High-Throughput Quantification of Glycoprotein Sialylation

by

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B.S. Chemical Engineering
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Submitted to the Department of Chemical Engineering
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Chemical Engineering
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2011

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Abstract

Sialic acid can improve qualities of therapeutic glycoproteins, such as circulatory half-life, biological activity, and solubility. In production of therapeutic glycoproteins, a high-throughput method (HTM) is required for process monitoring and optimization to ensure consistent and optimal sialic acid content. The HTM is also required for cell clone screening in cell line development. Current methods for quantifying sialic acid, however, require chromatographic separation that is time consuming and cannot rapidly analyze many samples in parallel.

Here we develop a novel HTM for quantifying glycoprotein sialylation. Using chemical reduction, enzymatic release of sialic acid, and chemical derivatization, the HTM can accurately, rapidly (15 min), and specifically analyze many samples in parallel. It requires only 45 μL of sample and has a quantitation limit of 2 μM sialic acid. We validated the HTM for monitoring sialylation of recombinant interferon-gamma (IFN-γ) produced in Chinese Hamster Ovary (CHO) cell culture. The HTM was accurate in monitoring sialylation of IFN-γ in batch CHO cell cultures.

Furthermore, we used the HTM to study the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialylation of glycoproteins produced in CHO-IFN-γ cell cultures. We found that feeding these chemicals increased sialylation from 20 to 36 mg sialic acid/g protein in batch CHO cell cultures. Moreover, a quadratic least square model predicts that the feeding 2 mM ManNAc and 100 μM Cu^{2+} will increase the sialylation to 41 ± 4 mg sialic acid/g protein, close to the experimental value of 35 ± 5 mg sialic acid/g protein. We also used the HTM to study intraclonal variability in glycoprotein sialylation. We found that there was significant variability in sialic acid content and productivity. The sialic acid content varied from 1 to 70 mg sialic acid/g protein, and was negatively correlated with the productivity.

Overall, we have developed a novel HTM and demonstrated its versatility for various applications in bioprocesses. The HTM can measure sialic acid content of hundreds of samples in 15 minutes, while conventional methods require more than one day per sample. Thus, the HTM is an important analytical tool for producing therapeutic proteins with consistent and optimum sialylation.

Thesis Supervisor: Daniel I. C. Wang
Title: Institute Professor

Dedicated to
Mama and Papa

Acknowledgements

In the past five years, many people helped me on challenges that I could not solve by myself. Although I will not be able to acknowledge every single contribution, I am very thankful for all of the favors that I have received.

First and foremost, I would like to thank my thesis advisor Prof. Daniel I.C. Wang. I am really grateful for the freedom, guidance, support, and trust that he gave me in working on my thesis. I learned a lot from him on solving complex problems using quick and simple solutions. His effort in providing collaboration with Bioprocessing Technology Institute (BTI) in Singapore and Instituto de Biologia Experimental Tecnologica (IBET) in Portugal is invaluable. I am also very thankful for his career advising and support in my job search. Working with him was the best part of my experience at MIT.

I am very thankful to my thesis committee Prof. J. Christopher Love, Prof. Kristala L.J. Prather, and Prof. Patrick S. Doyle for their thoughtful comment and feedback throughout my thesis development. I thank Prof. Love for sharing his aspiration in medical research and tips on developing highly collaborative and productive teams. I am also grateful to Prof. Prather for sharing her academic and industrial experience. Our one-hour meeting was very informative and helpful in deciding which path I would like to take.

My learning and working experience in the lab has been enjoyable because of colleagues inside and outside Wang lab. Ricardo Perdigao first taught me all cell culture techniques and HPLC. I thank Dr. David McClain for teaching a lot of cell culture skills and related analytical methods. Arranging our hectic hood schedules was very easy thanks to his flexibility and understanding. Dr. Fritz Simeon, Dr. Yusdy Phan, and Dr. Meiliana Tjandra generously shared their experience with chemical and enzymatic reactions. I appreciate Dr. James Leung for giving a lot of thoughtful feedback on my research and advice on careers in biotech industries. I also thank Dr. Andy Wijaya for allowing me to use a fluorometer in his lab and introducing me to nice Asian restaurants in Boston areas; this information was very helpful especially when my experiments did not work properly. I appreciate Griffith lab members and BPEC for allowing me to use their facilities. The working environment in the lab and office has been very nice because of Andres Abin, Peipei Han, Zhou Kang, Wang Wen, Sohan Patel, Xiao Wenhai, and Zhang Wei. Andres, I will probably miss your unique songs after I leave MIT. I also acknowledge Michelle Lu and Jose Echevarria for working diligently on their UROP projects. I hope that the projects were not boring for you, and you learned something that would be useful for your future. I would also like to thank Singapore MIT Alliance and MIT Portugal Program for providing us with a significant amount of funding for my project.

I also thank my collaborators at BTI and IBET. I sincerely thank Prof. Miranda Yap, Executive Director of BTI, for generously supporting our collaboration, and allowing me to use the facilities at BTI for three months. This experience was very helpful for my thesis, and I learned a lot during that time. All the people in the Analytics and Animal Cell Technology Groups at BTI have been very patient in helping me. Dr. Niki Wong, Dr. Lee May May, Dr. Muriel Bardor, and

Dr. Feng Huatao helped me a lot in many different ways with glycosylation analysis. Hoi Kong Meng did the IFN-γ purification and site occupancy analysis. Ong Boon Tee carried out TAA and a modified HTM on purified IFN-γ. I hope the HTM has made your life easier these days. Gavin Teo and Corrine Wan prepared samples for HPAEC glycoform analysis. Gavin Teo also did HPLC quantification of IFN-γ and HPAEC glycoform analysis. I do appreciate Dr. Say Kong Ng, Dr. Niki Wong, and Lydia Wati for developing and providing me with a CHO cell line. This cell line saved me from a lot of troubles during my thesis. I also thank Dr. Ana Teixeira at IBET for performing batch cultures of CHO-IgG4 cells and sharing her thoughts on how we could collaborate.

I also received a lot of assistance with administrative work from many people at MIT. I first thank Angela Birnbaum, Shawna MacDonald and Martha Adams at MIT EHS for helping with sending samples crucial for my thesis to Singapore. The way you prepared everything ensured me that my very fragile samples safely traveled across the Pacific Ocean and arrived at Singapore before the dry ice completely evaporated. I thank Sara Darcy and Renee LeBlanc for taking care of my packing slips. Sara, thank you for your quick response in approving expensive purchases; my experiments were never delayed due to purchase approval processes. Aaron Parillo and James Hardsog helped me a lot with computer problems. I had a nice experience working with Marc Rioult, Xi Xi Jia at the MIT Technology Licensing Office, and Dr. Sandra Brockman-Lee on a patent application. I also thank Suzanne Maguire, Katie Lewis, Mary Wesolowski, Iris Chang at MIT Chemical Engineering Department, Susan Lanza at MIT Brain and Cognitive Sciences Department, Daniel Darling, and Catherine Greene at BPEC for various administrative assistance.

I also thank all my friends whom I met during my time at MIT. Hsien-Chung Tseng has been a great friend and colleague. I will never forget our panic and clueless time with 10.40 problem sets, as well as interesting trips to 10.28 warehouse. I enjoyed fun and thoughtful discussions during lunches and dinners with Dr. Andy Wijaya, Dr. Fritz Simeon, Dr. Yusdy, Rezy Pradipta, Hendrata Dharmawan, Erfan Gunawan, Dr. Meilana Tjandra, Jessica Tanuwidjaja, Rhea Liem, Hsien-Chung Tseng, Andres Abin, Fei Liang, Tek Hyung Lee, and many others.

I give my special thanks to my sister, Eileen Lam, for her lovely support and cheering me up whenever I needed it. I really enjoyed her frequent visits to Boston and the delicious food that she cooked. I have to admit that her bulgogi tastes better than the bulgogi that I found in Korean restaurants here.

My very special gratitude goes to my Mom and Dad for their unconditional love, support, and wisdom. I am really blessed to have parents who dedicate their lives for the happiness of their children. Many of my parents' friends strongly suggest to my parents that I should go back to and work in my home country instead of doing research in the US. Nevertheless, my parents have always been open minded and given me the opportunity to pursue what is suitable for me. I doubt that I will ever be able to pay back what they have done for me. As my sincere appreciation, I dedicate this thesis to my Mom and Dad.

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Chapter 1

Introduction

1.1. Motivation

Glycoproteins, such as erythropoietins, monoclonal antibodies, and hormones, constitute major classes of biologic drugs in the market (~$48 billion) today [1]. One of the properties that affect quality of therapeutic glycoproteins is N-acetylneuraminic acid (NANA), referred to in the followings as sialic acid content: the number of mol of sialic acid attached to one mol of glycoprotein. Sialic acid is important because it can increase circulatory half-lives of many therapeutic glycoproteins by ~10 to 1,000 fold [2]. It can also increase biological activity, such as the anti-inflammatory activity of immunoglobulin G (IgG) in the treatment of autoimmune diseases [3]. Moreover, it can improve physical properties of glycoproteins, such as solubility [4], resistance to protease attack, and thermal denaturation [5,6].

In production of therapeutic glycoproteins, many biological, chemical, and physical parameters can affect the sialic acid content of recombinant glycoproteins [7,8]. Changes in these parameters can decrease sialylation over time [2] and result in inconsistent sialic acid content of the proteins [8]. Process monitoring and optimization are therefore required to ensure consistent and optimal protein sialylation. In addition, development of cell lines producing the recombinant proteins typically requires screening of hundreds of cell clones. These tasks can be done if a high-throughput method that can rapidly measure sialic acid in many cell culture samples in parallel is available.

In the past few decades, many methods for analyzing sialylation of glycoproteins have been developed. For example, elegant methods using chemical ligation and metabolic labeling have been developed for *in vivo* imaging of sialylated proteins expressed on the surface of a cell or model organism [9-11]. For quantifying percent sialylation of glycans, High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) combined with Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) have been widely used [2,12-14]. For quantifying concentration of sialic acid, many colorimetric, chromatographic, enzymatic, and fluorescence methods have been developed [15]. Among the quantitative methods, Thiobarbituric Acid Assay (TAA) [16], fluorescence method using o-phenylenediamine-2HCl (OPD) [17] or malononitrile [18,19] derivatization, and enzymatic kits (Sigma, QA Bio, and Prozyme) are some of the methods that can be used for quantifying sialic acid content of recombinant proteins produced in cell culture [2,13,14]. These methods, though powerful, require protein purification that typically takes one day to purify one sample. In addition, some of these methods take several hours to quantify the sialic acid and cannot analyze many samples in parallel. Thus, these methods are not suitable for high-throughput analyses, and a new high-throughput method is required.

1.2. Thesis Objectives

This thesis aims to overcome the bottlenecks in high-throughput quantification of sialic acid content of glycoproteins, and perform high-throughput analyses of sialylation of recombinant proteins produced in mammalian cell culture. First, we will develop a novel high-throughput method (HTM) for parallel and rapid (few minutes) quantification of sialic acid content of glycoproteins. We will study the specificity, accuracy, and sensitivity of the HTM. In addition,

the HTM will be optimized to achieve high sensitivity and speed. Second, we will use the HTM to monitor sialic acid content of recombinant proteins produced in mammalian cell culture. Monitoring sialylation is important because sialic acid content usually decreases over time in mammalian cell culture. This study will demonstrate the applicability of HTM for quality control in bioprocesses. Here, Chinese Hamster Ovary (CHO) cell cultures producing recombinant human interferon-gamma (IFN-γ) and immunoglobulin G-4 (IgG4) will be used as model systems. Third, we will use the HTM to study the effects of feeding carbohydrates and metal ions on sialic acid content of proteins produced in IFN-γ-CHO cell culture. This study will provide a proof-of-concept for the use of HTM in bioprocess characterization and optimization, in which the effects of many parameters on product quality are investigated. Fourth, we will use the HTM to measure intraclonal variability in sialic acid content of proteins produced in IFN-γ-CHO cell culture. This clonal screening is required in developing cell lines for producing therapeutic recombinant proteins. Overall, these studies will demonstrate the versatility of the novel HTM in upstream and downstream bioprocesses, as well as its advantages over the existing methods.

1.3. Thesis Organization

This thesis consists of six chapters. Chapter 2 provides a literature review on protein sialylation and various methods for analyzing sialylation. Chapter 3 describes the materials and methods used in this thesis. Chapter 4 demonstrates the specificity, accuracy, and sensitivity of the new HTM developed and optimized in this thesis. Chapter 5 shows various applications of the HTM in bioprocesses, including monitoring sialylation, cell culture optimization, and clonal screening. Chapter 6 provides conclusions and recommendations for future studies.

Chapter 2

Literature Review

2.1. Protein sialylation

2.1.1. Overview of sialylation processes

Glycosylation is a biological process in which short polymers of carbohydrates (glycans) are attached to biomolecules, such as proteins and lipids. In living organisms, glycans can be found on, for example, cell membrane, extracellular matrix, hormone, and antibody. Glycosylation is very important for proper interactions among biomolecules in living organisms, and has significant implications in many biological areas. In particular, sialic acid, typically attached at the outermost part of glycosylated molecules (Fig. 2.1), has significant effects on many biological processes, such as cancer progression [20,21], cell migration [22], and activity of therapeutic recombinant proteins [3,4,6,8].

Glycans can be attached to proteins in many different ways. Some glycans are attached to the Asn residue in the tripeptide sequence Asn-X-Ser/Thr (where X is any amino acid except Pro). These glycans are called N-linked glycans. In O-linked glycosylation, the glycans are attached to Ser or Thr residues. There are also other types of glycoproteins, such as GPI-anchored proteins and proteoglycans. This review focuses on N-linked glycosylation as it is the major type of glycosylation found in therapeutic glycoproteins. Further details on other types of glycosylations can be found in previous reviews [20,21].

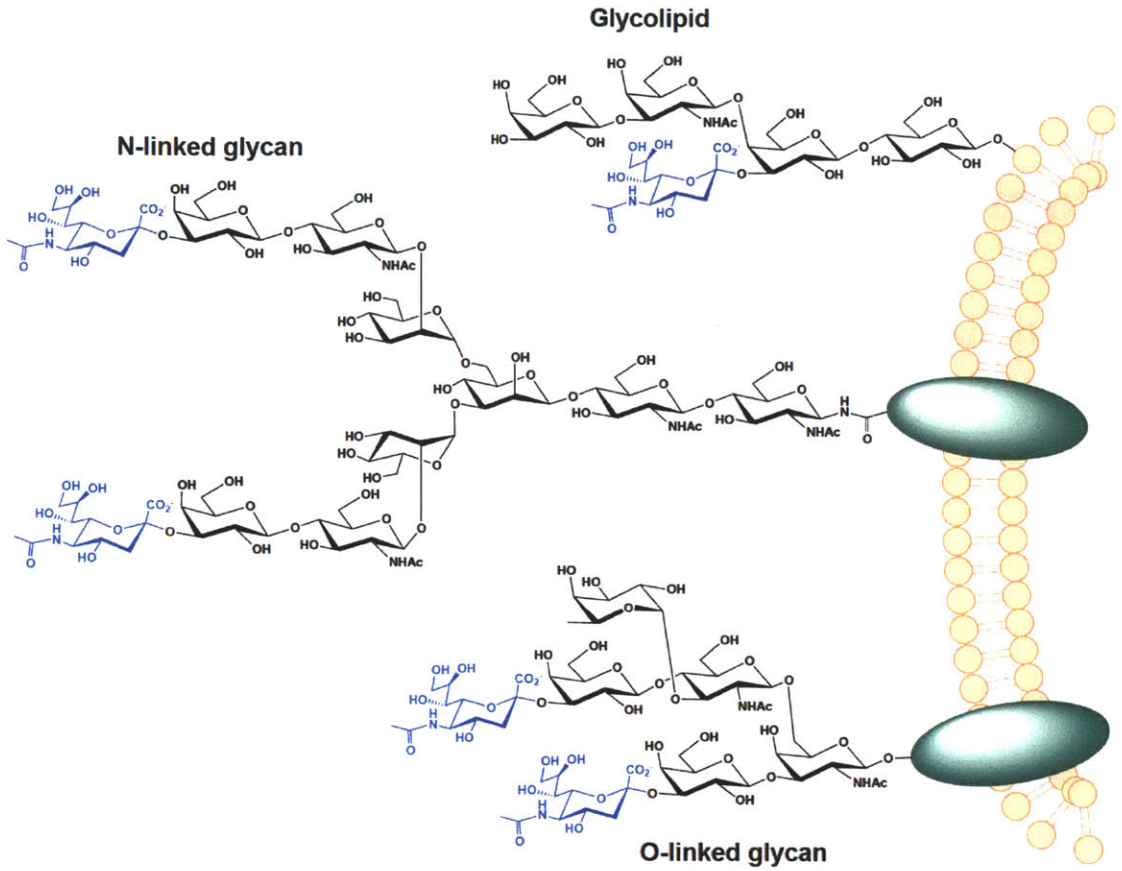


Figure 2.1. Structures of glycosylated proteins and lipids in vertebrates. Glycans can be attached to lipids (orange) or proteins (green). In protein glycosylation, the glycans can be linked to Asparagine residue (N-linked glycosylation) and Serine or Threonine residue (O-linked glycosylation). Sialic acid (blue) is typically attached at the outermost part of the glycans.

N-linked glycosylation involves many steps that take place in the rough endoplasmic reticulum (ER) and golgi (Fig. 2.2) [8,23-26]. The first step is the synthesis of lipid-linked oligosaccharides (LLO). Here, two N-acetylglucosamine (GlcNAc), nine mannose (Man), and three glucose (Gluc) residues are added one at a time to a dolichol phosphate (Dol-P). These carbohydrates are transferred from nucleotide sugars, including UDP-GlcNAc, GDP-Man, and UDP-Gluc, to the LLO.

In the second step, an enzyme called oligosaccharyl transferase (OGT) transfers the oligosaccharides from Dol-P to an asparagine residue on a protein in the ER lumen. This transfer can take place while the protein is being synthesized by the ribosome [27] or after it is fully synthesized [28]. The transfer of the oligosaccharides depends on many factors, including protein tertiary structure [29], availability of cofactors of OGT [30-33], LLO, polypeptide substrate, and OGT [34]. As a result, not all Asn residues in the tripeptide sequence Asn-X-Ser/Thr are occupied by the oligosaccharides. This type of heterogeneity is called macroheterogeneity (Fig. 2.3).

In the following steps, oligosaccharides are then trimmed by glycosidases, and several carbohydrates are added to the trimmed glycans by glycosyltransferases. Specifically, three glucose residues are removed by α-glucosidase I and α-glucosidase II, and six mannose residues by ER α(1,2)mannosidase, cis-golgi α-mannosidase I, and golgi α-mannosidase II. In addition, two GlcNAc residues are added to the trimmed glycans by N-acetylglucosaminyltransferase II, two galactose residues by β(1,4)galactosyltransferase, one fucose residue by α(1,6)fucosyltransferase, and two sialic acid residues by α(2,3)sialylatransferase.

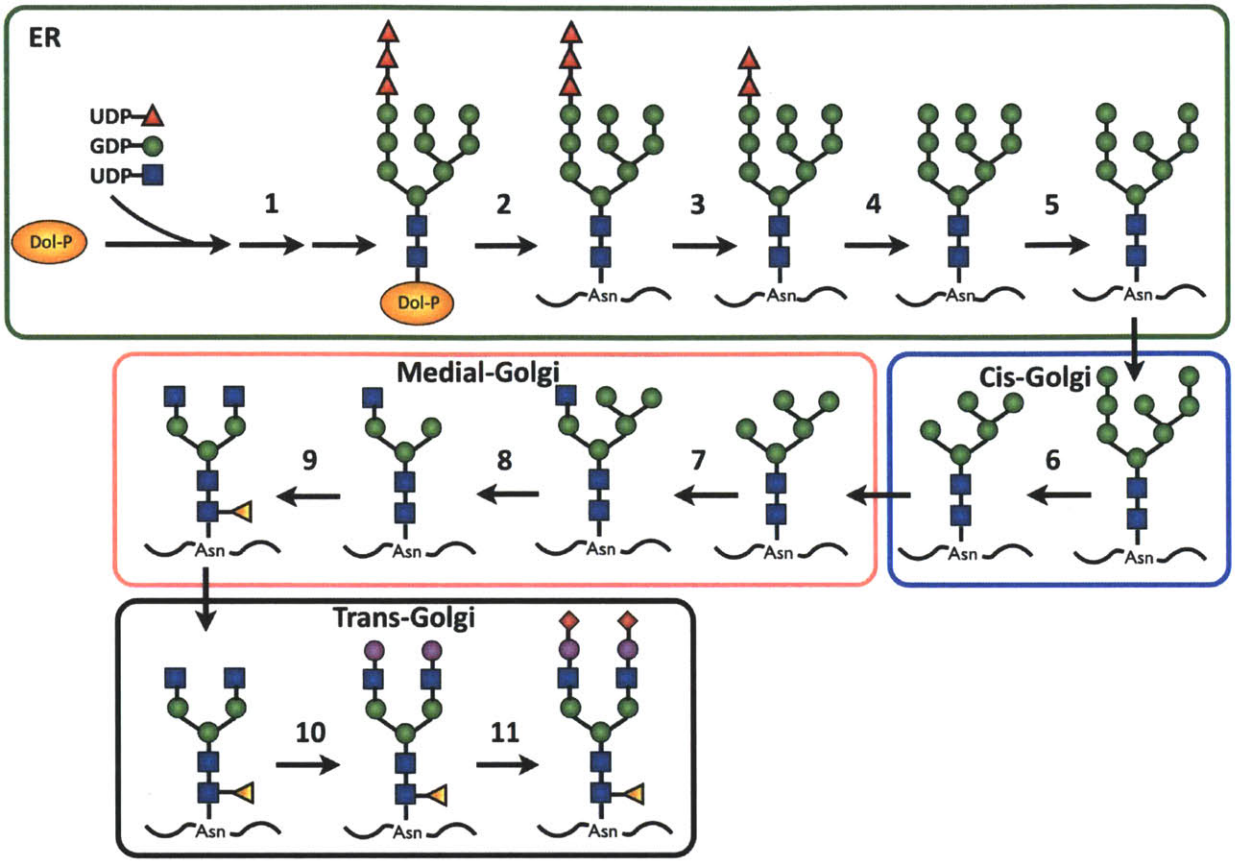


Figure 2.2. Schematic diagram of N-linked glycosylation pathway in endoplasmic reticulum (ER), cis, medial, and trans-golgi. This pathway consists of 4 major steps. First, lipid-linked oligosaccharide (LLO) is assembled within the ER (1). This step involves addition of N-acetylglucosamine (blue square), mannose (green circle), and glucose (red triangle) to dolichyl phosphate (Dol-P). The additions of N-acetylglucosamine and some mannoses take place outside the ER. The intermediate LLO is flipped from the outside to inside of the ER during the synthesis. Second, the LLO is transferred by oligosaccharyltransferase to nascent polypeptide being synthesized by ribosome in the ER (2). Third, the oligosaccharide is trimmed by α-glucosidase I (3), α-glucosidase II (4), ER α(1,2)mannosidase (5), and cis-golgi α-mannosidase I (6). Fourth, the oligosaccharide undergoes further processing in medial and trans-golgi. Here, N-acetylglucosaminyltransferase I & II adds GlcNAc (7,9), mannose is removed by golgi α-mannosidase II (8), α(1,6)fucosyltransferase adds fucose (yellow triangle) (9), β(1,4)galactosyltransferase adds galactose (purple circle) (10), and α(2,3)sialylatransferase adds sialic acid (red diamond) (11). This figure is adapted from previous review [23,26,35].

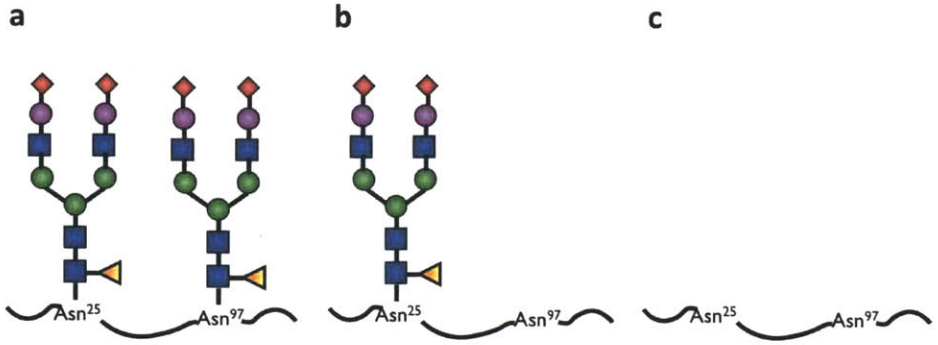


Figure 2.3. Macroheterogeneity of N-linked glycosylation. The site occupancy of recombinant proteins produced in cell culture is not always homogeneous. IFN-γ, used as an example here, has two sites of glycosylation: Asn-25 and Asn-97. Recombinant IFN-γ produced in cell culture may have two sites occupied (a), one site occupied (b), and zero site occupied (c) by the glycans. These figures are adapted from previous review [23].

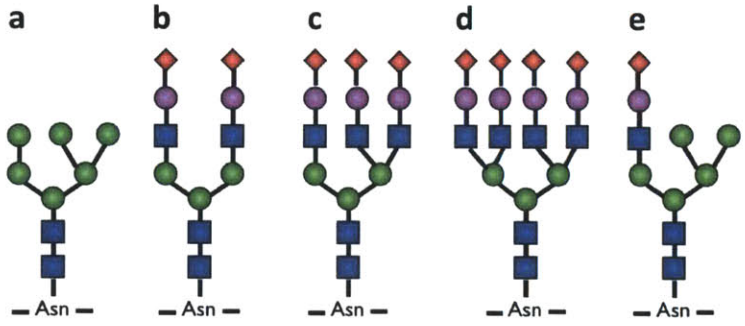


Figure 2.4. Microheterogeneity of N-linked glycosylation. There are many glycan structures found on glycoprotein, and they can be classified into three classes: high mannose (a), complex (b,c,d), and hybrid structures (e). All of them have the same core structures consisting of two GlcNAc (blue square) and three mannoses (green circles). The high mannose structure has additional manoses. The complex structures have additional GlcNAc, galatcose (purple circle), and sialic acid (red diamond). The complex structure with two branches is called biantennary structure (b), that with three branches is triantennary structure (c), and that with four branches is tetraantennary structure (d). The hybrid structure has a combination of both high mannose and complex structures. These figures are adapted from previous review [23].

If each step of the glycosylation process goes to completion, the structure of the glycans will be a complex biantennary structure (Fig. 2.4). It is the major structure found in recombinant therapeutic proteins, especially those produced in mammalian cell cultures [12,13,36]. In reality, some of these steps do not go to completion, and there are other glycosyltransferases and glycosidases that can add and remove carbohydrates from the glycans, respectively. These variations result in more than 7500 possible glycan structures [37-39], and this type of heterogeneity is called microheterogeneity. These glycan structures can be classified into 3 major categories: high-mannose, complex, and hybrid glycan structures (Fig. 2.4). Fortunately, only some of these glycans are expressed in therapeutic proteins, and the typical number of structures detected by current analytical technologies are ~ 50.

The microheterogeneity of the glycans depends not only on the activity of glycosyltransferases and glycosidases, but also on the availability of nucleotide sugars. CMP-sialic acid is a substrate for sialylation, and is synthesized from glucose or mannose (Fig. 2.5). These carbohydrates are converted to sialic acid by a series of enzymatic reactions in the cytosol. Inside the nucleus, the sialic acid is converted to CMP-sialic acid, which is then transported to the trans-golgi for sialylation [40,41]. This pathway is complex and tightly regulated. For instance, an increase in intracellular UDP-GlcNAc concentration is correlated to a decrease in sialylation [42-45]. This effect is likely due to inhibition of the transport of CMP-sialic acid from cytosol to trans-golgi [41]. Similarly, CMP and UMP can inhibit the transport of CMP-sialic acid [44]. Thus, increases in UDP-GlcNAc, UMP, and CMP concentration can cause accumulation of CMP-sialic acid outside the trans-golgi and reduce sialylation of glycoproteins. Furthermore, the CMP-sialic acid can inhibit the conversion of UDP-GlcNAc to ManNAc, a precursor for CMP-sialic

acid [41]. These inhibitions may function to keep the synthesis of CMP-sialic acid under tight control. In addition to inhibiting CMP-sialic acid transporter, UDP-GlcNAc inhibits upstream reactions, including conversion of GlcNAc to GlcNAc-6-P and conversion of fructose-6-P to glucosamine-6-P [41]. This complex interaction implies that feeding certain precursors of CMP-sialic acid may have complex effects on sialylation. The effects of feeding CMP-sialic acid precursors on sialylation are reviewed in Section 2.1.3.

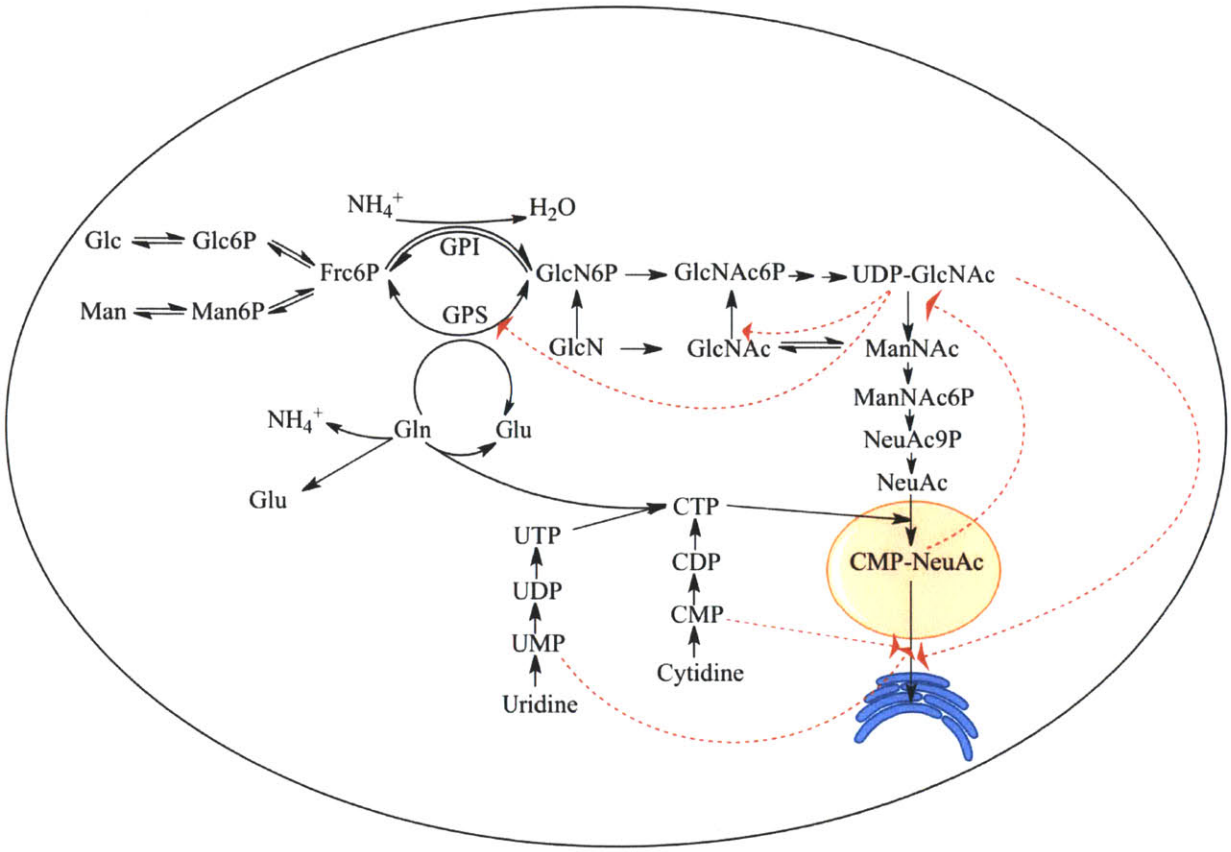


Figure 2.5. Sialylation pathway in mammalian cells. Glucose and mannose are converted by a series of enzymes to sialic acid (NeuAc). In the nucleus (orange), sialic acid is converted to CMP-sialic acid, which is then transported into the trans-golgi (blue). Red dashed arrows indicate inhibition of specific reactions by the nucleotides and nucleotide sugars. Abbreviations: Frc: fructose, GPI: glucosamine-6-phosphate isomerase, GPS: glucosamine-6-phosphate synthase, GlcN: glucosamine, Glc: glucose, GlcNAc: N-Acetylglucosamine, Man: mannose, ManNAc: N-Acetylmannosamine, NeuAc: N-Acetylneuraminic acid (sialic acid). This figure is adapted from previous reviews [40,41].

2.1.2. Implications of sialic acid on therapeutic proteins

Sialic acid affects the properties of various therapeutic glycoproteins in many different ways. One example is recombinant human erythropoietin (rhEPO), which has been successfully used to treat anemia and had 2009 US sales of $6.3 billion [1]. Previous studies showed that sialic acid protected rhEPO from clearance by asialoglycoprotein receptors in the liver. As a result, the circulatory half-life of sialo-rhEPO was 5.6 hours, while that of asialo-rhEPO was 1.4 minutes [46-48]. In addition, the *in vivo* activity of rhEPO also depended on sialic acid; hemoglobin concentration was increased by administration of sialo-rhEPO, but not asialo-rhEPO [46]. Moreover, asialo-rhEPO exerted various neuroprotective activities without increasing the hemoglobin concentration, suggesting that asialo-rhEPO could potentially be used for treatment of various neurological diseases [46]. In addition to effects on biological activity, sialic acid also increased solubility [4], resistance to protease attack and thermal denaturation [6,49]. These evidences show that quantifying sialic acid content is important in defining the therapeutic applications of rhEPO.

Sialic acid also affects the properties of therapeutic recombinant monoclonal antibody (MAb). MAbs have been widely used to treat cancers and autoimmune diseases, and had US sales of $16.9 billion in 2009 [1]. Although the current FDA-approved MAbs are mostly not sialylated [50], a recent study showed that sialic acid affected the inflammatory activity of immunoglobulin G (IgG) [3]. Specifically, they found that sialo-human IgG had greater anti-inflammatory activities than asialo-IgG did in a mouse model of rheumatoid arthritis. Therefore, the sialic acid may improve the efficacy of IgG in treating autoimmune diseases.

Moreover, sialic acid affects the efficacy of interferon-β (IFN-β). IFN-β has been used to treat multiple sclerosis (MS), hepatitis B, and C [51], and had US sales of approximately $3 billion in 2009 [1]. Similar to rhEPO, the circulatory half-life of sialo-IFN-β was longer than that of asialo-IFN-β; the former was 1.4 hours, while the latter was 0.8 hours [52]. Nonetheless, asialo-IFN-β was more effective than sialo-IFN-β in inhibiting the DNA replication of hepatitis B virus (HBV) *in vitro* [52,53], and decreasing the number of serum HBV virion *in vivo* [53]. This may be due to higher uptake of the asialo-IFN-β by asialoglycoprotein receptor in the liver. Therefore, sialic acid affects not only the circulatory half-life, but also the biological activity of IFN-β. The above examples and many others (Table 2.1) show the various effects of sialic acid content on glycoprotein properties. Therefore, monitoring and controlling sialic acid content during the production of therapeutic glycoproteins are very important to ensure that the products meet the required quality.

Table 2.1. Sialic acid increases the circulatory half-lives of glycoproteins.

Glycoprotein	Circulatory half-life		Reference
	Sialylated	Desialylated	
Acetylcholinesterase	12 hours	15.3 minutes	calculated from [54]
α1-antitrypsin	4.6 days	4.1 minutes	[55]
Butyrylcholinesterase	14.1 hours	11 minutes	calculated from [54]
CTLA4Ig	85 hours	0.9 hours	[56]
Erythropoietin	5.6 hours	1.4 minutes	[46]
Follicle Stimulating Hormone	~50 minutes	~ 1 minute	calculated from [57]
Human Chorionic Gonadotropin	25 minutes	< 1 minute	[58]
Human Factor VIII	4 hours	5 minutes	[59]
Human Luteinizing Hormone	1 hour	8.6 minutes	[60]
Interferon-β	1.4 hours	0.8 hours	[52]

2.1.3. Cell culture conditions affecting glycoprotein sialylation

Many biological, chemical, and physical parameters in bioprocesses producing therapeutic glycoproteins can affect their sialylation [7,8]. In addition, the sialylation pathway is very complex and highly regulated, implying that increasing the sialylation is not as simple as feeding sialic acid precursors (Section 2.1.1). Understanding the effects of these parameters on the sialylation is therefore crucial for producing glycoproteins with consistent and optimum sialylation. This section focuses on the effects of feeding cell culture with various chemicals on the sialylation of recombinant proteins produced in mammalian cell culture. This strategy is simpler and faster than biological engineering of cells, such as transfection of glycosyltransferase and sugar nucleotide transporter genes into the cells. Moreover, feeding of certain chemicals, such as ManNAc and galactose, may have more specific effects on sialylation than changing physical parameters, such as temperature, and shear stress, does.

Feeding ManNAc can increase sialylation. ManNAc is a precursor of CMP-sialic acid, and feeding ManNAc increased intracellular CMP-sialic acid concentration and sialylation of IFN-γ [61,62]. The increase in sialylation, however, was not reproducible in CHO and NS0 cell cultures producing human tissue inhibitor of metalloproteinases 1 (TIMP-1) [45], as well as GS-NS0 cell culture producing humanized IgG1 [63] despite increases in intracellular CMP-sialic acid in these cell cultures. This finding implies that there may be other factors, such as transport of CMP-sialic acid to trans-golgi [13] and protein structure, limiting the sialylation.

An increase in intracellular UDP-GlcNAc concentration has been correlated with a decrease in sialylation. This correlation may be because UDP-GlcNAc can inhibit the transport of CMP-

sialic acid to trans-golgi [41,44], thereby limiting the sialylation (Fig. 2.5). The UDP-GlcNAc concentration can be increased by feeding ammonia [36,64], glucosamine [36], and uridine [42,65]. Feeding ammonia has been found to decrease sialylation, and this effect may occur due to several possible mechanisms. First, ammonia can increase intracellular pH of trans-golgi. Second, it can increase intracellular concentration of UDP-GlcNAc. Third, it can also decrease the gene expressions of β1,4-galactosyltransferase and α2,3-sialyltransferase, which can in turn decrease sialylation. Using well-designed experiments, Gawlitzek et al. found that the decrease in sialylation was specifically due to an increase in intracellular pH of trans-golgi [14]. The detrimental effect of ammonia on sialylation may suggest that reducing the accumulation of ammonia in cell culture, such as using glutamine controlled fed batch culture, can increase sialylation. Nevertheless, Wong et al. found that glutamine limitation decreased sialylation of IFN-γ [66]. Since glutamine is involved in many other pathways, such as synthesis of amino acids and α-ketoglutarate used for TCA cycle, limiting glutamine may have non-specific effects that eventually decrease the sialylation.

Similarly, feeding glucosamine has various effects on sialylation. Previous studies found that feeding glucosamine decreased sialylation of human interleukin-2 (IL-2) produced in BHK-21 cell culture [36], EPO in CHO cell culture [43], TIMP-1 in CHO and NS0 cell cultures [45]. These effects may be due to increases in intracellular UDP-GlcNAc, which can inhibit transport of CMP-sialic acid. In CHO cell culture producing tumor necrosis factor-IgG (TNF-IgG) [14] and BHK cell culture producing IL-2 [67], feeding glucosamine did not affect sialylation. Wong et al., however, found that feeding glucosamine increased sialylation of IFN-γ produced in CHO cell culture [62]. These various effects were thought due to differences in recombinant

protein products, cell lines, and cell culture conditions. In addition, glucosamine can inhibit glucose transport into the cells and thus, cause other effects on cell metabolism [43].

Feeding galactose has also been used for increasing sialylation. Galactose is a substrate for galactosylation, a step prior to sialylation. Thus, feeding galactose may indirectly increase sialylation by providing more galactose available for sialylation. This strategy successfully increased sialylation of IFN-γ produced in CHO cell cultures [62]. Nonetheless, in CHO cell cultures producing recombinant IL-4/13 cytokine trap fusion, feeding galactose did not affect the sialylation. This may be due to an increase in the gene expression of sialidase observed in this galactose supplemented cell culture [68].

An alternative strategy to increase sialylation is feeding uridine and cytidine, precursors for the synthesis of CMP-sialic acid (Fig. 2.5). Wong et al. found that feeding uridine and cytidine increased intracellular concentrations of uridine and cytidine nucleotides, respectively, in CHO cell cultures producing IFN-γ [62]. Although CMP may inhibit CMP-sialic acid transporter [41,44], they found that feeding cytidine further increased sialylation of IFN-γ in ManNAc-fed culture. On the other hand, feeding uridine slightly decreased the sialylation in galactose-fed culture, but increased it in glucosamine-fed culture. Based on these results, it will be interesting to study the effects of feedings these compounds in combination because feeding galactose and glucosamine may increase the availability of galactosylated glycans, and feeding ManNAc may increase sialylation of the glycans.

Similarly, manganese, a cofactor for OGT, can also be fed into cell culture to increase sialylation. Feeding manganese can increase the glycosylation site-occupancy of rhEPO [30] and recombinant tissue plasminogen activator (t-PA) [31] produced in CHO cell cultures. As a result, more glycans are available for sialylation, and the glycoproteins have higher sialic acid content. In addition to manganese, iron is a cofactor for OGT and can increase the site-occupancy of recombinant t-PA [31]. Moreover, other metal dications, including calcium, cobalt, copper, magnesium, and zinc, are cofactors for OGT [32,33]. In fact, these ions are also cofactors for galactosyltransferase [69,70], and manganese has been found to increase galactosylation of rhEPO in CHO cell culture [30]. Furthermore, these ions have been found to increase the activity of enzymes involved in the synthesis of LLO [71,72] and CMP-sialic acid [73], both of which are precursors for sialylation of glycoproteins. Therefore, it would be interesting to investigate whether the ions other than manganese and iron can be combined with other strategies, such as feeding ManNAc, to increase siaylation. This problem is studied in this thesis, and the results are presented in Section 5.2.

Recently, glucocorticoides (GC) have also been used to increase sialylation [74]. Dexamethasone (DEX), one type of GC, increased gene expression of β1,4-galactosyltransferase and α2,3-sialyltransferase in CHO cells producing recombinant CTLA4-Ig fusion protein. Consequently, DEX increased sialylation of CTLA4-Ig protein. DEX also reduced cell death and extracellular sialidase activity, thereby reducing the loss of sialic acid from the protein. Moreover, other types of GC, including hydrocortisone (HYC) and prednisolone (PRD), also increased the sialylation. Through studies using GC receptor antagonist, Jing et al. found that the mechanism behind these effects might involve GC receptor [74].

Overall, these studies show that various compounds can be added to mammalian cell culture to modulate the sialylation of recombinant proteins. Identifying the best composition of supplements, therefore, requires screening of hundreds or thousands of cell culture conditions. A high-throughput method for quantifying sialic acid will be a very important tool to perform this screening. In addition to screening, the high-throughput method can provide quantitative knowledge for studying how the cell culture responds to changes in culture conditions and developing robust bioprocesses.

2.2. Methods for analyzing sialylation

2.2.1. Quantitative methods

In the past few decades, many colorimetric, chromatographic, enzymatic, and fluorescence methods have been developed for quantifying sialic acid [15]. Several examples of colorimetric methods include orcinol method [75], resorcinol method [76], thiobarbituric acid assay (TAA) [16], and periodic acid/methyl-3-benzothiazolone-2-hydrazone method [77]. The main disadvantage of these methods is interference by, for instance, hexoses, pentoses, and unsaturated fatty acids [15]. Therefore, cell culture media will have significant interference from these compounds, especially glucose, because it is typically dissolved in cell culture media at ~25 mM, more than 1000 times higher than the typical concentration of sialic acid released from recombinant proteins. In order to minimize the interference, chromatographic protein purification is required. This purification is, however, a bottleneck for high-throughput analysis because it takes almost one day to purify one sample, and it can purify only one sample at a time.

In addition to colorimetric methods, there are several enzymatic methods for measuring sialic acid concentration (Fig. 2.6). All of these methods use N-acetylneuraminic acid aldolase (NANA

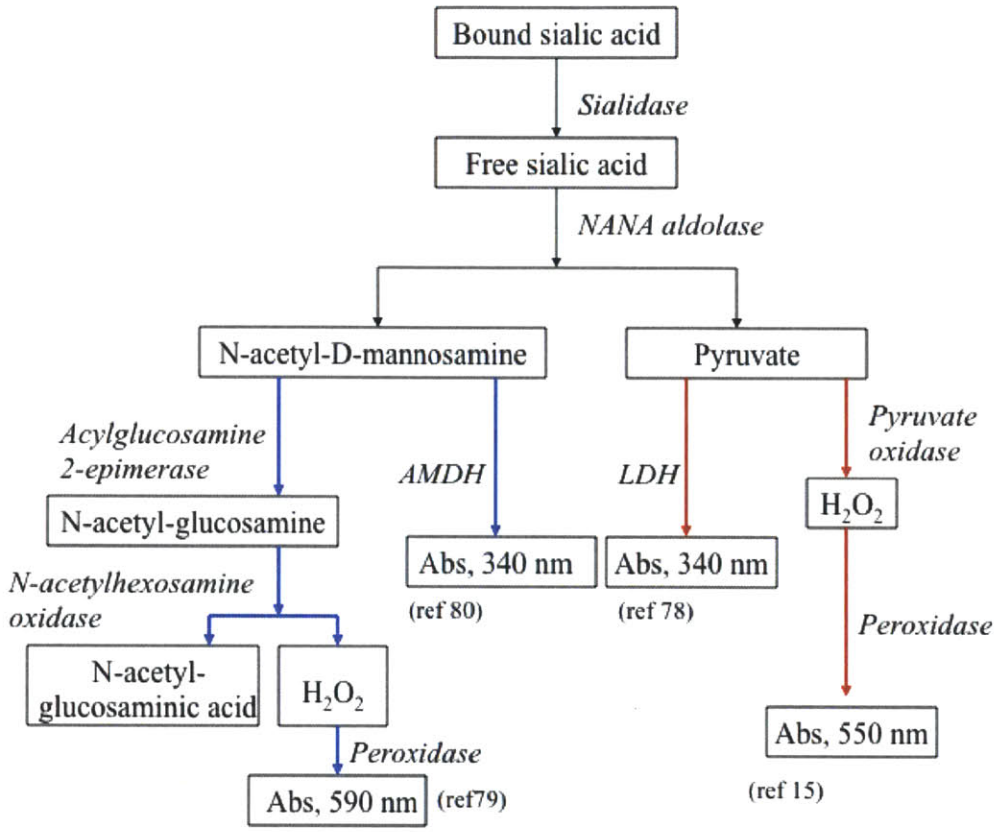


Figure 2.6. Enzymatic methods for measuring sialic acid content of glycoproteins. The blue arrows indicate methods that measure sialic acid based on products derived from N-acetyl-D-mannosamine. The red arrows indicate methods that measure products derived from pyruvate. The enzyme used in each step is shown next to the corresponding arrow. NANA aldolase: N-acetylneuraminicacid aldolase, AMDH: N-acetylmannosamine dehydrogenase, LDH: lactate dehydrogenase. "Abs, λ nm" indicates that the sialic acid is measured based on the absorbance of product mixture at λ nm. The figure was adapted from [78].

aldolase) to convert sialic acid to ManNAc and pyruvate. Some of these methods measure products derived from pyruvate [15,79]. They require protein purification because cell culture medium contains pyruvate at concentration from 1 to 10 mM, approximately 100 to 1000 times the typical concentration of sialic acid released from recombinant proteins produced in cell culture. The other enzymatic methods measure products derived from ManNAc [80,81]. These methods also require protein purification if the cell culture is supplemented with ManNAc. Otherwise, the sialic acid will not be detectable because ManNAc is typically supplemented at 1 to 10 mM. Thus, these enzymatic methods cannot be used for high-throughput analysis.

Several fluorescence methods have also been developed. These methods include derivatization of sialic acid by malononitrile [18,19], o-phenylenediamine-2HCl [17], and pyridoxamine [82] to produce fluorescent compounds. The major disadvantage of these methods is interference by, for instance, lipids, 2-deoxy riboses, ketoacids, and many others [15,18]. In addition, OPD is by itself fluorescent. To minimize the interference, these methods require High-Performance Liquid Chromatography (HPLC) to separate labeled sialic acid from free labels and other labeled molecules. In addition, these methods also require protein purification to prevent clogging of the HPLC column by culture supernatant. As chromatographic protein purification and HPLC can run only one sample at a time, these methods cannot rapidly analyze many samples in parallel.

Overall, the current methods for measuring sialic acid content of glycoproteins produced in cell culture suffer from interference by chemicals in culture medium, and require protein purification and HPLC to minimize the interference. As a result, the methods cannot analyze many samples

in parallel and are very time consuming. These drawbacks motivate us to develop a novel high-throughput method for quantifying sialylation of glycoproteins produced in cell culture.

2.2.2. Imaging methods

There are many chemical tools that have been developed for imaging sialylated molecules in live cell cultures and animals. These methods are reviewed here because some of these methods may be modified in the future to develop high-throughput methods for analyzing sialic acid. There are two major classes of methods that have been recently developed for imaging sialylated molecules (Fig. 2.7). The first class involves feeding unnatural N-Acetylneuraminic acid (NANA, sialic acid) or peracetylated N Acetylmannosamine (Ac4ManNAc), a precursor for biosynthesis of sialic acid, that possesses chemically reactive groups or "chemical reporter" (Fig. 2.7a). This reporter consequently appears on the sialic acid of glycosylated biomolecules and is covalently linked to imaging probes. This so called "metabolic labeling" approach can work only if the chemical reporter is small enough to be tolerated by cellular machineries and inert to biochemical reactions inside the cells.

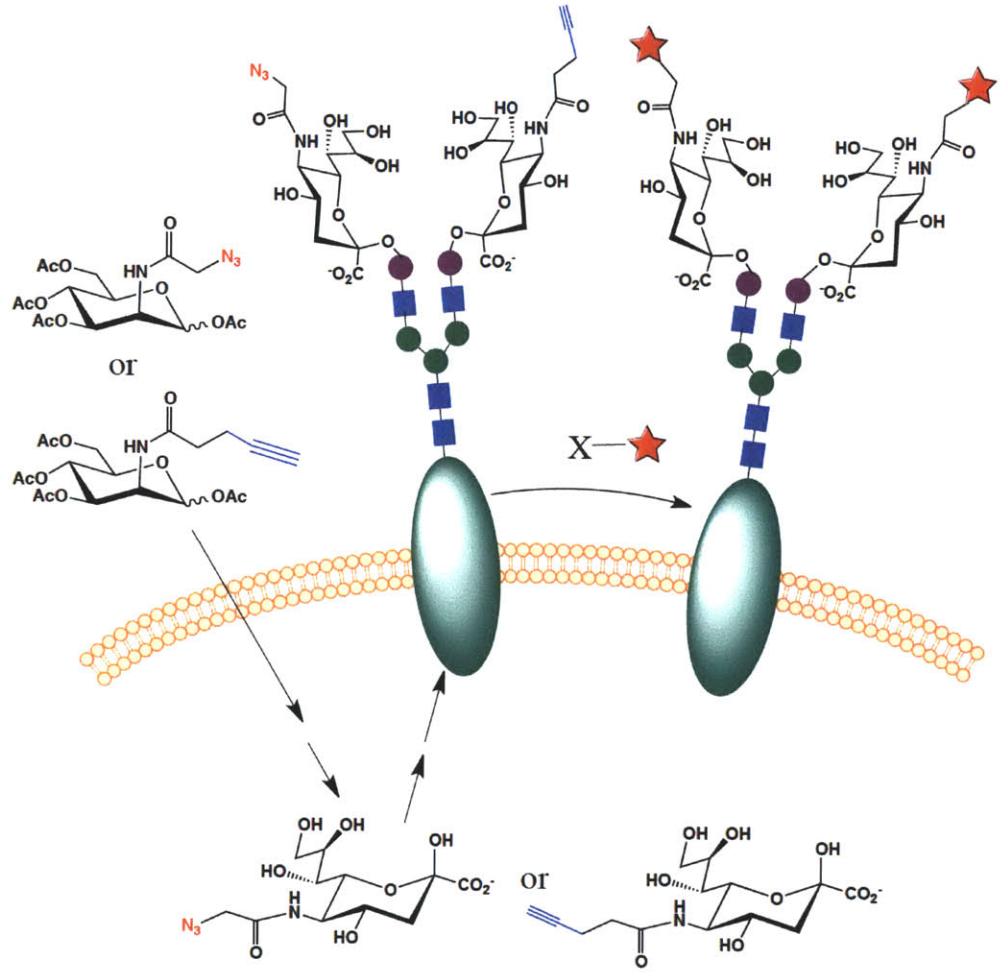
Various reporters and probes have been used for imaging sialylated molecules (Fig. 2.8). Recent methods use azide and alkyne reporters, and covalently link them to probes by Staudinger ligation, Cu-catalyzed azide-alkyne cycloaddition (CuAAC), or strain-promoted azide-alkyne cycloaddition (Cu-free click chemistry). In Staudinger ligation, the azide reacts with phosphine groups of various imaging probes. For example, Hangauer and Bertozzi used a phosphine-tethered fluorescein linked to a fluorescence resonance energy transfer (FRET) quencher [83]. The quencher was released upon reaction between azide and phosphine, thereby minimizing background fluorescence due to fluorescein that did not bind to sialic acid. Similarly,

Cohen et al. used firefly luciferin linked to a phosphine; the luciferin was released upon Staudinger ligation, diffused into cells expressing luciferase, and was converted into oxyluciferin via a luciferase-catalyzed reaction that released light [84].

In CuAAC (Fig. 2.8), the azide or alkyne reporters react with alkyne or azide linked to probes, respectively [85-87]. For instance, Hsu et al. linked alkyne reporter with biotin azide, which was then labeled with fluorescein-conjugated streptavidin [86]. They also showed that the alkyne could be linked to 3-azido-7-hydroxycoumarin, which became fluorescent after the ligation. Chang et al. followed the same biotin labeling approach, but used horseradish peroxidase anti-biotin conjugate (HRP-anti biotin) for Western blot analysis [87].

In Cu-free click chemistry (Fig. 2.8), the azide reporter reacts with cyclooctynes linked to imaging probes. For instance, Chang et al. "clicked" the azide with cyclooctine-FLAG peptide conjugates, which were labeled by HRP anti-FLAG antibody conjugate (HRP-anti-FLAG) for Western blot analysis [88]. The HRP can be replaced with, for instance, fluorescence probes for imaging. In the latest generation of Cu-free click chemistry method, Chang et al. modified their method by feeding cells with Ac_4ManNAz in which the 6-hydroxy group was conjugated to a peptide substrate for the prostate-specific antigen (PSA) protease [89]. This caged metabolic precursor can be taken by the cells and converted to azido-sialic acid only if it is released from the peptide by the protease. This approach renders the labeling specific to cells secreting PSA protease, and can be readily made specific to other types of cells by replacing the peptide substrate with the appropriate biomolecules.

a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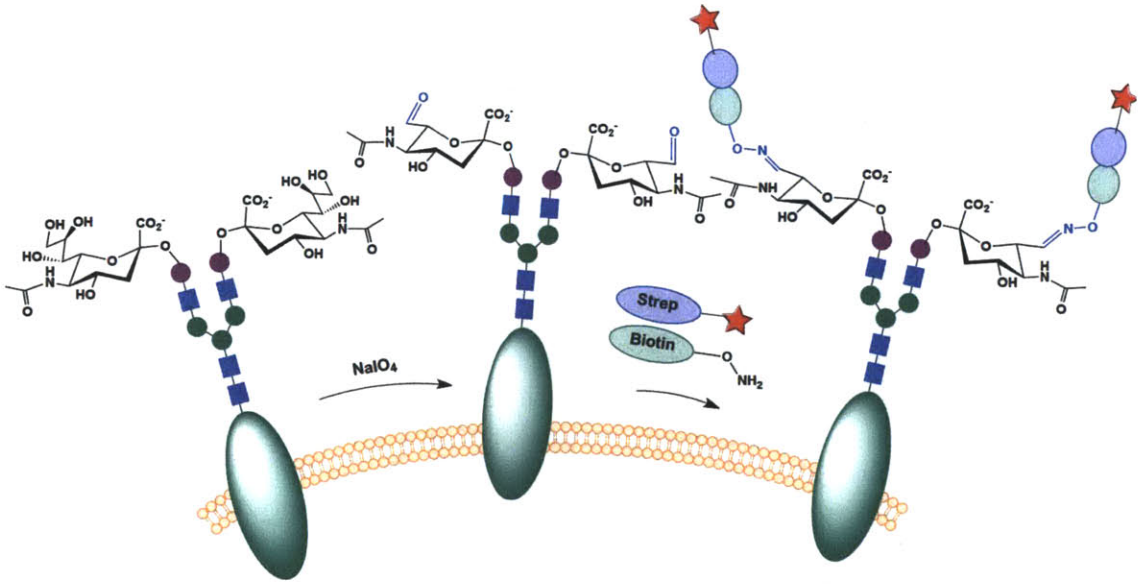
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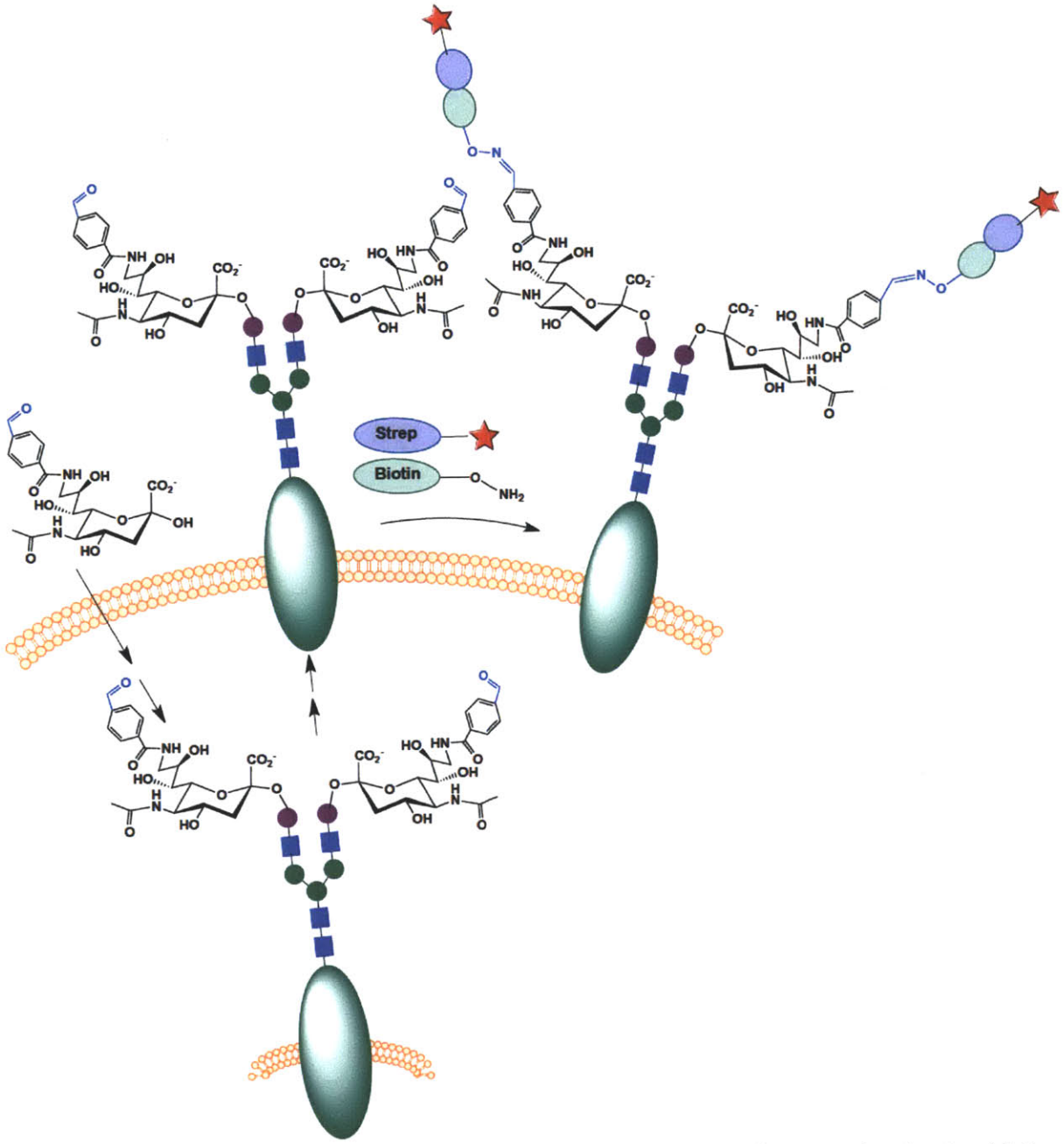


Figure 2.7. Chemical methods for imaging sialylation in live cell cultures and animals. (a) In metabolic labeling, $Ac_4ManNAz$ (red) or $Ac_4ManNAl$ (blue) is fed to cell culture or animal and metabolically converted to azido or alkynyl sialic acid, respectively. The azide and alkyne groups are then presented on cell surface and covalently react with chemical groups (X) of imaging probes (red star) using various reactions presented in Fig. 2.8. (b) BA-NeuAc can also be fed to cell culture or animal and linked to incorporate aldehyde into sialic acid on cell surface. The aldehyde is then linked to imaging probes using aniline catalyzed oxime ligation. (c) Alternatively, sialic acid presented on cell surface can be converted to aldehyde by periodate oxidation and the aldehyde is linked to imaging probes by aniline catalyzed oxime ligation.

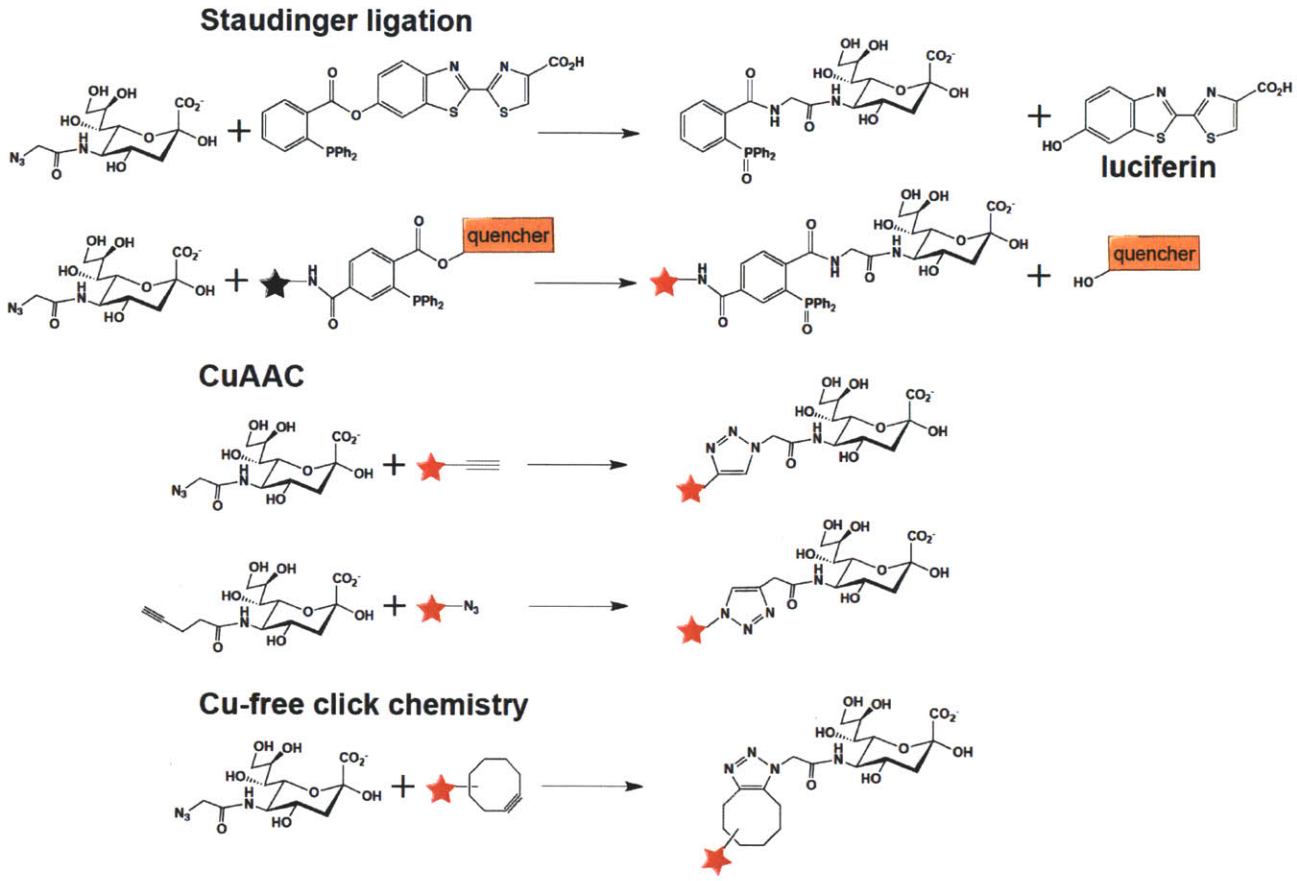


Figure 2.8. Chemical reporters and probes for imaging sialylation in live cell cultures and animals. Black star represents a fluorophore quenched by FRET quencher, and red star represents an unquenched fluorophore.

In addition to azide and alkyne, aldehyde can also be used as a reporter. Zeng et al. fed human B-cell line with an unnatural sialic acid, 9-deoxy-9-N-carboxy-benzaldehyde-neuraminic acid (BA-NeuAc), and linked the BA-NeuAc presented on the cell surface to aminooxybiotin by aniline-catalyzed oxime ligation [10]. The cells were then labeled with dichlorotriazinyl amino fluorescein (DTAF)-streptavidin for imaging (Fig. 2.7b).

The second class of imaging method does not require feeding of unnatural sugars. The only method in this category is a periodate oxidation and aniline-catalyzed oxime ligation (PAL) method developed by Zeng et al. [10]. It uses periodate oxidation to convert the polyhydroxy group of sialic acid to an aldehyde. The aldehyde is then covalently linked to aminooxybiotin using aniline-catalyzed oxime ligation, and the biotin is labeled with DTAF-streptavidin (Fig. 2.7c). Overall, the variety of methods presented here show that there are plenty of options that can be used for imaging sialylated molecules in live cell culture and animals.

The methods described above have been successfully applied for imaging live cell culture and live animals. Some of them offer unique advantages compared with the others. Hangauer and Bertozzi used their FRET-based Staudinger ligation approach to image live HeLa cells [83]. As a result of FRET quenching of unbound probes, they could obtain images with low background. Similarly, Cohen et al. used their bioluminescence probe to image a prostate cancer cell line stably transfected with firefly luciferase [84]. This method has much higher sensitivity than fluorescence-based methods. It requires only ~ 0.01 μM probes, while fluorescence methods typically require 10 μM probes. Nevertheless, the bioluminescence approach requires the presence of luciferase. Thus, this approach can be used only for transfected cells and transgenic

animals. These evidences show the versatility of Staudinger ligation methods for various modes of imaging sialylated molecules. These techniques, however, have several weaknesses. The phosphine reagents are prone to oxidation that can limit their shelf-lives and cause rapid metabolism by liver. In addition, Staudinger ligation is relatively slow and may not capture rapid biological processes in live animals.

The CuAAC method is faster than Staudinger ligation, and has been used for imaging fixed Jurkat cells [86]. This method seems to be more sensitive than the Staudinger ligation methods. The signal to noise ratio obtained by Hsu et al. with 50 μM Ac_4ManNAl and ~0.2 μM fluorescein-streptavidin conjugate was higher than by Hangauer and Bertozzi with 100 μM Ac_4ManNAz and 25 μM FRET-based probe. This higher sensitivity may be partly explained by Chang et al.'s studies [87]. They found that the uptake and conversion of Ac_4ManNAl to alkynyl sialic acid was higher than those of Ac_4ManNAz in six cell lines. The higher incorporation of alkynyl group was also observed in mice. Despite the fast kinetics and higher uptake of Ac_4ManNAl, CuAAC requires Cu catalyst, which is toxic and may have limited applications for imaging live cells and animals.

Cu-free methods have been more widely used than other methods for imaging live cells and organisms. They are as fast as CuAAC and do not require toxic Cu catalyst. Chang et al. used several phosphine tags to image Jurkat cells and mice [88]. They found that difluorinated cyclooctyne (DIFO), which was designed to have high reactivity towards azide reporter, gave highest signal among other cyclooctynes in imaging live Jurkat cell cultures. When used for *in vivo* imaging mice, dimethoxy azacyclooctyne (DIMAC), which was designed to be hydrophilic,

gave the highest signal. DIFO gave slightly lower signal in mice and had significant non-specific binding to mouse serum albumin even in the absence of Ac_4ManNAz feeding. Similar approach using DIFO has also been used to image live zebrafish [9,90] and *Caenorhabditis elegans* [91]. Although these studies use the Cu-free methods to visualize O-linked glycans, the method can be used to image sialylation by feeding Ac_4ManNAz instead of peracetylated N-azidoacetylgalactosamine (Ac_4GalNAz), a precursor for O-linked glycosylation. The latest generation of Cu-free click chemistry method using caged metabolic precursor has also been applied by Chang et al. to label Chinese Hamster Ovary (CHO) cells [89]. They found that the CHO cells could be labeled only if they were incubated with PSA, thereby showing the specificity of the method to PSA secreting cells.

The PAL method has also been successfully used for imaging sialylation in CHO and K20 cells [10]. Recently, Baskin et al. used PAL to image sialylation and combined it with Cu-free click chemistry to image O-linked glycans [90]. With this combination, they were able to image both sialylated glycans and O-linked glycans in zebrafish embryo development. The advantage of PAL is that it does not require feeding of unnatural sugars and therefore, should theoretically be applicable to any cell lines and organisms that have sialic acid.

Overall, the studies presented here provide proof-of-concepts for imaging sialylated biomolecules in live cell cultures and animals. Among the metabolic labeling methods, Cu-free click chemistry seems to be the best imaging method because it has faster rate than Staudinger ligation, does not require Cu catalyst used in CuAAC, and has been validated for imaging various live cell cultures and animals. In addition, PAL method can be used in combination with

Cu-free click chemistry to simultaneously image two types of glycans, such as sialylated glycans and O-linked glycans.

While the focus of this section is on *in vivo* imaging of sialylated molecules, the methods discussed here may be modified for high-throughput analysis of sialic acid. At present, however, they cannot be used for high-throughput quantification of protein sialylation because they need separation of the free labels from the labeled sialylated molecules to avoid the intereference by the free labels. Thus, a new concept for separating the labeled sialic acid from free labels is needed in order to use these imaging methods as high-throughput methods.

2.3. Model system - CHO cells producing recombinant human IFN-γ

In this thesis, CHO cell was used as a model system because it is the major production system for recombinant proteins [92,93], and a lot of studies have been done on the sialylation of recombinant proteins produced in CHO cell cultures (Section 2.2). The CHO cell line used in this thesis produces recombinant human IFN-γ. This protein is a homodimer. Each monomer has 143 amino acids and two glycosylation sites at Asn-25 and Asn-97 [94]. Most of the glycans have biantennary structures, and a few have tri- and tetraantennary structures [23,26,95-97]. As discussed in Section 2.1.3, CHO cells are capable of producing sialylated recombinant proteins. The typical sialic acid content of IFN-γ ranges from 2.6 to 2.8 mol sialic acid/mol IFN-γ [13,26,66]. The sialic acid content of IFN-γ changes over time in batch cultures [2] and can be increased by feeding ManNAc [61]. Therefore, CHO-IFN-γ can be used for our proof-of-concept studies: using HTM for monitoring sialylation of recombinant proteins and studying the effects of culture conditions on sialylation of recombinant proteins. In addition, analytical tools for

protein purification, protein quantification, sialic acid quantification, and glycoform analysis for IFN-γ are well established, and can be used to validate the HTM [2,13,61,62,66].

Chapter 3

Materials and Methods

3.1. Cell Culture

3.1.1. CHO-IFN-γ cell culture

Chinese Hamster Ovary (CHO-DG44) cell line producing a recombinant human interferon gamma (IFN-γ) was generated by Dr. Say Kong Ng (Bioprocessing Technology Institute, BTI, Singapore) through methotrexate (MTX) amplification [98], amplified, and cloned by Dr. Niki S.C. Wong (BTI) and Lydia Wati (BTI). CHO cells were grown in a 1:1 mixture of HyQ-PF CHO (Hyclone) and CD-CHO (Invitrogen) supplemented with 6 mM Glutamine (Invitrogen), 0.1% Pluronic (Invitrogen), and 500 nM MTX (Sigma) at 37°C (shake flask, 115 rpm, 5% CO_2). The cells were split every 2 to 3 days under sterile conditions for maintenance. The seeding concentration was 3 x 10^5 cells/ mL. Trypan blue exclusion method and hemacytometer were used to measure viable cell density and viability. Culture supernatant was obtained by centrifuging cell suspension at 8,000 rpm for 10 min and stored at -20°C for further analyses. In feeding experiment, 80 mM ManNAc, 8 mM $CuCl_2$, and 0.8 mM $MnSO_4$ were added at various volumes when the cells were seeded at 3 x 10^5 cells/ mL. Using these concentrated stocks minimized dilution of cell culture due to feeding the chemicals.

3.1.2. CHO-IgG4 cell culture

Three GS-CHO cell lines producing a recombinant IgG4 monoclonal antibody were kindly provided by Dr. J. Birch and Dr. R. Alldread (Lonza Biologics, UK). The cells were seeded at

4 x 10^5 cells/mL and cultured in CD CHO (Invitrogen) supplemented with 25 μM methionine sulphoximine (Sigma). Batch culture of these cells were carried out in 1L bioreactors (Sartorius) at 37°C, 30% dissolved oxygen, aeration rate of 0.01 vvm, agitation rate of 110 rpm, starting volume of 650 mL, and pH of 7.2.

3.1.3. Single cell culture of CHO-IFN-γ cells and expansion of subclones

A batch culture of CHO cells was serially diluted to ~0.7 cells/2 μL, and 2 μL of cell suspension was deposited onto 96-well plates (Fig. 3.1). 200 μL mixture of 50% CHO culture supernatant and 50% fresh medium (Section 3.1.1) was added into wells that contain single cells, and these single cells were expanded in 96-well plates for 10 days. The supernatant was harvested from batch cultures of CHO cells on the second or third day. It was added in order to enable CHO cell growth at very low viable cell densities (less than 3 x 10^5 cells/ mL). The culture supernatant was filtered with 0.2 μm filter to completely remove the CHO cells from the supernatant. Glutamine was supplemented at 6 mM at the time of seeding. All of these subclones were transferred into 6-well plates and expanded for five days in 3 mL 50:50 fresh medium : culture supernatant. These subclones were then transferred to shake flasks and expanded for three days in 30 mL 50:50 fresh medium : culture supernatant. Afterwards, all of the cells were centrifuged and suspended in 100% fresh medium in shake flasks. The cells were cultured for four to six days until the viable cell density reached approximately 8 to 10 x 10^6 cells/ mL, and the culture supernatant was collected for further analysis.

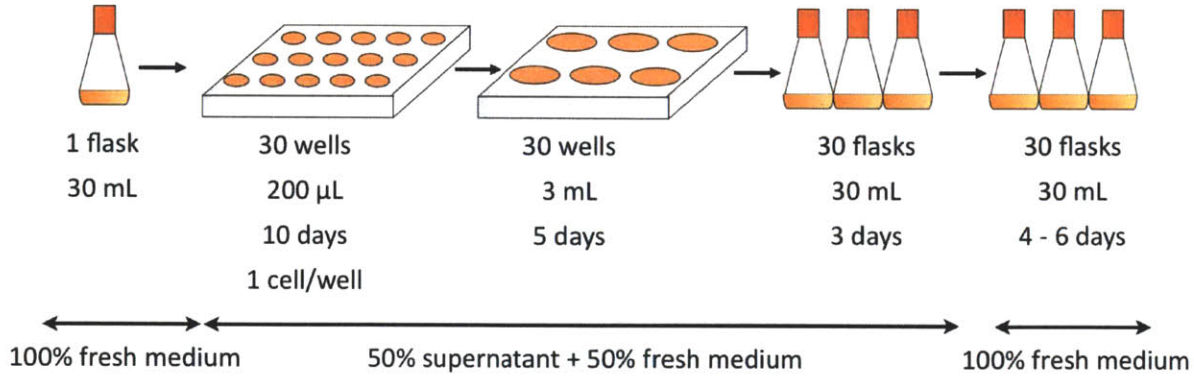


Figure 3.1. Schematic diagram of single cell culture and expansion of subclones. A batch culture of CHO cells was serially diluted and deposited onto 96-well plates. These subclones were further expanded in 6-well plates and shake flasks to obtain sufficient amount of proteins for sialic acid quantification by HTM. The volumes of the cell culture, cell density, duration of the cell culture, and media used in the cell culture are indicated in the figure.

3.1.4. Maintenance of CHO-IFN-γ cell bank

CHO cell culture was centrifuged at 1,000 rpm for 5 min, and the cell pellet was suspended at 10^7 cells/mL in a fresh culture medium (Section 3.1.1) supplemented with 7.5% dimethyl sulfoxide (DMSO). 1.5 mL of the cell suspension was then aliquoted into cryopreservation vials (Nalgene). The vials were then stored in a Cryo 1°C Freezing Container, "Mr. Frosty" (Nalgene) at -80°C. The vials were then transferred into a liquid nitrogen tank on the following day.

3.2. IFN-γ and protein analytical methods

3.2.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA kit for human IFN-γ (Invitrogen) was used as described in the manufacturer's protocol in quantifying the concentration of IFN-γ in CHO cell culture supernatant. Each sample was serially diluted using fresh culture media and sample diluent buffer provided in the kit. Each sample was measured in duplicate, and the optical density was measured using a SpectraMax M2 plate reader with SoftMax Pro 5.2 software (Molecular Devices).

3.2.2. Coomassie plus assay (CPA)

CPA reagent and Bovine Serum Albumin standard (Thermo Scientific) were used as described in the manufacturer's protocol in quantifying total proteins in CHO culture supernatant. Each sample was measured in duplicate, and the optical density was measured using SpectraMax M2 and M3 plate readers (Molecular Devices).

3.2.3. Purification and HPLC quantification of IFN-γ

The purification of IFN-γ was carried out on AKTA Explorer 100 chromatographic system (Amersham Biosciences) as described in detail previously [66]. Briefly, CHO culture supernatants were loaded with a loading buffer (20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2; Merck, Darmstadt, Germany) into an immunoaffinity column made from purified mouse antihuman IFN-γ clone B27 (BD Pharmigen, San Diego, CA). The captured IFN-γ was eluted with an elution buffer (10 mM HCl, 150 mM NaCl, pH 2.5; Merck). The purified IFN-γ was quantified using a Shimadzu LC-10ADvp HPLC (Shimadzu Corporation, Kyoto, Japan) and IFN-γ standards (Fitzgerald Industries International Inc., Concord, MA) as described previously [13]. The samples and standards were injected into a reverse phase Vydac C18 column (Grace Vydac, Hesperia, CA). Elution was carried out using a gradient of buffer A (0.1% (v/v) trifluoroacetic acid (TFA) in HPLC grade water) and buffer B (0.1% (v/v) TFA in HPLC grade acetonitrile).

3.3. Sialic acid analysis

3.3.1. High-throughput method (HTM)

The HTM consists of three steps. First, 30 μL of sample was mixed with 30 μL of $Bu_4N(BH_4)$/tetrahydrofuran (THF) (0.1 M) in a PCR plate (Bio-rad) at room temperature (5 min). Second, 30 μL mixture of acetate buffer (0.1 M, pH 5.0) and hydrochloric acid (HCl) (1.21 M) (Mallinckrodt) was added to set the pH to ~5.2, and sialidase (0.0125 U, from *Arthrobacter ureafaciens*, Roche) was added to release sialic acid at 37°C (5 min). Third, 90 μL of borate buffer (0.15 M) and 12 μL malononitrile (8 g/L) were added to derivatize sialic acid at 80°C (5 min). The derivatization was stopped by incubation in ice bath. These three steps were

run in duplicates to obtain ~400 μL mixture, 300 μL of which was transferred to a 96-well-plate for fluorescence measurement using the SpectraMax M2 and M3 plate readers at excitation wavelength of 357 nm. Unless stated otherwise, the chemicals were bought from Sigma.

3.3.2. Thiobarbituric acid assay (TAA)

Total sialic acid was measured using a modified version of TAA [99] as described in detail previously [66]. Briefly, purified IFN-γ sample was desialylated by sialidase (Roche) in 50 mM acetate buffer, pH 5.2 (Sigma) at 37°C for 24 h. 25 mM periodic acid (Sigma) in 0.125 N H_2SO_4 (J.T. Baker) was then added, and the mixture was incubated at 37°C for 30 min. Arsenite solution (Fluka) was added to remove excess periodic acid. In the next step, thiobarbituric acid (Sigma) was added and the mixture was incubated at 98°C for 7.5 min. The samples were then cooled for a few minutes, and mixed with a mixture of n-butanol and HCl (Merck) to extract the fluorophore. The clear organic phase was transferred to a cuvette or a 96-well plate, and the fluorescence intensity was measured by Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA) (λ_{ex} = 550 nm, λ_{em} = 570 nm) or the SpectraMax M2 plate reader (λ_{ex} = 530 nm, λ_{em} = 570 nm).

3.3.3. Enzymatic kits

Three enzymatic kits for quantifying sialic acid, including SIALIC-Q (Sigma), SialiQuant (QA bio), and GlykoScreen (Prozyme) were used according to the manufacturers' protocols to quantify concentration of sialic acid standards. Fluorescence and optical density measurement in these assays were carried using the SpectraMax M2 plate reader. The quantitation limits of these kits were obtained from the standard calibration curves.

3.4. Statistical analysis and design of experiment (DOE)

3.4.1. Statistics software

DOE and statistical analysis of the feeding experiment data were carried out using JMP® 8.0.2 software from SAS Institute (Cary, NC). The theoretical analysis and background behind the DOE and statistical analysis are covered in detail by Mason et al. [100].

3.4.2. t-test

Differences between sialic acid contents obtained from HTM, TAA, and Sigma product specification were assessed by unpaired two-tailed Student's t-test, and $P < 0.05$ was considered statistically significant. The assumption that the population distribution of sialic acid content data was at least approximately normal was supported by the linearity of the normal probability plots of the data.

Chapter 4

Achievements of a Novel HTM for Quantification of Glycoprotein Sialylation

Sialic acid is important for the quality of therapeutic proteins; it can increase serum half-life [2], biological activity [3], solubility [4], resistance to thermal denaturation, and protease attack [5,6]. Current methods for measuring sialic acid are time consuming because they require protein purification to remove interference by chemicals, such as glucose and pyruvate, in cell culture media (Section 2.2.1). In order to quickly (few minutes) analyze many samples in parallel, a new HTM is required. In this chapter, we describe the development of a novel HTM for analyzing sialic acid content of glycoproteins produced in cell culture. Section 4.1 describes how the HTM works, and why it does not require protein purification to remove the interference by cell culture media. Section 4.2 demonstrates the achievements of HTM: its specificity, accuracy, and sensitivity. The key results are summarized in Section 4.3. The development of HTM was published in *Analytical Biochemistry* [101].

4.1. Novel Concept for HTM

The HTM can rapidly (15 min) analyze many samples in parallel without using any chromatographic separation or purification. It consists of 3 steps (Fig. 4.1a): chemical reduction of the cell culture supernatant by tetrabutylammonium borohydride, $Bu_4N(BH_4)$, enzymatic cleavage of sialic acid from the glycoproteins using sialidase (EC 3.2.1.18), and chemical derivatization of sialic acid by malononitrile to produce a fluorescent molecule (Fig. 4.1b). The fluorescence intensity is proportional to the concentration of sialic acid released from the

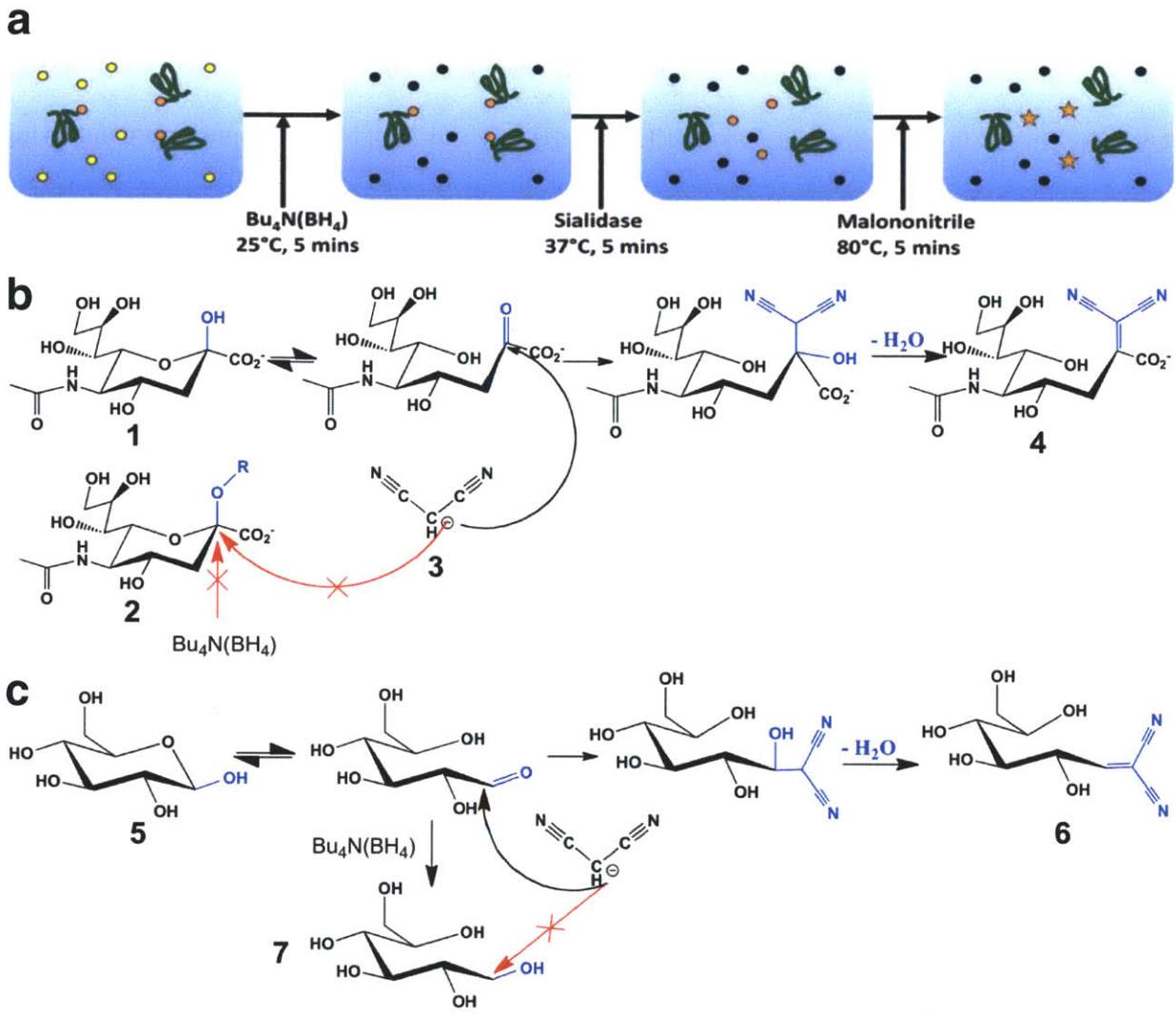


Figure 4.1. High-throughput method (HTM) is designed to specifically measure glycoprotein sialylation. **(a)** First, the molecules in cell culture supernatant that can interfere with the sialic acid measurement (○) are reduced by $Bu_4N(BH_4)$. Second, the sialic acid (●) bound to glycoproteins ( ) is released by sialidase. Third, the released sialic acid is derivatized by malononitrile to produce a fluorescent molecule (★). The reduced molecules (●) cannot react with malononitrile to become fluorescent molecules. The fluorescent intensity is used to estimate the concentration of sialic acid released from glycoprotein. This value is then divided by glycoprotein concentration to obtain the sialic acid content. **(b)** Proposed mechanism of HTM based on derivatization of glucose with malononitrile [102]. Nucleophilic attack by deprotonated malononitrile **3** on the ketone group of free sialic acid **1** produces a fluorescent molecule **4**. The ketone group of sialic acid bound to glycoprotein **2** is protected by a glycosidic bond and does not go through reduction by $Bu_4N(BH_4)$ or reaction with malononitrile. **(c)** Glucose **5** can also react with malonitrile to become a fluorescent molecule **6**, but reduced glucose **7** cannot react with malononitrile.

glycoproteins. To obtain the sialic acid content, this value is then divided by the concentration of the glycoprotein measured using the appropriate methods, such as Coomassie Plus Assay, Enzyme Linked ImmunoSorbent Assay (ELISA), HPLC, and a high-throughput microengraving method [103].

In the first step, $Bu_4N(BH_4)$ reduces molecules that can interfere with sialic acid measurement, such as ketones, aldehydes, or other electrophilic groups [18] (Fig. 4.2). In the CHO culture medium used in our study, this background fluorescence is most likely due to glucose (Fig. 4.1c); it is typically present in mammalian cell culture medium at ~5 to 25 mM, ~1000x higher than the typical concentration of sialic acid released from the glycoproteins produced in a batch CHO cell culture. This hypothesis is supported by the decrease in background fluorescence of CHO culture supernatant over time in a batch culture (Fig. 4.2). During the reduction, sialic acid bound to the glycoprotein is not reduced by $Bu_4N(BH_4)$ because its ketone group is protected by a glycosidic bond (Fig. 4.1b). On the other hand, free sialic acid in culture medium, such as that released by intracellular sialidase [2,104], is reduced. This $Bu_4N(BH_4)$ reduction eliminates the need for chromatographic purification of glycoproteins. We have also investigated if other reducing agents, including LAH/THF, $NaBH_4$/EtOH, $(C_2H_5)_4N(BH_4)$/THF, $(CH_3)_4N(BH_4)$/THF, $LiBH_4$/THF, can be used for the first step. We found that these reducing agents did not completely dissolve in their respective solvents at 0.1 M and a lot of white powders were observed after addition to culture samples. Although these reducing agents may be able to reduce the background fluorescence, we did not use them because the presence of white powders may disrupt the method. In contrast, $Bu_4N(BH_4)$ dissolved instantly in THF and no white powder was observed in all the three steps.

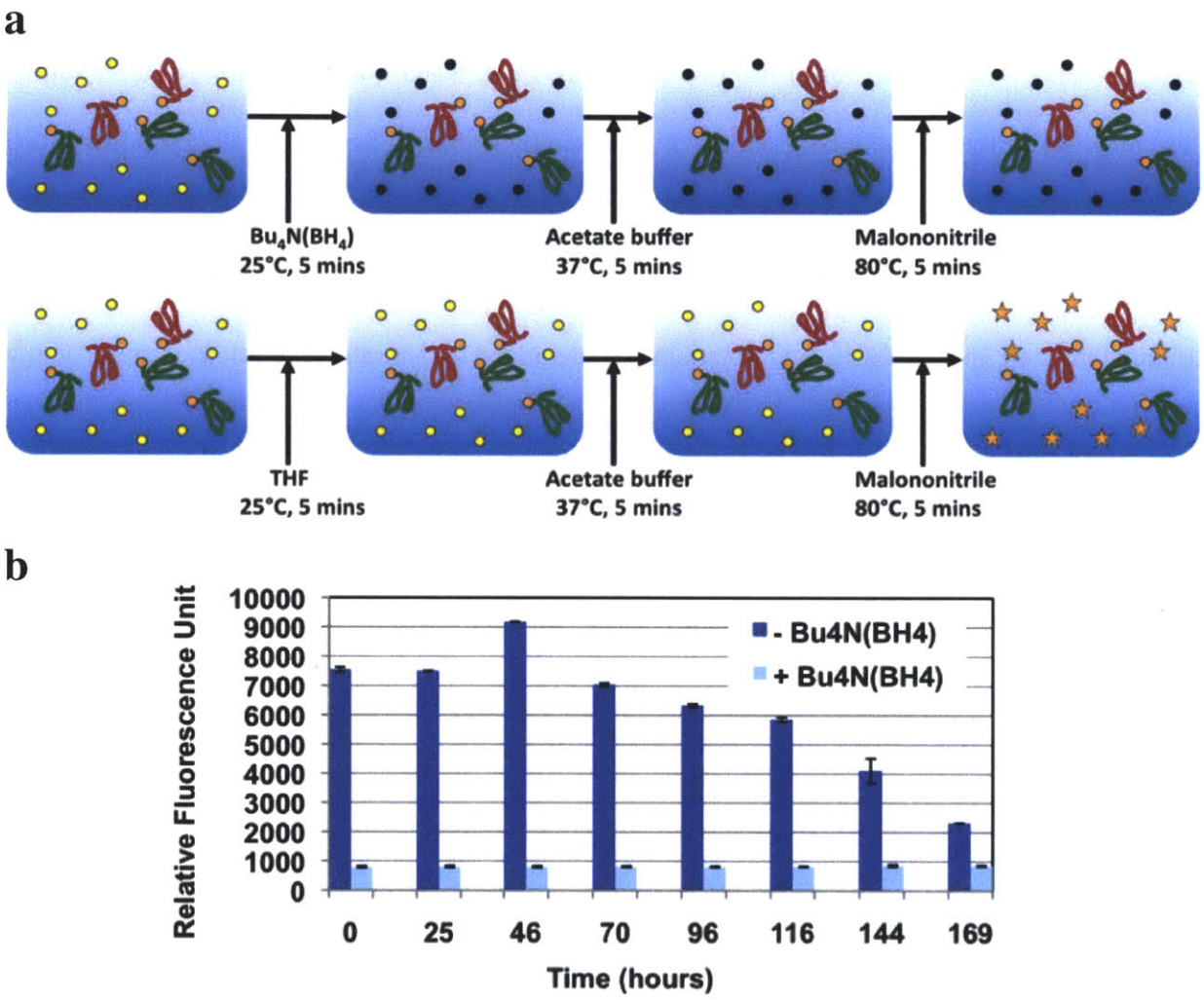


Figure 4.2. Background fluorescence of CHO culture supernatant decreased over time. (**a**) A schematic diagram of the experiment. $Bu_4N(BH_4)$ was replaced with THF to measure the background fluorescence without reduction. Sialidase was not added in the 2nd step so that the sialic acid ( ) was not released from IFN-γ ( ) and other glycoproteins ( ). Malononitrile was added to convert the interfering compounds ( ) to fluorescent molecules ( ). (**b**) Fluorescence intensity of CHO culture supernatants collected during a batch culture measured by the modified HTM. Significant fluorescence was detected when the culture supernatant was not reduced by $Bu_4N(BH_4)$, but the fluorescence intensity was low when it was reduced.

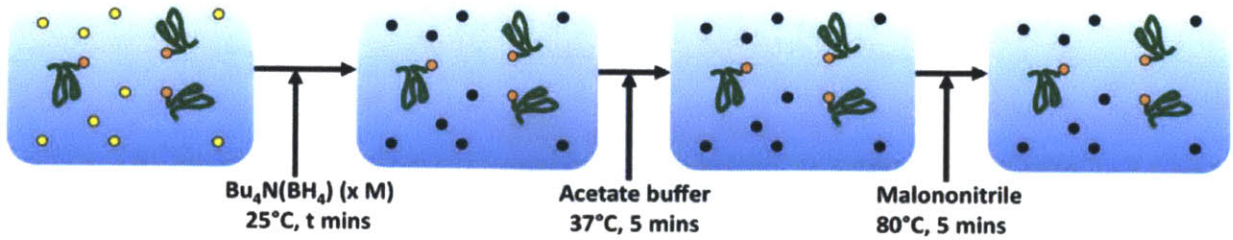
In the second step, sialidase and acetate buffer are added to release sialic acid bound to glycoproteins. The acetate buffer has two functions. First, water quenches $Bu_4N(BH_4)$ reduction and prevents further reduction of sialic acid released by sialidase. Second, it sets the pH to ~ 5.5, the optimum pH for sialidase [104].

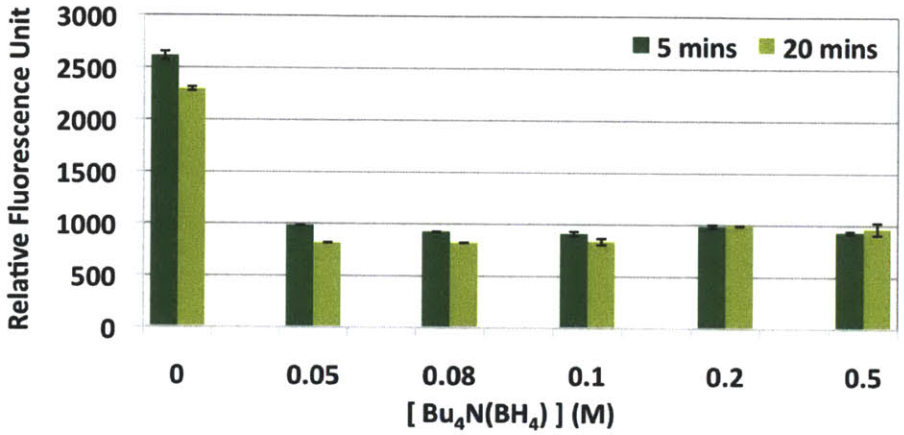
In the third step, malononitrile and borate buffer are added to derivatize the released sialic acid to a fluorescent molecule [18,19]. The borate buffer increases the pH to ~9.4 and has two functions. First, it stops the second step as sialidase is inactive at this pH [104]. Second, it facilitates the derivatization reaction. In basic conditions, the malononitrile is deprotonated and attacks the ketone group of sialic acid (Fig. 4.1b). Since the derivatized sialic acid is the only fluorescent molecule in this reaction, the fluorescence intensity of the sample can be used to measure the concentration of sialic acid bound to glycoproteins using the Beer-Lambert law. The sialic acid concentration is then divided by the glycoprotein concentration in order to calculate the sialic acid content. Therefore, the HTM does not require any chromatographic purification or separation, and can analyze many samples in parallel. The reaction time and reagent concentration used in the HTM were obtained from an optimization of the method (Fig. 4.3).

4.2. Specificity, accuracy, and sensitivity of HTM

We found that the HTM was specific to the sialic acid bound to glycoproteins (Figs. 4.4a,b). As a positive control (Sample 1), 150 μM sialic acid standard was added to the CHO culture medium after $Bu_4N(BH_4)$ reduction. In a negative control (Sample 2), the sialic acid was replaced with water. As a model sample (Sample 3), fetuin standard was dissolved in the CHO-IFN-γ culture medium (Section 3.1.1) at 1.5 g/L. Sialidase was not added in Sample 4 to confirm the specificity

a





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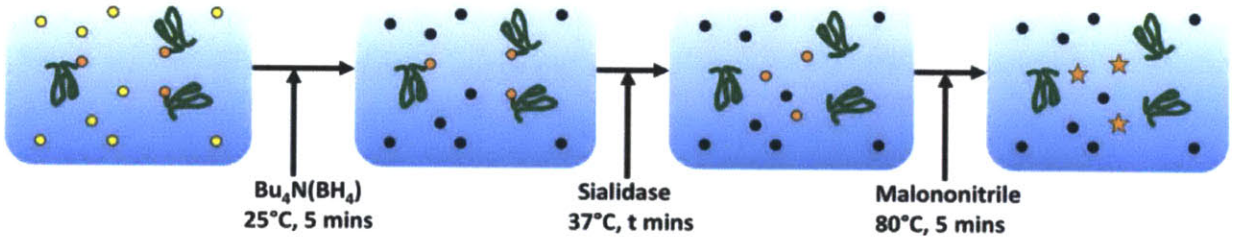


Fetuin (g/L)	Sialic acid content (mol sialic acid/mol fetuin)		t-test
	5 mins incubation	80 mins incubation	*P*
1.5	7.8 ± 0.4	7.6 ± 0.4	0.08

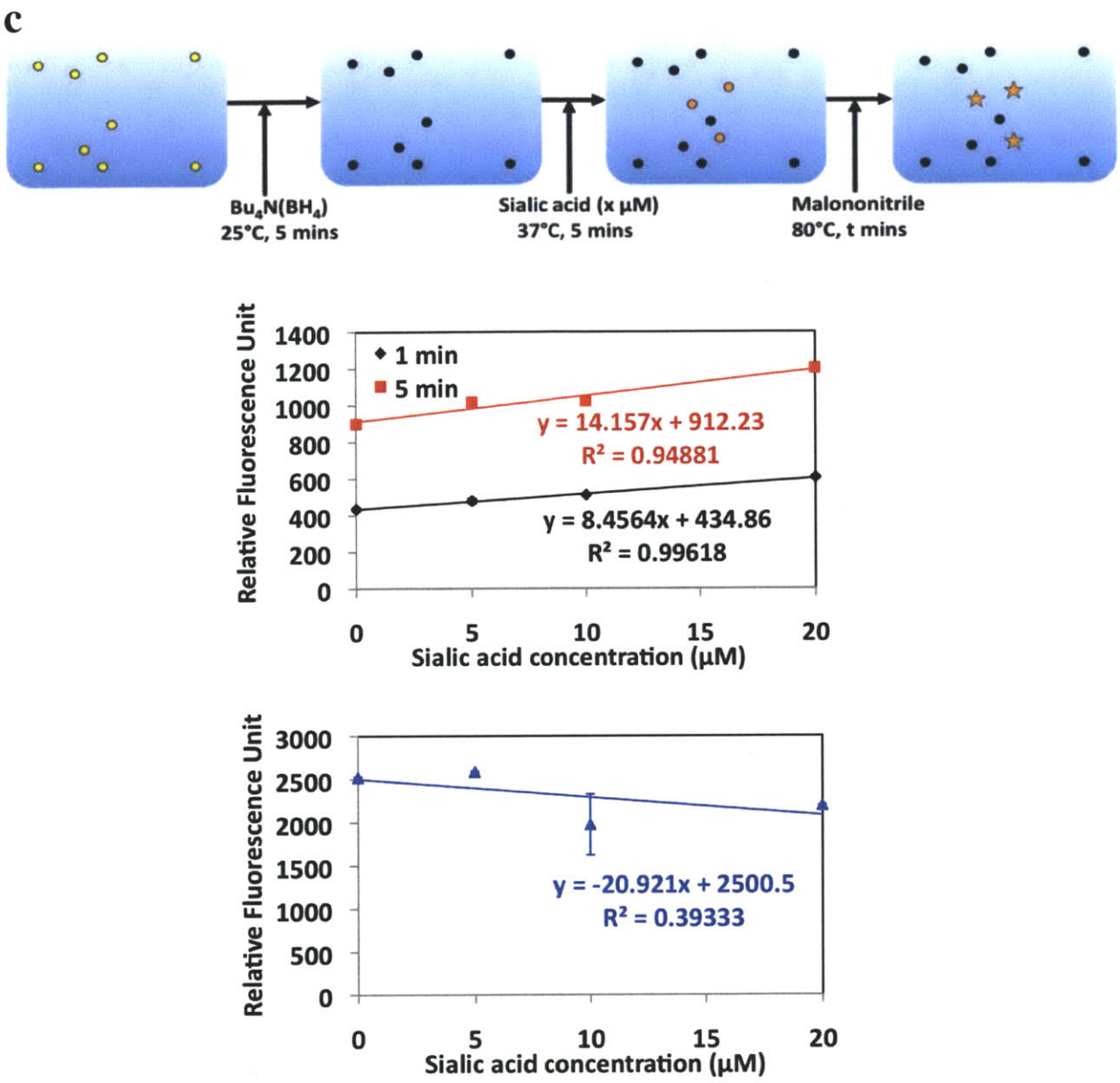
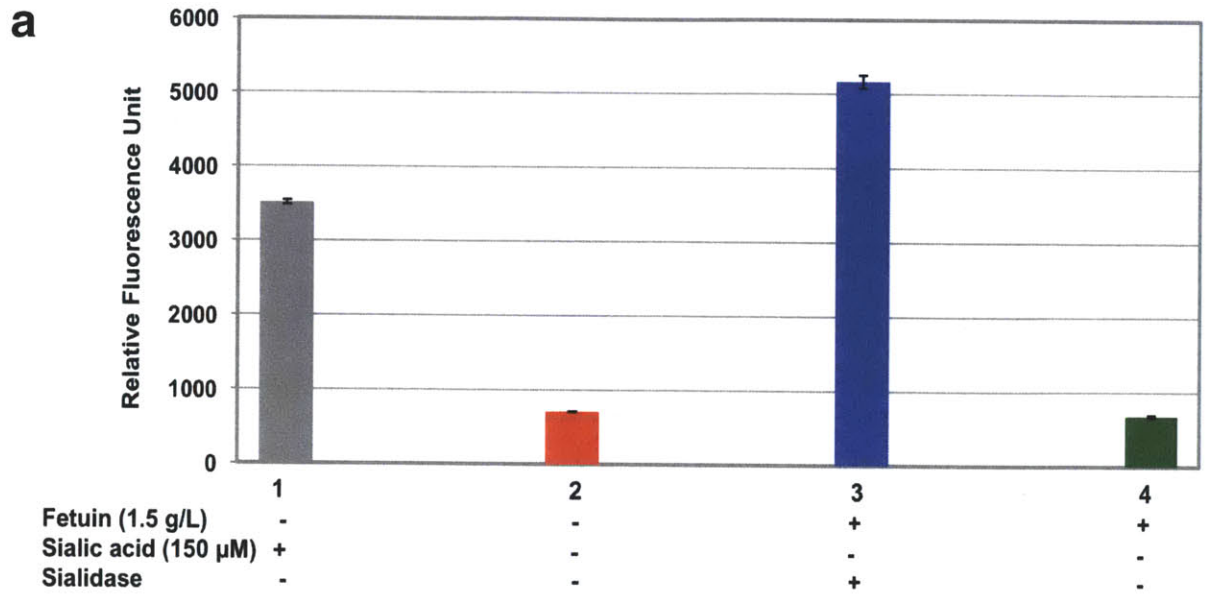


Figure 4.3. Optimization of the HTM. **(a)** The first step was optimized by varying the concentration of $Bu_4N(BH_4)$ and reduction time. 5 and 20 min of reduction gave similar result, indicating that 5 min were sufficient to reduce the interfering molecules. 0.1 M was chosen to avoid incomplete reduction when lower concentration was used. 0.1 M and 5 min were selected as the optimum concentration and reaction time. Error bars = SE, n = 2. **(b)** The second step was optimized by changing the incubation time from 5 to 80 min (SE, n = 5). The sialic acid contents were similar, indicating that 5 min were sufficient to release the sialic acid from the glycoprotein. **(c)** The third step was optimized by varying the derivatization reaction time. Both 1 and 5 min of derivatization gave linear calibration curve, but 5 min gave higher sensitivity. Prolonging the reaction to 30 min, however, resulted in high background and nonlinear calibration curve. These effects might be due to some unknown reactions that took place during the 30 min incubation at 80°C and disrupted the assay. Error bars = SE, n = 2.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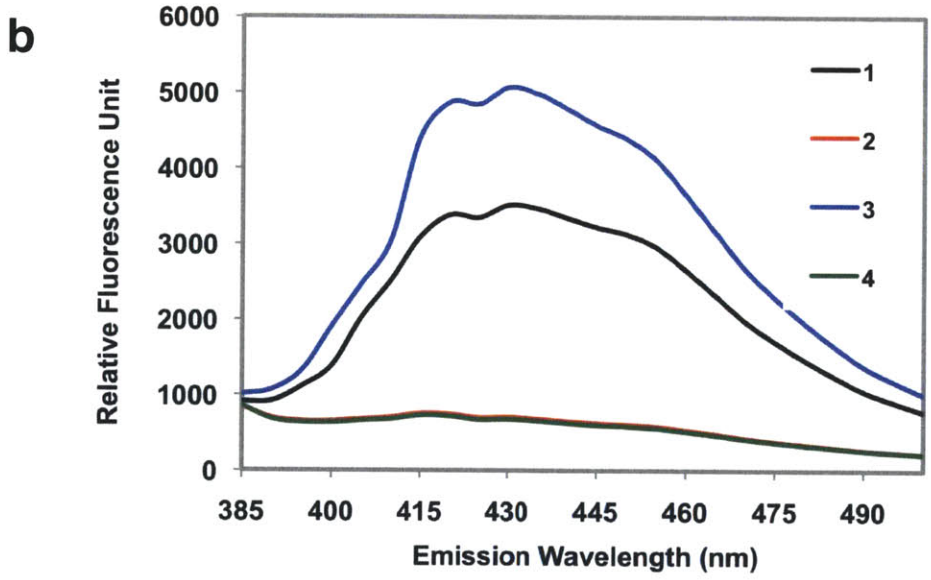
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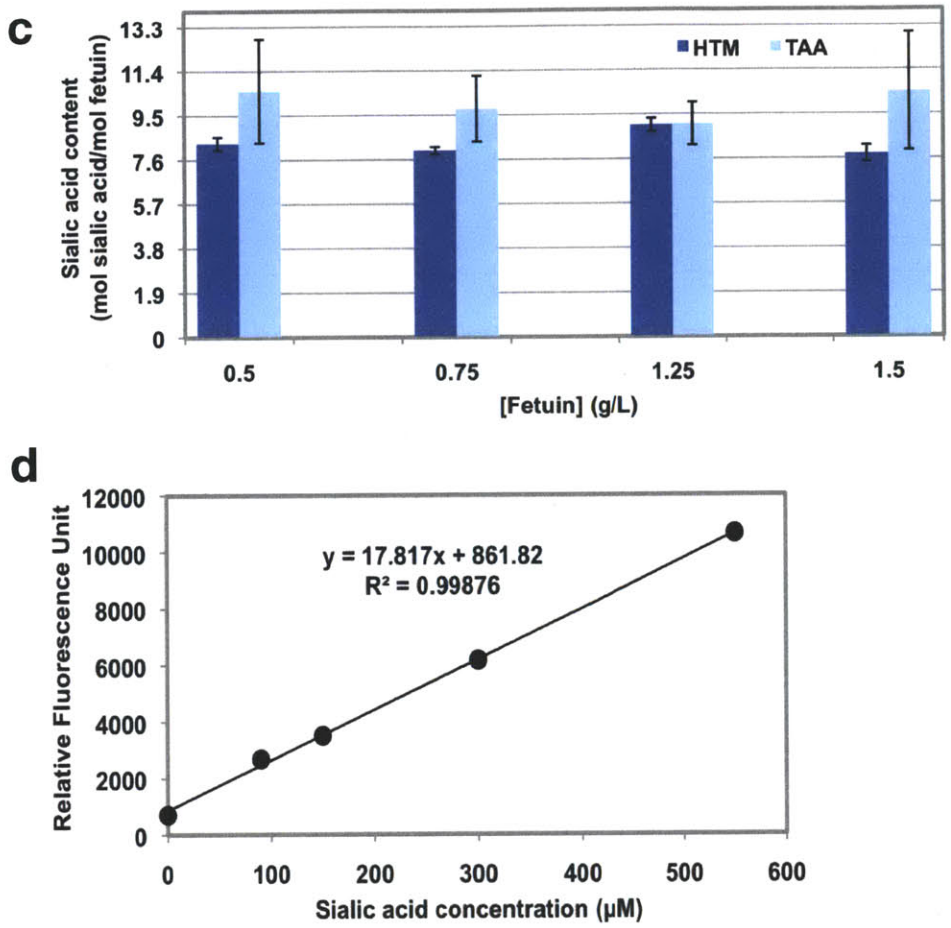


Figure 4.4. HTM was specific, accurate and sensitive. **(a)** Fluorescence intensity of controls (1, 2, 4) and model sample (3) was measured by HTM. Sample 1 was a positive control; 150 μM sialic acid standard was added to the CHO culture medium after $Bu_4N(BH_4)$ reduction. Sample 2 was a negative control; it was similar to sample 1, but the sialic acid standard was replaced with water. Sample 3 was a model sample; a fetuin standard was dissolved in the medium at 1.5g/L. Sample 4 was similar to 3, but sialidase was not added to release the sialic acid. Error bars = SE, n = 3. **(b)** Average fluorescence emission spectra of samples 1, 2, 3, 4. The emission spectra of samples 1 and 3 are similar, and those of sample 2 overlap with sample 4. **(c)**. The sialic acid contents of fetuin quantified by HTM are statistically similar ($P_1 \geq 0.05$) to those by TAA, but slightly higher ($P_2 < 0.05$) than the Sigma product specification, 7.6 mol sialic acid/ mol fetuin (error bars = SD, n = 5). The values of the sialic acid content are provided in Table 4.1. The assumption that the population distribution of sialic acid content data was at least approximately normal was supported by the linearity of the normal probability plots of the data (Fig. 4.5). **(d)** Calibration curve of HTM in which sialic acid standard was added to the CHO culture medium after reduction by $Bu_4N(BH_4)$. The quantitation limit of HTM was 2 μM. Error bars (unseen) = SE, n = 3.

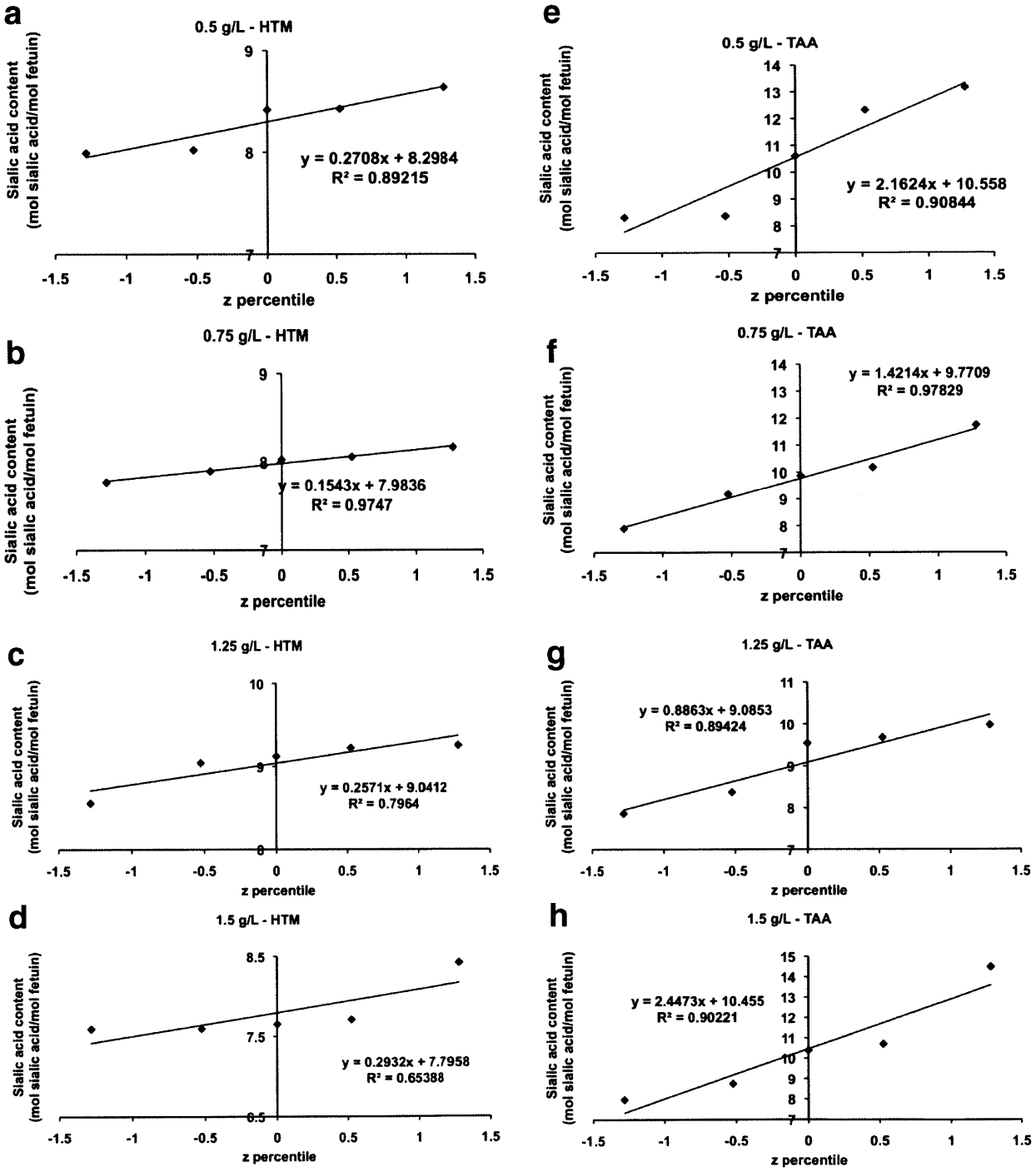


Figure 4.5. Linearity of normal probability plots of sialic acid content measured by HTM and TAA validates the use of t-test in analyzing the accuracy of HTM. The sialic acid contents of 0.5 g/L (**a**), 0.75 g/L (**b**), 1.25 g/L (**c**), and 1.5 g/L (**d**) of fetuin in CHO culture medium measured by HTM were ordered and the i^{th} smallest sialic acid content was set as the $[100(i\text{-}0.5)/n]^{th}$ sample percentile. The sialic acid contents of 0.5 g/L (**e**), 0.75 g/L (**f**), 1.25 g/L (**g**), and 1.5 g/L (**h**) of fetuin in water measured by TAA were ordered similarly. The linearity of the plots shows that the distribution of the data in Fig. 2c is at least approximately normal.

of the HTM. Sample 1, but not sample 2, was fluorescent, indicating that the fluorescence in sample 1 was due to the sialic acid. The presence of fluorescence in sample 3 and the absence in sample 4 showed that the fluorescence was specific to sialic acid released from the fetuin. The similarity between the fluorescence emission spectra of samples 1 and 3 confirmed that the fluorescence was due to the sialic acid. In addition, we found that the fluorescence emission spectra and intensities of N-glycolylneuraminic acid (NGNA) are identical to those of NANA (Fig. 4.6). This is an important issue to consider when using the HTM for mammalian cell culture because some cell lines can produce glycoproteins that contain NGNA, which is immunogenic [105].

We also found that the HTM was accurate relative to TAA and a commercial enzymatic method (Figs. 4.4c, 4.5, and Table 4.1). We used the HTM to measure the sialic acid contents of fetuin standard dissolved in CHO culture medium at 0.5, 0.75, 1.25, and 1.5 g/L. These values were compared with sialic acid contents of fetuin standard dissolved in water and measured by TAA. The fetuin was dissolved in water because some chemicals in CHO culture medium can interfere with TAA [106,107]. t-test showed that there was no statistically significant difference between the results obtained by the HTM and TAA ($P_1 \geq 0.05$). We also compared our results with the sialic acid content of 7.6 mol sialic acid/mol fetuin measured by Sigma using an enzymatic method consisting of sialidase, N-Acetylneuraminic acid aldolase, and lactate dehydrogenase. We found that our results were slightly higher than 7.6 mol sialic acid/ mol fetuin ($P_2 < 0.05$). Thus, the values obtained by the HTM are between 7.6 mol sialic acid/ mol fetuin and TAA values, indicating that the HTM is accurate. We also found that the HTM could accurately measure sialic acid content of orosomucoid (α_1-acid glycoprotein). The sialic acid content

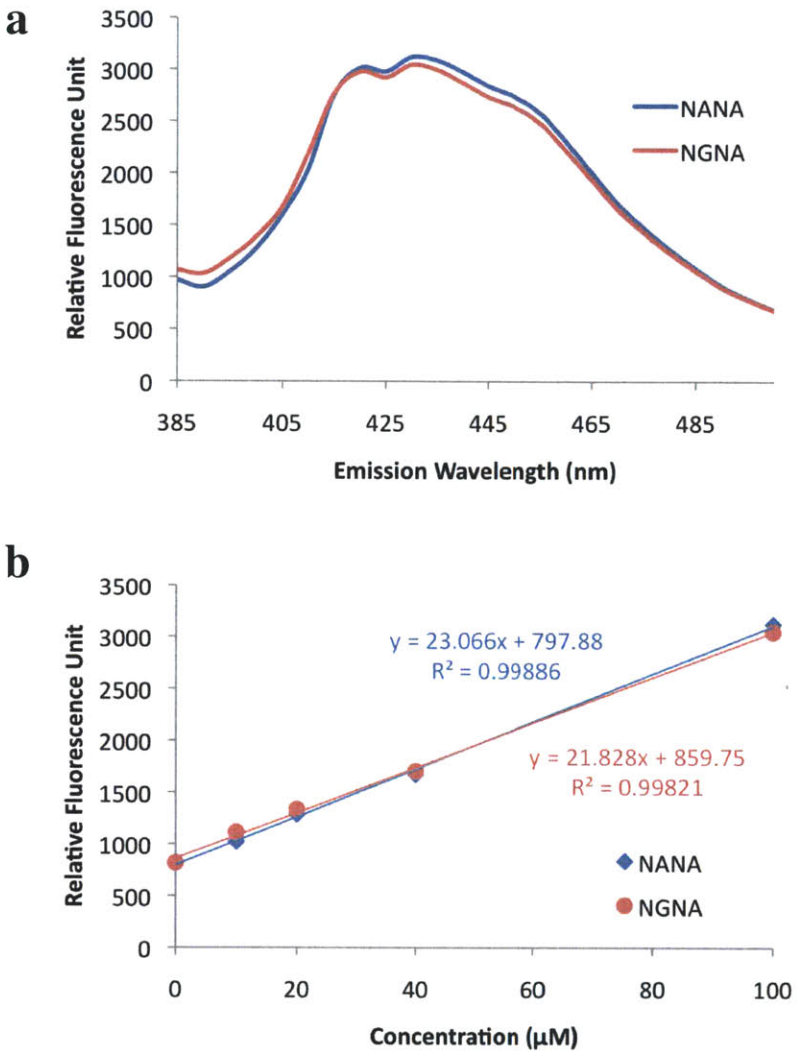


Figure 4.6. Fluorescence emission spectra (**a**) and intensities (**b**) of NANA and NGNA are identical. NANA and NGNA standards were added to CHO culture medium after $Bu_4N(BH_4)$ reduction, and fluorescence intensity was measured after malononitrile derivatization at λ_{ex} = 357 nm and λ_{em} = 430 nm. The spectra correspond to averages of 3 independent 100 μM NANA and NGNA samples. Error bars (unseen) = SE, n = 3.

Table 4.1. The HTM is accurate in quantifying glycoprotein sialylation. Sialic acid content of a fetuin standard dissolved in CHO culture medium was measured by the HTM. The values are similar to those measured by TAA. These values correspond to Fig. 4.4c. P_1 corresponds to t-test between HTM and TAA, while P_2 between HTM and 7.6 mol sialic acid/mol fetuin specified by Sigma. Error bars = SD, n = 5.

Fetuin (g/L)	Sialic acid content (mol sialic acid/mol fetuin)		t-test	
	HTM	TAA	P_1	P_2
0.5	8.3 ± 0.3	10.6 ± 2.2	0.09	0.005
0.75	8.0 ± 0.2	9.8 ± 1.4	0.05	0.005
1.25	9.0 ± 0.3	9.1 ± 0.9	0.92	0.0003
1.5	7.8 ± 0.4	10.5 ± 2.5	0.08	0.285

Table 4.2. Minimum glycoprotein concentrations required to detect 5 μM sialic acid. The serum half-lives of the proteins listed below are significantly higher when they are sialylated than desialylated (Table 2.1). The minimum concentrations were calculated based on molecular weights and typical sialic acid contents of the proteins. The titer values range from 0.02 to 0.75 g/L, which are within industrial capacities. These values indicate that the HTM is sufficiently sensitive for industrial applications.

Protein	Sialic acid content	MW (kDa)	Minimum titer (g/L)
Acetylcholinesterase	49 nmol SA/mg protein		0.10
α1-antitrypsin	3 mol SA/mol protein	44.3	0.07
Butyrylcholinesterase	137 nmol SA/mg protein		0.04
EPO	8 mol SA/mol protein	34	0.02
FSH	4 mol SA/mol protein	30	0.04
Human chorionic gonadotropin	10 mol SA/mol protein	36.7	0.02
Human factor VIII	28 mol SA/mol protein	200	0.04
Human luteinizing hormone	2.5 mol SA/mol protein	28	0.06
IFN-β	2 mol SA/mol protein	20	0.05
IFN-γ	2 mol SA/mol protein	17	0.04
IgG	1 mol SA/mol protein	150	0.75

measured by the HTM was 17.0 ± 0.3 mol sialic acid/mol orosomucoid, while that by TAA was 16.2 ± 0.6 mol sialic acid/mol orosomucoid. These values are close to the value of 14.2 mol sialic acid/mol orosomucoid reported in the literature [108], indicating that the HTM is is also accurate for this protein.

In addition, we found that the HTM was sensitive. The sensitivity of the HTM was analyzed based on its quantitation limit, defined as 10 times standard deviation of the fluorescence intensity corresponding to 0 μM sialic acid (Fig. 4.4d). The quantitation limit of HTM was 2 μM sialic acid. This results show that the HTM is more sensitive than commercially available kits, such as SIALIC-Q (Sigma) and SialiQuant (QA bio), which have quantitation limit of 45 and 264 μM sialic acid, respectively. In addition, these kits require protein purification in order to remove pyruvate, typically present at ~ 1 mM in mammalian cell medium. We also found that the quantitation limit of TAA was 60 μM sialic acid. Therefore, the HTM is more sensitive than these methods. Another commercial kit, GlykoScreen (Prozyme), has higher sensitivity (quantitation limit of 0.4 μM sialic acid) than HTM. This kit, however, require protein purification to remove the pyruvate. In addition, we found that although the HTM is less sensitive than GlykoScreen kit, it is sufficiently sensitive for industrial applications (Table 4.2).

4.3. Conclusions

Overall, we have developed a novel HTM that is specific, accurate, and sensitive for quantifying sialic acid content of glycoproteins produced in cell culture. The HTM has three major advantages over existing methods. First, it can quantify glycoprotein sialylation in 15 min. Current methods with prior protein purification may need at least one day. Second, the HTM can analyze many samples in parallel. In contrast, the use of chromatographic purification and

separation limits the number of samples analyzed to one at a time. Third, the plate reader required by the HTM is cheaper than the chromatographic protein purification system.

Chapter 5

Applications of HTM in Bioprocesses

HTM for quantifying glycoprotein sialylation is important for various applications in bioprocesses. In bioreactor operation, for instance, many parameters can affect the sialylation of recombinant proteins (Section 2.1.3). Therefore, analyzing hundreds of culture conditions is required to study the effects of the parameters on glycoprotein sialylation, as well as optimize the bioreactor operation. Moreover, sialylation decreases over time due to release of intracellular sialidase [2,23,104] and accumulation of ammonia [14]. Thus, monitoring sialylation of glycoprotein in cell culture is crucial for ensuring consistent product quality. In addition, the HTM is required for rapid screening of hundreds of cell clones generated in cell line development. This chapter demonstrates the versatility of HTM for these applications in bioprocesses. Section 5.1 shows the use of HTM for monitoring sialylation of glycoproteins produced in CHO cell cultures. In addition, we used the HTM to study the effects of culture conditions on sialylation of glycoproteins produced in CHO cell cultures. The results of this study are described in detail in Section 5.2. Section 5.3 shows the application of the HTM for cell line development. Here, we used the HTM to study intraclonal variability in sialylation of glycoproteins. The key results are summarized in Section 5.4.

5.1. Monitoring sialylation of recombinant proteins

Monitoring sialic acid content of recombinant proteins produced in mammalian cell cultures is important for product quality control. As a proof-of-concept, we validated the HTM for monitoring sialic acid content of recombinant IFN-γ produced in a batch culture of CHO

cells (Fig. 5.1). The viable cell density reached a maximum of 8.8 x 10^6 cells/ml at 144 h, and the viability dropped below 90% at 169 h (Fig. 5.1a). The CHO cells produced 48 ± 3 mg/L IFN-γ (SE, n = 2) at 169 h as measured by ELISA. The CHO cells also secreted other proteins. The total protein concentration was 435 ± 12 mg/L (SE, n = 3) as measured by Coomassie Plus Assay, and the secretion rate of total protein was similar to that of IFN-γ (Fig. 5.1b).

The HTM was used to measure the sialic acid released from not only IFN-γ but also other proteins secreted by the CHO cell because the HTM could not distinguish sialic acid bound to IFN-γ from other proteins. This overall sialylation was used as a semi-quantitative estimate of the sialic acid content of IFN-γ. The HTM could detect significant amount of sialic acid as early as 96 h (Fig. 5.1c). The sialic acid concentration measured by the HTM was then divided by the total concentration of proteins measured by Coomassie Plus Assay to calculate the sialic acid content (Fig. 5.1d). The HTM showed a decrease in sialic acid content from 43 ± 3 mg sialic acid/g protein at 96 h to 11 ± 1 mg sialic acid/g protein at 169 h (SE, n = 2). The accuracy of the HTM was validated by TAA. Here IFN-γ in CHO culture supernatant was purified by affinity chromatography, and the sialic acid released from the purified IFN-γ was measured by TAA. The desialylation rate of total protein was 0.39 ± 0.09 mg sialic acid/(g protein.h), while that of purified IFN-γ was 0.029 ± 0.002 mol sialic acid/(mol IFN-γ.h) (SE). Normalization of the sialic acid contents of total protein and IFN-γ showed that there was no significant difference between these two rates (Fig. 5.2); the desialylation rate of total protein was 0.009 ± 0.002 h^{-1} and that of IFN-γ is 0.011 ± 0.001 h^{-1} (SE). The similarity between these two normalized rates demonstrates

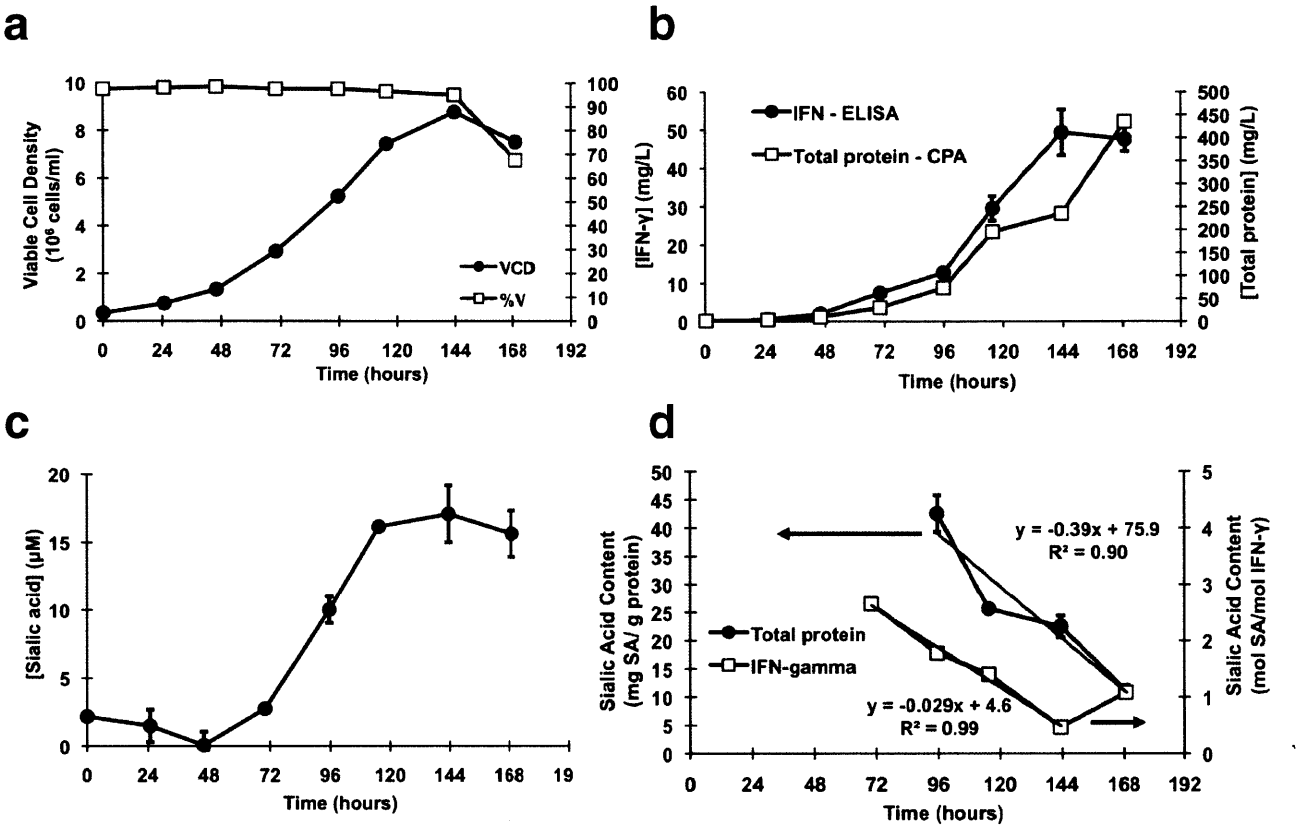


Figure 5.1. HTM is accurate for semi-quantitative measurement of sialic acid content of IFN-γ produced in CHO cell culture. **(a)** Viable cell density (VCD) and viability of CHO cells in a batch culture. Error bars (unseen) = SE, n = 2. **(b)** Concentration of IFN-γ (error bars = SE, n = 2) and total proteins (error bars = SE, n = 3) produced by CHO cells in the batch culture. **(c)** Concentration of total sialic acid released from glycoproteins produced by CHO cells measured by HTM. Error bars = SE, n = 2. **(d)** Sialic acid content of total proteins and IFN-γ produced in CHO cell culture. Sialic acid content of total proteins was obtained from the ratio of sialic acid and total protein concentration in CHO culture supernatant measured by HTM and Coomassie Plus Assay (CPA), respectively. Sialic acid content of IFN-γ was obtained from ratio of sialic acid and IFN-γ concentration in purified IFN-γ sample measured by TAA and HPLC, respectively. Data from day 3 to 6 were used to obtain the trendline for IFN-γ sialylation, while that from day 4 to 7 were used for total protein sialylation. Error bars = SE, n = 2.

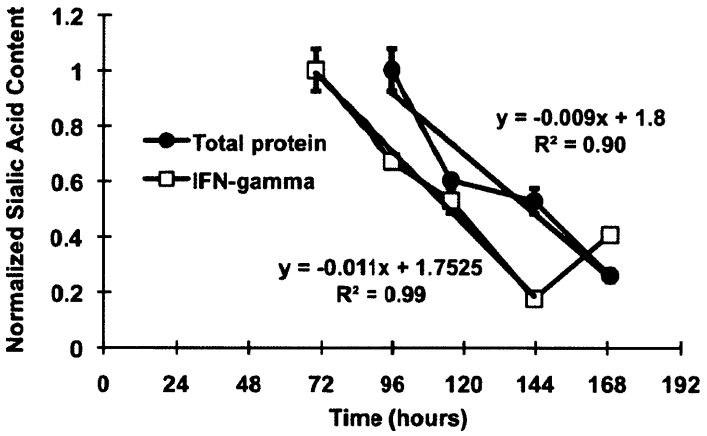


Figure 5.2. Total protein and IFN-γ have similar normalized desialylation rates. The sialic acid contents of total protein and IFN-γ shown in Fig. 5.1d were normalized by their maximum values on day 4 and 3, respectively. Data from day 3 to 6 were used to obtain the trendline for IFN-γ sialylation, while that from day 4 to 7 were used for total protein sialylation. The normalized desialylation rate of total protein was $0.009 \pm 0.002\ hr^{-1}$ and that of IFN-γ is $0.011 \pm 0.001\ hr^{-1}$ (SE). These rates were obtained using LINEST command in Microsoft Excel.

that the overall sialylation measured by HTM can be used to monitor the sialylation of IFN-γ. The decrease in sialic acid content over time is a well known phenomenon that is primarily caused by release of intracellular sialidase by dead cells [2,104] and accumulation of ammonia in cell culture [14].

In addition, we also used the HTM for monitoring glycoprotein sialylation in CHO cell cultures producing IgG4 (Fig. 5.3). The clones had similar growth rates (Fig. 5.3a); the viable cell density of the three clones reached a maximum of ~8 x 10^6 cells/ml at 119 h. There were significant differences in the IgG4 productivity among these three clones. The maximum concentration of IgG4 produced by the high producer was 658 ± 99 mg/L, medium producer was 369 ± 55 mg/L, and low producer was 230 ± 35 mg/L (Fig. 5.3b). In addition, the maximum concentration of total proteins produced by the high producer was 613 ± 23 mg/L, medium producer was 539 ± 18 mg/L, and low producer was 466 ± 14 mg/L (Fig. 5.3c). Comparison of the IgG4 and total protein concentration shows that most of the proteins produced by the high producer was IgG4, while there were a lot of other proteins than IgG4 secreted by the medium and low producers (Fig. 5.4). These results imply that the HTM is best used with high-producer cell lines because the overall sialylation will be similar to the product sialylation. For high-throughput screening of cell clones, the HTM is therefore, best used as a second stage screening after screening for high producer cell clones.

The overall sialylation of proteins secreted in these three clones had different dynamics (Fig. 5.3d). The overall sialylation in high producer cell culture decreased from 7 ± 2 to 4 ± 1 mg

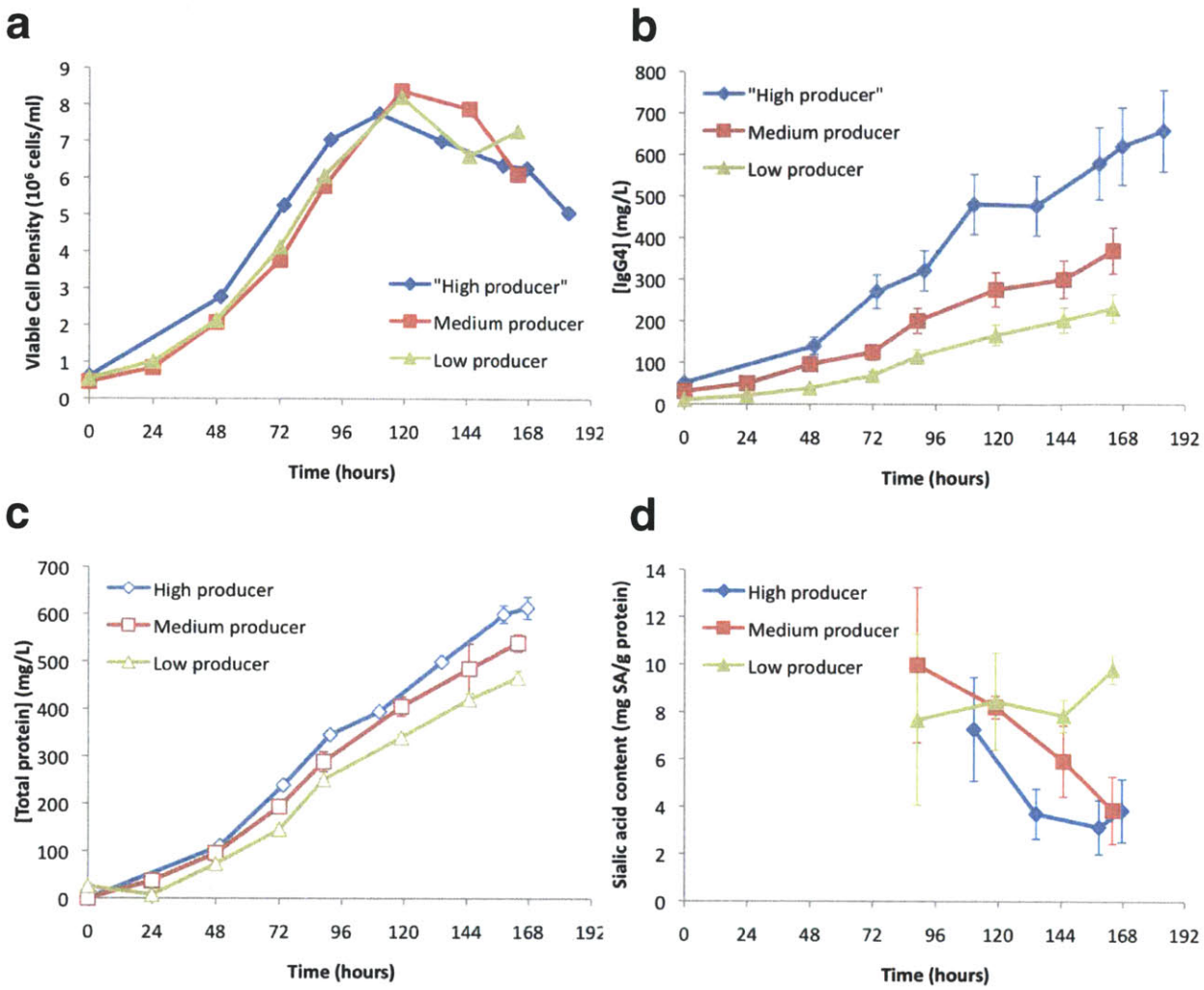


Figure 5.3. HTM can be used for monitoring sialic acid content of proteins in CHO cell culture producing IgG4. **(a)** Viable cell density of three CHO cell clones in three batch cultures. Concentration of IgG4 (**b**) and total proteins (**c**) produced by CHO cells in the batch cultures. **(d)** Sialic acid content of total proteins and IFN-γ produced in CHO cell culture. Sialic acid content of total proteins was obtained from the ratio of sialic acid and total protein concentration in CHO culture supernatant measured by HTM and Coomasie Plus Assay, respectively. Cell culture with higher productivity has lower sialic acid content, especially after five days of culture. Error bars: 15% relative errors (**b**), SE, n = 2 (**c,d**).

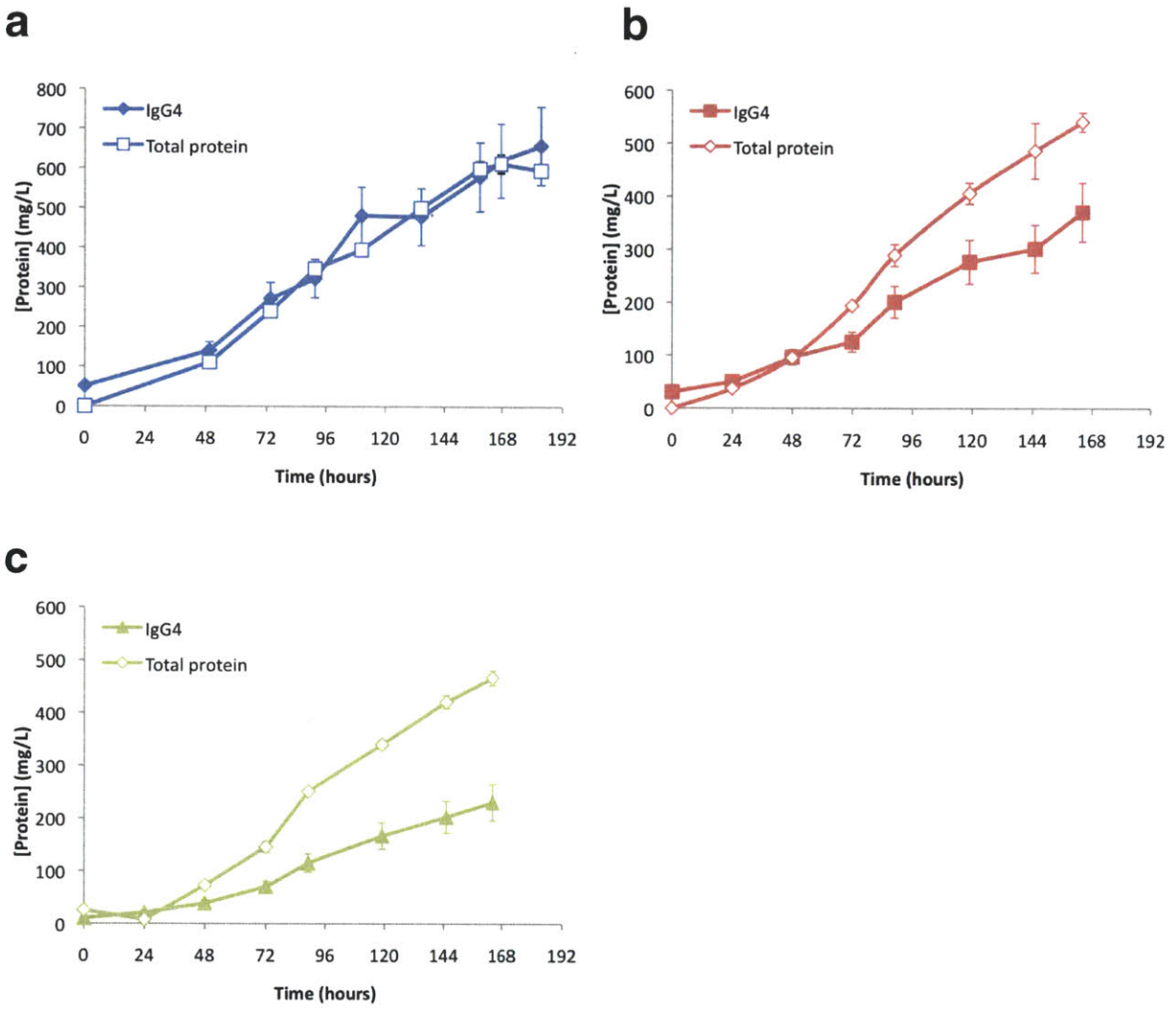


Figure 5.4. Comparison between IgG4 and total protein concentrations in three CHO cell cultures. The concentrations of IgG4 and total proteins are similar in high producer CHO cell culture (**a**), but not in medium (**b**) and low (**c**) producer CHO cell cultures. These results show that most of the proteins in high producer CHO cell culture is IgG4. Error bars: 15% relative errors (IgG4), SE, n = 2 (total protein).

sialic acid/g protein within 56 h. The desialylation rate in this cell culture was faster than that in medium producer cell culture, in which the overall sialylation decreased from 10 ± 3 to 4 ± 1 mg sialic acid/g protein within 75 h. In contrast, the sialylation in low producer cell culture remained approximately constant at 8 to 10 mg sialic acid/g protein.

Overall, these results show that the HTM can be used for monitoring sialylation of various recombinant proteins produced in mammalian cell cultures. The HTM can provide sialic acid content in 15 minutes. Thus, the cell culture can be quickly stopped if the sialic acid content approaches lowest value approved by regulations. In contrast, this action is not possible with conventional methods because they require more than one day, during which sialic acid content can decrease significantly (Figs. 5.1d, 5.2, 5.3d), to quantify the sialylation.

5.2. Effects of CHO cell culture conditions on sialylation

Many cell culture parameters can affect sialylation of recombinant proteins (Section 2.2). Here, we use the HTM to study the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on the overall sialylation of proteins secreted in CHO-IFN-γ cell cultures. As described in Section 2.2, feeding ManNAc can increase sialylation by increasing intracellular CMP-sialic acid [61,62]. In addition, many metal dications can increase the activity of OGT [30-33], galactosyltransferase [69,70], enzymes involved in the synthesis of LLO [71,72] and CMP-sialic acid [73]. It is, however, not known whether feeding these ions can enhance the increase in sialylation due to feeding ManNAc. Thus, we aim to study how the effects of feeding these chemicals interact with each other, and identify culture conditions that will maximize the sialylation.

We first identified which metal ions could increase sialylation. We screened six metal ions: Cu^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}, and found that only Cu^{2+} and Mn^{2+} could increase the sialylation (Fig. 5.5). The maximum concentrations of these ions were selected based on toxicity levels of the chemicals in CHO cell cultures.

In order to get a response surface, we used a rotatable Central Composite Design (CCD) (Figs. 5.6a,b). The design consists of 15 different conditions: eight corner points of a cube, six star points with $a = (2^3)^{1/4} = 1.68$, and a center point. Each of these points was repeated in three independent batch shake flask cultures. This design is chosen because it is rotatable, meaning that the design gives equal precision for model-fitted responses at points that have the same distance from the center of the cube. In addition, the experimental data can be used to fit a second order polynomial model. In contrast, 2^k factorial designs, in which each of the k factors, such as ManNAc and Cu^{2+} concentrations, have two factor levels, such as 0 mM and 2 mM, is rotatable, but the data cannot be used to fit non-linear models. Moreover, 3^k factorial designs, in which each of the k factors has three factor levels, can generate data that can be fitted to a second order polynomial, but is not rotatable [100]. All of the chemicals were added at the beginning of the batch cultures, and the maximum concentrations of these chemicals were determined based on their toxicity; the viability of the CHO cells was not affected by feeding the chemicals within the concentration ranges used in this study.

We found that feeding ManNAc, Cu^{2+}, and Mn^{2+} in combination increased sialylation from 20 to 36 mg sialic acid/g protein on the fifth day of batch CHO cell cultures (Fig. 5.6c). Out of the 15 conditions, the overall sialylation in 13 conditions was significantly higher than in the control

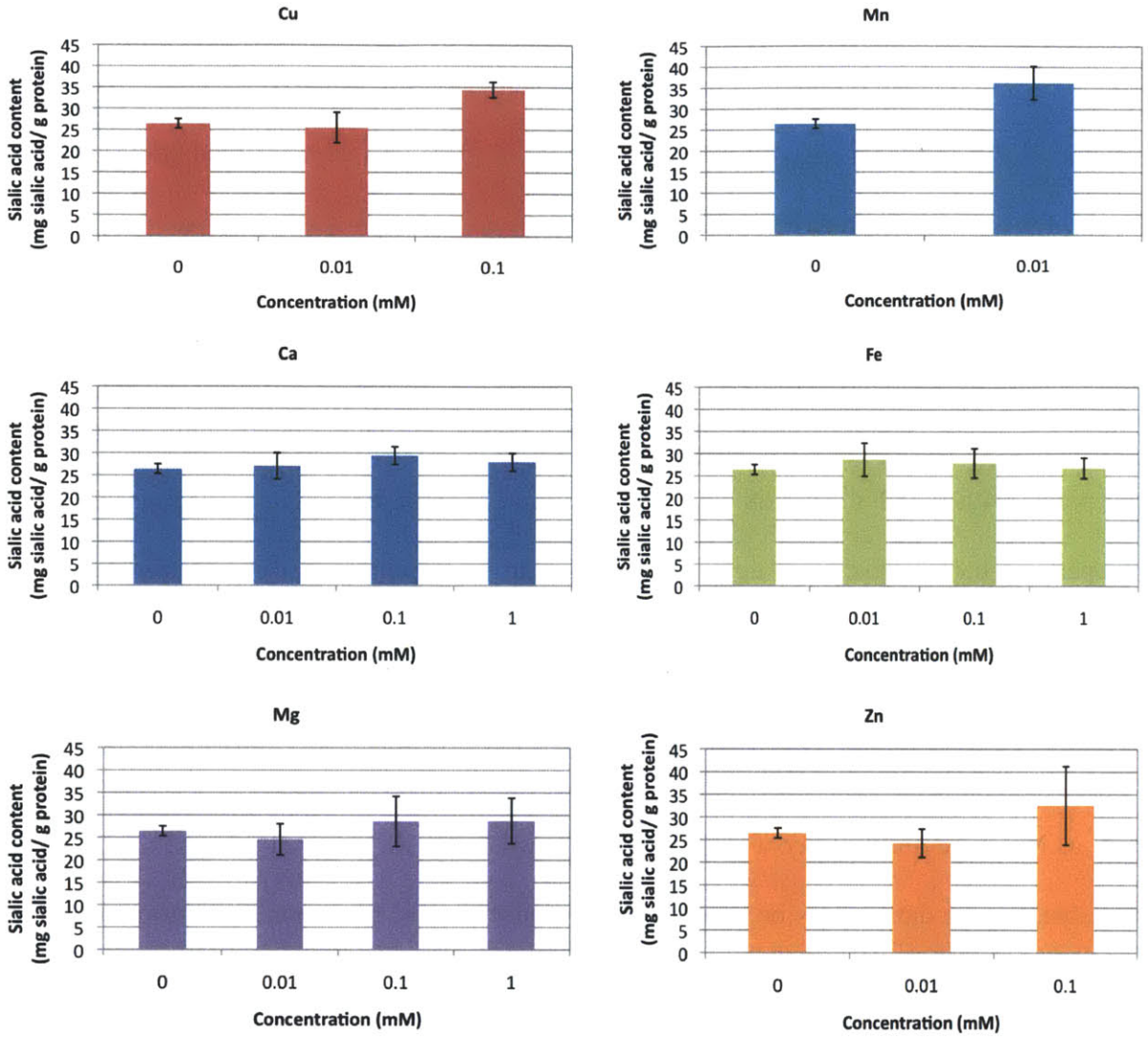
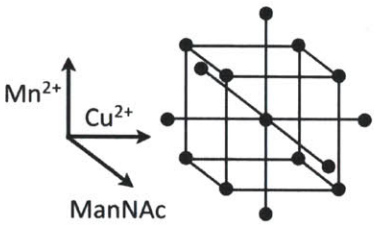


Figure 5.5. Effects of feeding Cu^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+} on sialic acid content of glycoproteins produced in CHO cell cultures. Feeding Cu^{2+} at 0.1 mM and Mn^{2+} at 0.01 mM significantly increased sialylation of proteins produced in the cell cultures. The other ions, however, did not significantly increase the sialylation. Error bars = SD, n = 3 independent batch cultures.

a



	Max concentration
ManNAc	2 mM
Cu^{2+}	100 μM
Mn^{2+}	10 μM

b

	[ManNAc] (mM(	[Cu2+] (μM)	[Mn2+] (μM)
1	0	50	5
2	0.4	20.3	2
3	0.4	20.3	8
4	0.4	79.7	2
5	0.4	79.7	8
6	1	0	5
7	1	50	0
8	1	50	5
9	1	50	10
10	1	100	5
11	1.6	20.3	2
12	1.6	20.3	8
13	1.6	79.7	2
14	1.6	79.7	8
15	2	50	5

c

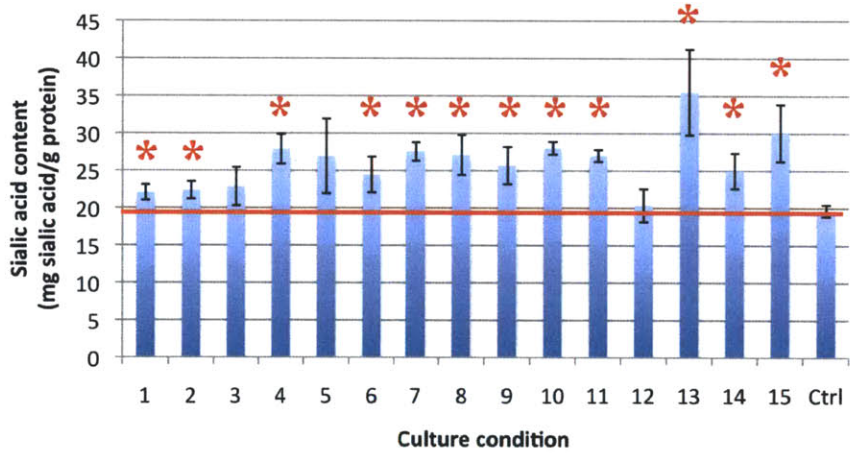


Figure 5.6. Feeding ManNAc, Cu^{2+}, and Mn^{2+} increased sialic acid content of glycoproteins produced in CHO cell culture. **(a)** Central composite design was used to obtain surface response of sialic acid content as a result of feeding ManNAc, Cu^{2+}, and Mn^{2+}. The maximum concentration for ManNAc was 2 mM, Cu^{2+} was 100 μM, and Mn^{2+} was 10 μM. **(b)** There were 15 different conditions, and each condition was carried out in three independent 30 mL batch cultures. **(c)** Sialic acid contents of proteins in 13 out of 15 culture conditions were significantly higher than in control cultures (Ctrl, red line), in which ManNAc, Cu^{2+}, and Mn^{2+} were not added. Cell cultures were harvested on the fifth day. Error bars = SD, n = 3 independent batch cultures. Stars indicate $P < 0.05$.

condition. Moreover, a quadratic least square model was fitted to the experimental data. The model is given by Eqs. 1 and 2, where [ManNAc] is in mM, $[Cu^{2+}]$, and $[Mn^{2+}]$ are in μM. Each factor is normalized in Eq. 1, and Eq. 2 shows a simplified form of the model.

Sialic acid content (mg sialic acid/g protein)

$$= 26.2 + 1.6 \times \left(\frac{[ManNAc] - 1}{0.6} \right) + 2.1 \times \left(\frac{[Cu^{2+}] - 50}{29.7} \right) - 1.5 \times \left(\frac{[Mn^{2+}] - 5}{3} \right) - 2.1 \times \left(\frac{[ManNAc] - 1}{0.6} \right) \times \left(\frac{[Mn^{2+}] - 5}{3} \right) \quad (1)$$

$$= 21.3 + 8.5 \times [ManNAc] + 0.07 \times [Cu^{2+}] + 0.7 \times [Mn^{2+}] - 1.2 \times [ManNAc] \times [Mn^{2+}] \quad (2)$$

All of the main effects, the difference in sialic acid content due to changing the concentration of one of the three chemicals, are statistically significant (Table 5.1), and have positive coefficients (Eq. 2). The positive coefficients imply that separately feeding these chemicals to the CHO cell cultures will increase the sialylation. Moreover, the interaction between ManNAc and Mn^{2+} is the only significant interaction. It has a negative coefficient, implying that if both ManNAc and Mn^{2+} are added at high concentration, the sialylation will not be increased. This negative effect may be because Mn^{2+} is also a cofactor of many other enzymes, such as oxidoreductases, hydrolases, and lipases [109], and may cause other effects that eventually decrease the sialylation. Lack of fit test shows that the model is statistically adequate in describing the data (Table 5.2).

Table 5.1. All the main effects and an interaction between ManNAc and Mn^{2+} are statistically significant. The effects of ManNAc, Cu^{2+}, and Mn^{2+} are fitted to a quadratic least square model (Eq.1). The values shown in the column "Estimate" are coefficients of the normalized effects. *t*-statistics was used to test the significance of all main effects and interactions. $P < 0.05$ is statistically significant. The statistically significant effects and interaction are shown below.

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	26.184444	0.42946	60.97	<.0001*
[ManNAc] (mM)(0.405,1.594)	1.5611948	0.450083	3.47	0.0013*
[Cu 2+] (uM)(20.273,79.726)	2.0997272	0.450083	4.67	<.0001*
[Mn 2+] (uM)(2.0273,7.9726)	-1.522708	0.450083	-3.38	0.0016*
[ManNAc] (mM)*[Mn 2+] (uM)	-2.083333	0.588062	-3.54	0.0010*

Table 5.2. Least square model is statistically adequate in predicting the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialic acid content of proteins produced in CHO cell culture. $P < 0.05$ is statistically significant. $P = 0.2811$ indicates that the difference between the model and experimental data is insignificant relative to the experimental errors.

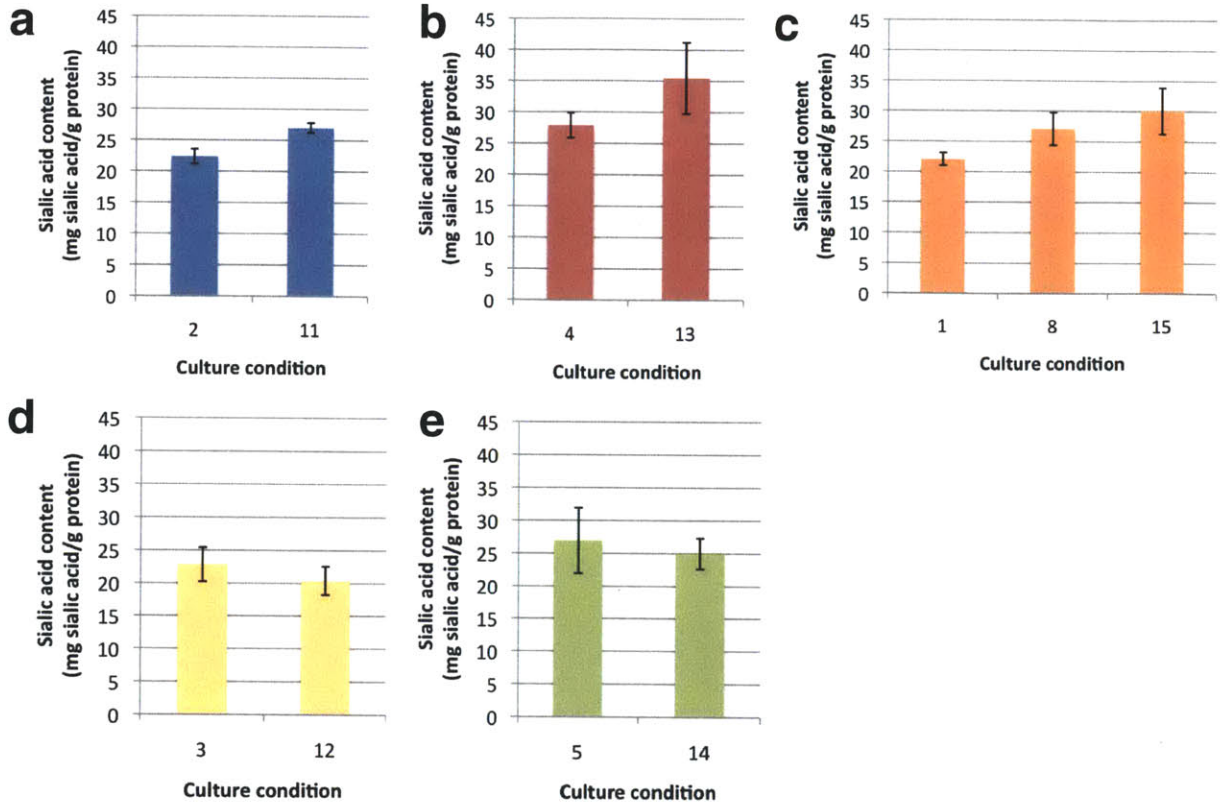
Lack Of Fit Test

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	10	99.71111	9.97111	1.2879
Pure Error	30	232.27333	7.74244	**Prob > F**
Total Error	40	331.98444		0.2811
				Max RSq
				0.7138

Further analysis of the data shows that the sialylation was higher when ManNAc and Cu^{2+} were fed together than separately. This effect was observed when the concentration of Mn^{2+} was fixed at 2 μM (Figs. 5.7a,b). This observation agrees with the model (Eq. 2). It also agrees with the rationale that feeding Cu^{2+} can enhance the enzymatic reactions prior to sialylation and therefore, further boost the increase in sialylation due to feeding ManNAc. Moreover, the increase in sialylation due to feeding ManNAc was also observed when Mn^{2+} was fixed at 5 μM (Fig. 5.7c). When Mn^{2+} was fixed at 8 μM, however, increasing ManNAc concentration did not increase sialylation (Figs. 5.7d,e). This result agrees with the model (Eq. 2), which estimates that feeding ManNAc decreases sialylation when the concentration of Mn^{2+} is more than 7.3 μM.

Similarly, feeding Cu^{2+} increased sialylation regardless of the concentrations of ManNAc and Mn^{2+} (Fig. 5.8). This result agrees with the model (Eq. 2), which estimates that there is no interaction between Cu^{2+} and either ManNAc or Mn^{2+}. Moreover, the increase due to Cu^{2+} was enhanced when ManNAc concentration was increased and Mn^{2+} was fixed at 2 μM (Figs. 5.8a,d). This observation also agrees with the rationale that feeding Cu^{2+} and ManNAc complement each other as decribed above. This enhancement, however, was not observed when Mn^{2+} was fixed at 8 μM (Figs. 5.8b,e). The reason may be due to the negative interaction between ManNAc and Mn^{2+} as described above.

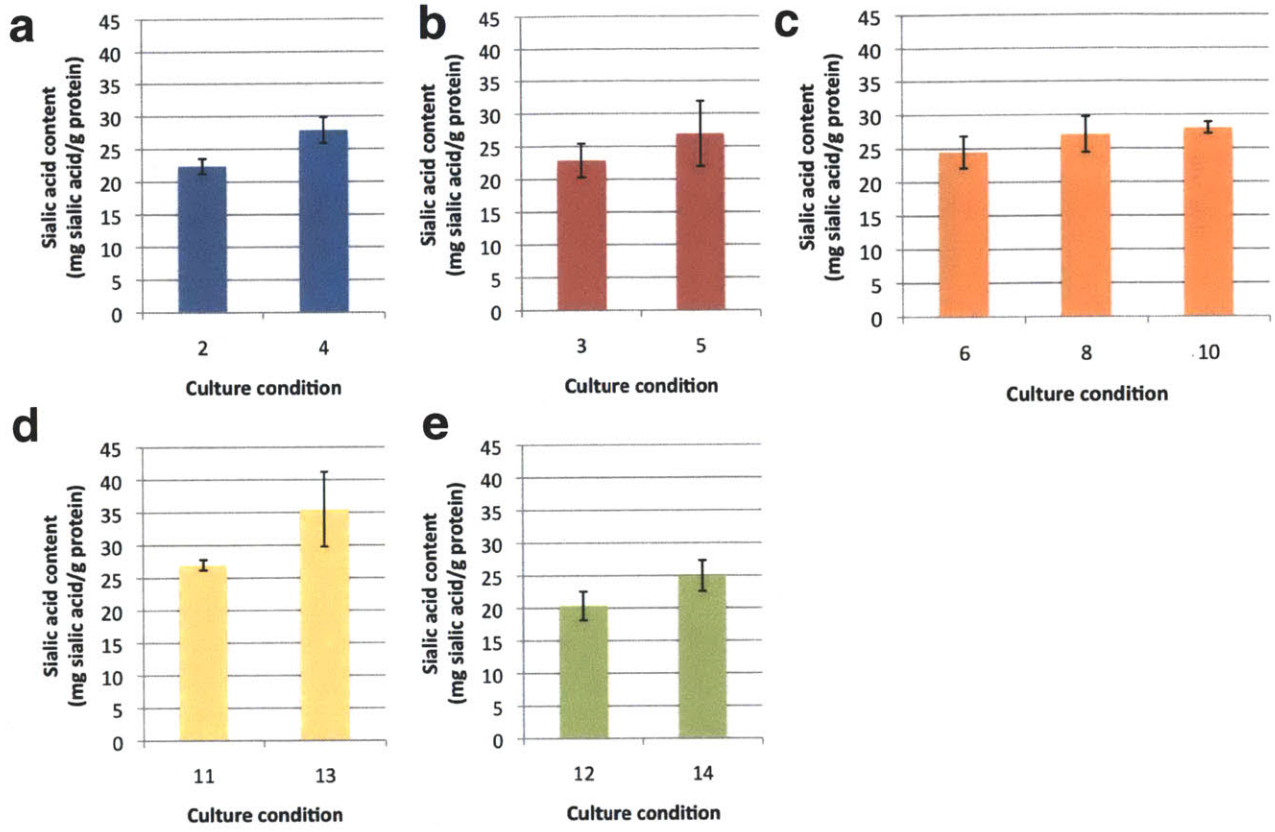
Feeding Mn^{2+}, however, did not increase sialylation in the culture conditions used in the CCD (Fig. 5.9). In fact, when ManNAc concentration was fixed at 1 and 1.6 mM, increasing Mn^{2+} concentration decreased the sialylation. This observation agrees with the model (Eq. 2), which estimates that feeding Mn^{2+} decreases sialylation when the concentration of ManNAc is higher



f

Culture condition	[ManNAc] (mM)	[Cu^{2+}] (μM)	[Mn^{2+}] (μM)
2	0.4	20.3	2
11	1.6	20.3	2
4	0.4	79.7	2
13	1.6	79.7	2
1	0	50	5
8	1	50	5
15	2	50	5
3	0.4	20.3	8
12	1.6	20.3	8
5	0.4	79.7	8
14	1.6	79.7	8

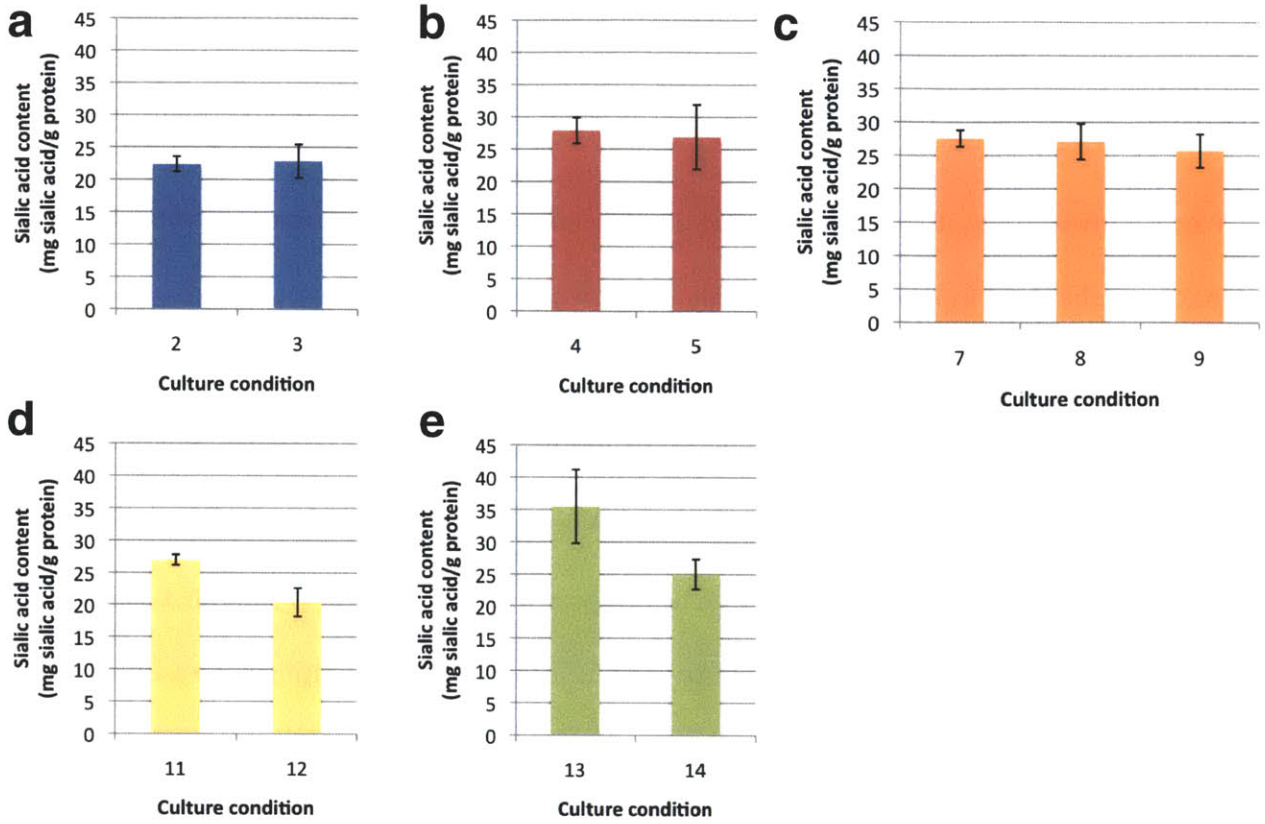
Figure 5.7. Effects of feeding ManNAc when [Cu^{2+}] and [Mn^{2+}] were fixed. Feeding ManNAc increased sialylation when [Mn^{2+}] was fixed at 2 μM **(a,b,f)** or 5 μM **(c,f)**. However, feeding ManNAc did not increase sialylation when [Mn^{2+}] was fixed at 8 μM **(d,e,f)**. This observation agrees with the least square model (Eq. 2), which predicts that feeding ManNAc decreases sialylation when [Mn^{2+}] > 7.3 μM. In addition, sialylation was higher when [Cu^{2+}] was fixed at 79.7 μM than 20.3 μM **(a,b,f)**, indicating that Cu^{2+} enhanced the increase in sialylation due to ManNAc feeding. Error bars = SD, n = 3 independent batch cultures.



f

Culture condition	[ManNAc] (mM)	[Cu^{2+}] (μM)	[Mn^{2+}] (μM)
2	0.4	20.3	2
4	0.4	79.7	2
3	0.4	20.3	8
5	0.4	79.7	8
6	1	0	5
8	1	50	5
10	1	100	5
11	1.6	20.3	2
13	1.6	79.7	2
12	1.6	20.3	8
14	1.6	79.7	8

Figure 5.8. Effects of feeding Cu^{2+} when [ManNAc] and [Mn^{2+}] were fixed. Feeding Cu^{2+} increased sialylation regardless of [ManNAc] and [Mn^{2+}]. This observation agrees with the least square model (Eq. 2). Error bars = SD, n = 3 independent batch cultures.



f

Culture condition	[ManNAc] (mM)	[Cu^{2+}] (μM)	[Mn^{2+}] (μM)
2	0.4	20.3	2
3	0.4	20.3	8
4	0.4	79.7	2
5	0.4	79.7	8
7	1	50	0
8	1	50	5
9	1	50	10
11	1.6	20.3	2
12	1.6	20.3	8
13	1.6	79.7	2
14	1.6	79.7	8

Figure 5.9. Effects of feeding Mn^{2+} when [Cu^{2+}] and [ManNAc] were fixed. Feeding Mn^{2+} did not increase sialylation when [ManNAc] was fixed at 0.4 mM (**a,b,f**). Moreover, it decreased sialylation when [ManNAc] was fixed at 1 and 1.6 mM (**c,d,e,f**). This observation agrees with the least square model (Eq. 2), which predicts that feeding Mn^{2+} decreases sialylation when [ManNAc] > 0.6 mM. Error bars = SD, n = 3 independent batch cultures.

than 0.6 mM. Overall, the above analysis shows that the model fits very well with the experimental data.

We also investigated if the model (Eq. 2) could accurately predict culture conditions that would maximize the sialylation. The model predicts that when Mn^{2+} concentration is fixed at 0 μM, the sialylation will be maximized when the ManNAc and Cu^{2+} concentrations are maximized at 2 mM and 100 μM, respectively (Figs. 5.10a). In this condition, the predicted maximum sialic acid content is 41 ± 4 mg sialic acid/g protein. When Mn^{2+} concentration is fixed at 5 μM, the maximum sialylation is 32 ± 2 mg sialic acid/g protein (Figs. 5.10b). This is also achieved by maximizing the concentrations of ManNAc and Cu^{2+}. In these two conditions, the model predicts that the increase in sialylation will be higher when both ManNAc and Cu^{2+} are added together than separately. On the other hand, when Mn^{2+} concentration is fixed at 10 μM, feeding Cu^{2+} will increase sialylation, but feeding ManNAc will decrease it due to the negative interaction (Figs. 5.10c). In addition, the predicted maximum sialylation is 30 ± 4 mg sialic acid/g protein. Overall, the predicted maximum sialylation within the concentration ranges used in this study (Fig. 5.6a) is 41 ± 4 mg sialic acid/g protein. This value can be obtained by taking partial derivatives of the model (Eq. 2) with respect to each factor. In order to test the accuracy of the model prediction, we fed 2 mM ManNAc and 100 μM Cu^{2+} to batch cultures of CHO-IFN-γ cells. This feeding increased overall sialylation from 24 ± 4 to 35 ± 5 mg sialic acid/g protein on the fifth day (Fig. 5.11). This experimental value is close to the predicted maximum value, indicating that the model prediction is accurate.

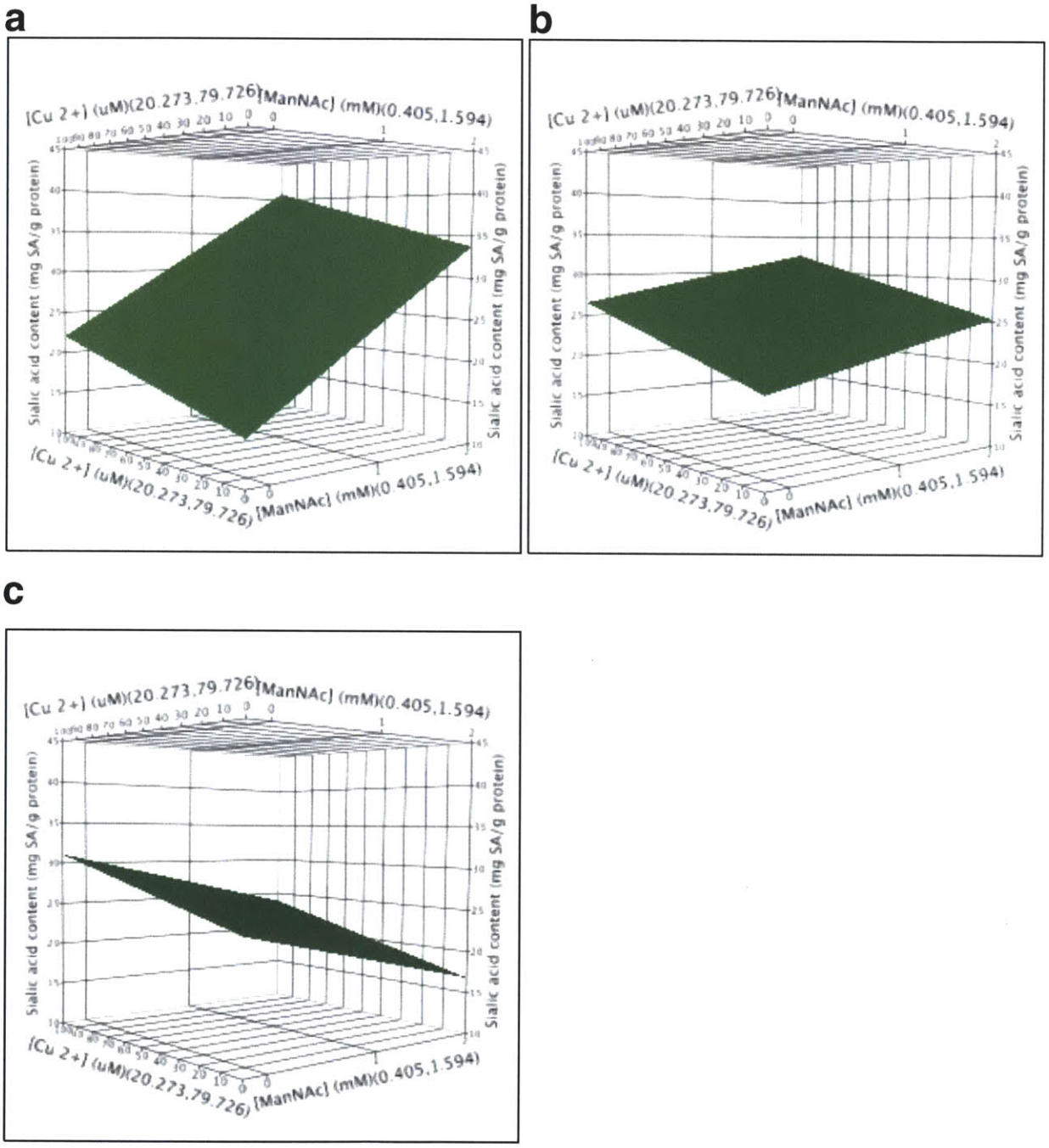


Figure 5.10. Model estimated effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialylation. The figures show overall sialylation of total proteins harvested from batch CHO cell cultures on the fifth day as a function of [ManNAc] and [Cu^{2+}], while [Mn^{2+}] is fixed at 0 μM (**a**), 5 μM (**b**), and 10 μM (**c**). Overall, the highest sialic acid content (41 ± 4 mg sialic acid/g protein) was achieved when [ManNAc] was 2 mM, [Cu^{2+}] was 100 μM, and [Mn^{2+}] was 0 μM.

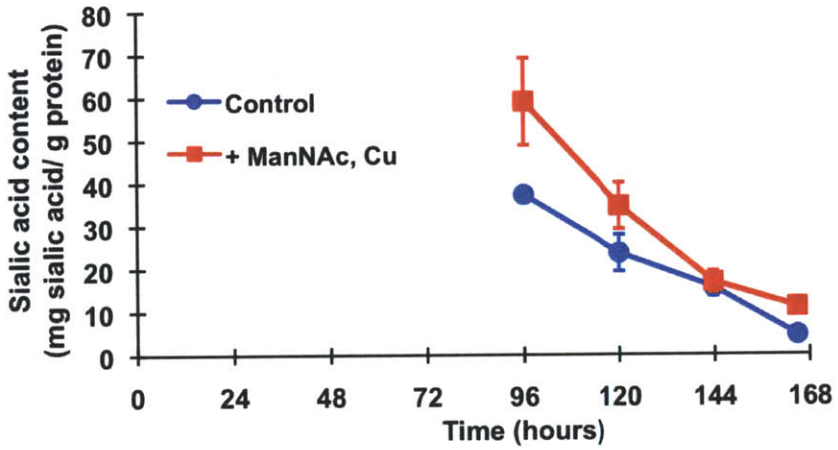


Figure 5.11. Effects of feeding ManNAc and Cu^{2+} on sialylation. Overall sialylation of proteins in batch CHO cell cultures supplemented with ManNAc at 2 mM and Cu^{2+} at 100 μM (red square) was significantly higher than control (blue circle). On the fifth day, the sialylation in supplemented culture was 35 ± 5 mg sialic acid/g protein, close to the model predicted value of 41 ± 4 mg sialic acid/g protein. The sialylation in control culture was 24 ± 4 mg sialic acid/g protein. On the fourth day, the sialylation in supplemented culture was 58 ± 7 mg sialic acid/g protein, while that in control culture was 37 ± 2 mg sialic acid/g protein. Error bars = SD, n = 2.

Overall, these studies show that the HTM can be used to rapidly analyze the effects of culture conditions on sialylation of proteins secreted in cell cultures. The HTM can finish the whole analysis within less than one day. In contrast, conventional technologies will require at least 50 days to analyze all of the 50 samples generated from the CCD experiment and studying the accuracy of model prediction.

5.3. High-throughput cell clone screening

Development of cell lines producing recombinant proteins require screening thousands of clones [110]. This task is even more complicated by the intraclonal variability that often arises within each clone [111], thereby requiring higher number of analyses for the screening. In order to rapidly perform this screening, high-throughput methods are required. Various high-throughput methods can be used for analyzing productivity of recombinant proteins, such as a microengraving method [103], ELISA, picking clones plated in semi-solid media [112,113], and fluorescence-activated cell sorting (FACS) [114]. For quantifying sialic acid, however, current methods are very time consuming and cannot be used for high-throughput screening. This task can be done using the HTM developed in this thesis.

Here, we aim to study intraclonal variability in sialic acid content of proteins secreted by CHO cells. A batch culture of CHO cells was serially diluted to ~0.7 cell/2 μL, and 2 μL of cell suspension was deposited onto 96-well plates. Some of the wells contained single cells, and these single cells were expanded into clones. These clones were cultured for approximately 22 to 24 days to obtain sufficient proteins to be analyzed by the HTM (Section 3.1.3). We found that there was significant intraclonal variation in the sialylation (Fig. 5.12a) and productivity (Fig. 5.12b) in 24 subclones. In addition, we found that the sialylation is negatively correlated with

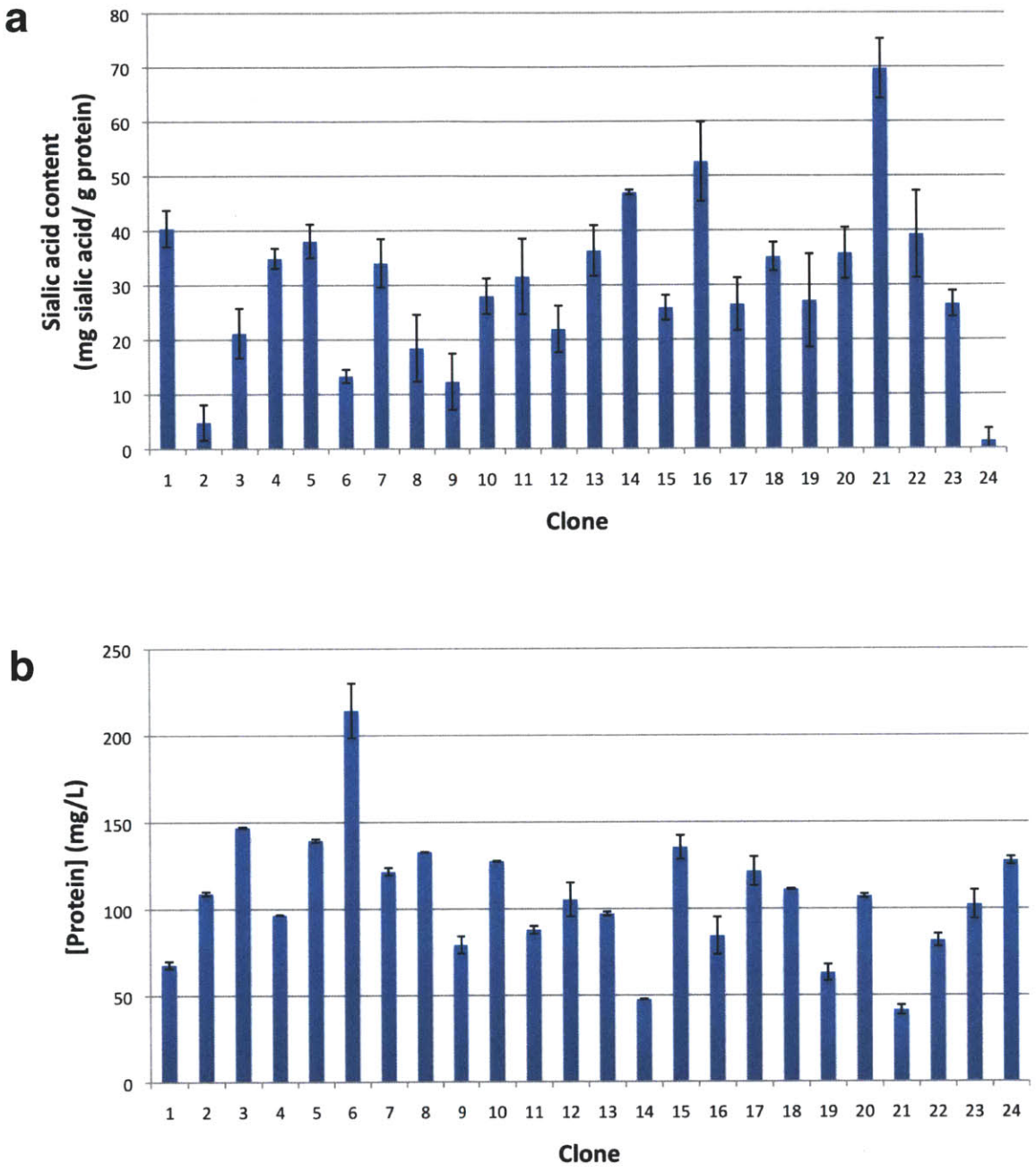


Figure 5.12. Intraclonal variability in CHO-IFN-γ cell cultures. Significant variability in overall sialylation (**a**) and concentration of total proteins (**b**) secreted by CHO cells was observed in 24 subclones. Error bars = SE, n = 2. The average sialic acid content among the 24 subclones is 30 ± 15 mg sialic acid/g protein (uncertainty = SD), and the average concentration of total protein is 106 ± 37 mg sialic acid/g protein (uncertainty = SD).

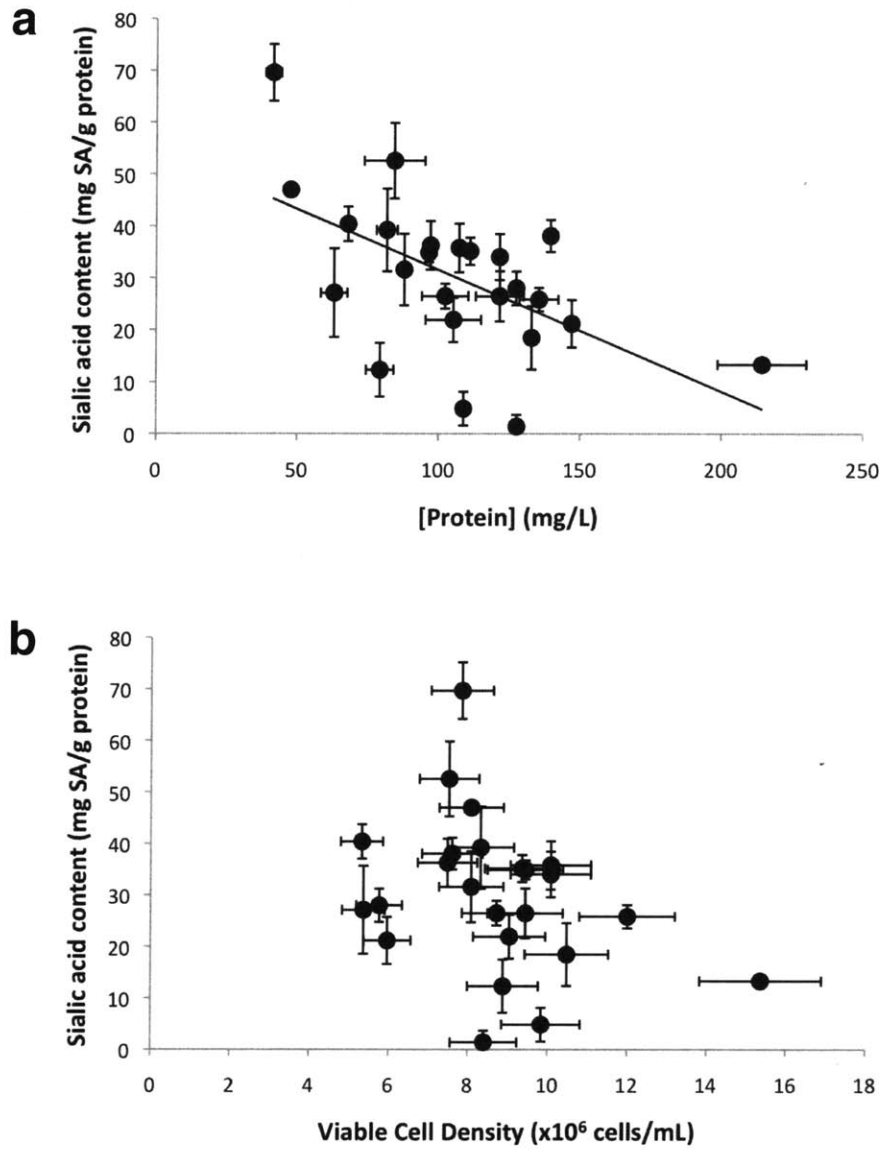


Figure 5.13. The overall sialic acid content is negatively correlated with total protein concentration (r = -0.6, P = 0.003) (**a**), but not viable cell density (r = -0.3, P = 0.126) (**b**). Error bars = SE, n = 2. $P < 0.05$ indicates that the correlation is statistically significant.

productivity (Fig. 5.13a), but not with viable cell density (Fig. 5.13b). The negative correlation agrees with previous studies showing that shorter golgi residence time, such as in high-producer cells, resulted in less sialylated proteins [38]. This variation is most likely due to epigenetics as the cells come from the same clone, and previous studies have shown that this intraclonal variability is also found in cell productivity [111,115]. In order to further investigate the reasons behind this variability, studies on the gene expressions of glycosyltransferases and nucleotide sugar transporters, as well as intracellular concentrations of nucleotide sugars are required.

Overall, HTM makes screening of thousands of cell clones based on protein sialylation feasible. The analysis of the subclones in this study could be finished in less than one day, while conventional methods will require at least 24 days. The HTM can therefore be used to rapidly analyze clonal and intraclonal variability for cell line development.

5.4. Conclusions

This chapter demonstrates that the HTM will be useful for process monitoring, characterization, and optimization to produce recombinant proteins with consistent and optimal sialylation. In addition, it can be used for screening cell clones and subclones in cell line development. It can be combined with protein quantification methods, such as Coomassie Plus Assay, for monitoring the sialylation of glycoproteins. Furthermore, the HTM can be used for studying the effects of various culture conditions on glycoprotein sialylation and identifying the optimum conditions. This study will be useful for analyzing the roles of many biological, chemical, and physical parameters on sialylation. Furthermore, molecular biology techniques [8,13,116,117] and computational analysis of glycosylation [37-39] provide strategies for engineering glycosylation pathways. The HTM can be used as a tool in further development of the biological techniques as

well as experimental validation of the computational analysis. Moreover, the HTM can accelerate cell screening in cell line development. Thus, the HTM has diverse applications in upstream and downstream bioprocesses.

Chapter 6

Conclusions and Recommendations

6.1. Conclusions

Sialic acid can affect many properties of therapeutic proteins, such as serum half-life [2], biological activity [3], solubility [4], resistance to thermal denaturation, and protease attack [5,6]. It is therefore important to ensure that the sialylation of proteins is consistent and optimum. To achieve these goals, high-throughput cell screening, monitoring, optimization, and characterization of bioprocesses are required. These tasks require high-throughput methods for analyzing sialic acid in hundreds of samples. Conventional methods for quantifying sialic acid, however, are time consuming because they require protein purification to remove interference by chemicals, such as glucose and pyruvate, in cell culture media. In order to rapidly analyze hundreds of samples, a high-throughput method (HTM) is required.

In this thesis, we develop a novel HTM for quantifying sialic acid content of glycoproteins produced in cell culture [101]. The HTM does not require protein purification to remove the interference by cell culture media, and can rapidly (15 min) analyze hundreds of samples in parallel. The HTM is specific to sialic acid bound to the glycoproteins. In addition, it is accurate relative to conventional methods, including TAA and an enzymatic kit. Moreover, it has quantitation limit of 2 μM sialic acid. This low quantitation limit means that the HTM is more sensitive than most of the sialic acid kits available today, and can be used for industrial applications.

We also used the HTM for various applications in bioprocesses. First, we used it for monitoring sialylation of sialic acid content of recombinant IFN-γ produced in a batch culture of CHO cell. Monitoring sialylation of recombinant proteins is crucial for bioprocesses because sialic acid content of the proteins usually decrease over time due to release of intracellular sialidase and accumulation of ammonia. The accuracy of the HTM was validated by TAA. We found that the normalized desialylation rates measured by HTM and TAA were similar, indicating that the HTM was accurate in monitoring sialylation of IFN-γ in CHO cell culture. In addition, we used the HTM for monitoring glycoprotein sialylation in CHO-IgG4 cell cultures. These studies demonstrate that the HTM can be used for monitoring sialylation of various recombinant proteins produced in mammalian cell cultures. The HTM can provide sialic acid content in 15 minutes, while conventional methods require more than one day, during which sialic acid content can decrease significantly.

Second, we used the HTM to study the effects of culture conditions on glycoprotein sialylation. In bioreactor operations, there are many chemical and physical parameters that can affect the sialic acid content of recombinant proteins. Understanding the effects of culture conditions on sialylation as well as bioprocess optimization are very important to produce proteins with consistent and optimum sialylation. We used the HTM to study the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialylation of glycoproteins produced in CHO cell culture. These compounds are precursors for several steps prior to sialylation of glycoproteins in the trans-golgi. Central composite design was used to obtain a surface response of CHO cell culture to feeding these compounds.

We found that feeding ManNAc, Cu^{2+}, and Mn^{2+} in combination increased sialylation from 20 to 36 mg sialic acid/g protein on the fifth day of batch CHO cell cultures. Moreover, feeding ManNAc and Cu^{2+} increased the sialylation, and the increase was higher when ManNAc and Cu^{2+} were fed together than separately. On the other hand, feeding ManNAc and Mn^{2+} in combination did not increase sialylation especially when both were added at concentrations more than 0.4 mM and 5 μM, respectively. Moreover, a quadratic least square model was used to estimate the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialic acid content of proteins produced in CHO cell culture. Lack of fit test shows that the model is statistically adequate in describing the data. The model also correctly estimates the above experimental observations. Furthermore, the model predicts that feeding 2 mM ManNAc and 100 μM Cu^{2+} can increase sialylation to 41 ± 4 mg sialic acid/g protein. This predicted value is close to the experimental value (35 ± 5 mg sialic acid/g protein). These results show that the HTM can be used for bioprocess characterization as well as fast bioprocess optimization. The HTM requires less than one day to finish the analysis, while conventional methods require more than 48 days.

Third, we used the HTM to perform high-throughput cell screening. In cell line development, screening hundreds of cell clones is required to obtain the best cell line for bioprocesses. Here, we used the HTM to measure intraclonal variability in sialic acid content of glycoproteins secreted in CHO cell culture. The CHO cell culture was diluted and then deposited in 96-well plates. 24 subclones were then expanded until sufficient amount of protein could be harvested for sialic acid quantification using HTM. We found that there was significant variability in sialic acid content and productivity. In addition, the sialic acid content was negatively correlated with productivity (r = -0.6), but not viable cell density (r = -0.3). This negative correlation agrees with

previous studies showing that shorter golgi residence time caused lower sialylation [38]. The HTM can finish the analysis within less than one day, while existing methods require more than 24 days. Overall, these studies demonstrate the versatility of HTM in bioprocesses.

6.2. Recommendations for future studies

6.2.1. Characterization of intraclonal variability

The results in Section 5.3 show that there was significant intraclonal variability in sialylation of proteins secreted in the CHO-IFN-γ cell cultures (Fig. 5.12). This intraclonal variability has also been found in CHO cell productivity [111,115]. In order to identify the reasons behind this invariability, further studies are required.

As described in Section 2.1.1, protein glycosylation depends on many factors, such as intracellular concentration of nucleotide sugar, availability of glycosyltransferases, nucleotide sugar transporters, and activity of glycosidases. Quantification of intracellular concentration of nucleotide sugars will explain whether the intraclonal variability was due to differences in the substrate availability. This study can be carried out using a well-established capillary electrophoresis method [62,118]. Furthermore, gene expressions of nucleotide sugar transporters, glycosyltransferases, and glycosidases are correlated with changes in glycosylation of recombinant IFN-γ produced in fed-batch CHO cell cultures [119]. Therefore, RT-PCR studies on these genes will reveal whether the intraclonal variability in the sialylation is due to differences in the gene expressions. Moreover, the activities of extracellular glycosidases can also affect protein glycosylation [2,23,104,119,120], and assays on the activities of the

glycosidases, such as sialidase, will describe whether the glycosidases can cause the intraclonal variability.

In addition to identifying the causes of the intraclonal variability, it is also important to study the dynamics of the variability. Pilbrough et al. found that intraclonal variability in CHO cell productivity was transient, and diminished significantly after approximately 30 days [111]. It is, therefore, important to study whether the intraclonal variability in sialylation was also transient. The results of this study will be important for cell line development, in which one needs to identify a stable cell line producing recombinant proteins with high productivity and proper glycosylation.

6.2.2. Responses of the subclones to feeding ManNAc, Cu^{2+}, and Mn^{2+}

The intraclonal variability measured by HTM varied significantly from 1 to 70 mg sialic acid/g protein (Fig. 5.12a). All of these subclones were derived from the same clone used in studying the effects of feeding ManNAc, Cu^{2+}, and Mn^{2+} on sialylation of proteins. The results in Section 5.2 show that feeding ManNAc, Cu^{2+}, and Mn^{2+} increased sialylation of proteins secreted in CHO-IFN-γ cell cultures. It will be interesting to identify how these subclones will respond to the feeding. In addition, studies on feeding these chemicals on sialylation of proteins by the subclone corresponding to 70 mg sialic acid/g protein may reveal the likely upper bound of the overall sialylation by the CHO-IFN-γ cell line. Studies proposed in Section 6.2.1 can also be carried out here to identify the causes and dynamics of the intraclonal variability in the responses to the feedings.

6.2.3. Effects of feeding Cu^{2+}, cytidine, and DEX on glycoprotein sialylation

The feeding strategy discussed in Section 5.2 focuses on the effects of ManNAc and metal ions on sialylation of proteins produced in CHO-IFN-γ cell cultures. As discussed in Section 2.1.3, there are other chemicals capable of increasing sialylation, such as cytidine [62] and DEX [74]. Cytidine can increase intracellular concentration of CMP-sialic acid, and DEX can increase the gene expression of β1,4-galactosyltransferase and α2,3-sialyltransferase, as well as reduce extracellular sialidase activity. In addition, feeding cytidine and DEX is cheaper than feeding ManNAc. ManNAc costs $28/g and is typically supplemented at 4.4g/L [61,62], while cytidine costs ~$7/g and is typically supplemented at 2.4 g/L [62], and DEX costs $0.2/mg and is typically supplemented at 3.9 mg/L [74]. Thus, feeding Cu^{2+}, cytidine, and DEX may be a better strategy than feeding ManNAc and Cu^{2+} to increase sialylation. Moreover, further studies should be carried out to examine the reasons behind the effects of feeding cytidine and DEX on sialylation. The intracellular concentration of CMP-sialic acid can be quantified using capillary electrophoresis [62,118] to confirm that cytidine can increase sialylation by increasing CMP-sialic acid. In addition, extracellular sialidase activity assay, RT-PCR on gene expressions of β1,4-galactosyltransferase, and α2,3-sialyltransferase will explain whether the DEX can increase sialylation by the mechanism described above.

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