." Acta Paediatrica 96: 17-22.
Whitford, W. (2003). "NS0 Serum-Free Culture and Applications." BioProcess International: 36-47.
Wilkens, C. A.*et al.* (2011). "Comparative metabolic analysis of lactate for CHO cells in glucose and galactose." Biotechnology and Bioprocess Engineering 16(4): 714.
Winterbourn, C. C. (1995). "Toxicity of iron and hydrogen peroxide: the Fenton reaction." Toxicol Lett 82-83: 969-974.
Wong, N. S. C.*et al.* (2010). "An investigation of intracellular glycosylation activities in CHO cells: Effects of nucleotide sugar precursor feeding." Biotechnology and Bioengineering 107(2): 321-336.
Wright, A. and Morrison, S. L. (1997). "Effect of glycosylation on antibody function: implications for genetic engineering." Trends Biotechnol 15(1): 26-32.
Wright, A. and Morrison, S. L. (1998). "Effect of C2-associated carbohydrate structure on Ig effector function: studies with chimeric mouse-human IgG1 antibodies in glycosylation mutants of Chinese hamster ovary cells." J Immunol 160(7): 3393-3402.
Yang, M. and Butler, M. (2002). "Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms." Biotechnol Prog 18(1): 129-138.
Yarema, K. J. (2005). Handbook of Carbohydrate Engineering, CRC Press.
Yin, B. W., Q.; Chung, C. Y.; Bhattacharya, R.; Ren, X.; Tang, J.; Yarema, K. J.; Betenbaugh, M. J. (2017). "A novel sugar analog enhances sialic acid production and biotherapeutic sialylation in CHO cells." Biotechnol Bioeng 114(8): 1899-1902.

Yokoyama, M. T. a. S. (2011). Bioreactors Designed for Animal Cells. Mammalian Cell Biotechnology in Protein Production. Hauser, H. r. a. W. R. Berlin, Boston: De Gruyter**:** 277-317.

Yorke, S. C. (2013). The application of N-acetylmannosamine to the mammalian cell culture production of recombinant human glycoproteins**:** 18-20.

Yu, C. G., Kai ; Zhu, Lei ; Wang, Wenbo ; Wang, Lan ; Zhang, Feng ; Liu, Chunyu ; Li, Meng ; Wormald, Mark R ; Rudd, Pauline M ; Wang, Junzhi (2016). "At least two Fc Neu5Gc residues of monoclonal antibodies are required for binding to anti-Neu5Gc antibody." Scientific reports, 29 January 2016, Vol.7, pp.20029 7(29): 20029.

Zanghi, J. A.*et al.* (1998). "Role of nucleotide sugar pools in the inhibition of NCAM polysialylation by ammonia." Biotechnol Prog 14(6): 834-844.

Zhang, J.*et al.* (2009). "Transient expression and purification of chimeric heavy chain antibodies." Protein Expr Purif 65(1): 77-82.

Zhang, P.*et al.* (2016). "Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs." Drug Discov Today 21(5): 740-765.

Zielke, H. R.*et al.* (1976). "Growth of human diploid fibroblasts in the absence of glucose utilization." Proc Natl Acad Sci U S A 73(11): 4110-4114.

Zielke, H. R.*et al.* (1978). "Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts." J Cell Physiol 95(1): 41-48.