APPLICATION OF MASS SPECTROMETRY IN QUANTITATIVE GLYCOMICS: QUANTITATIVE ISOBARIC LABELING (QUIBL)

by

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(Under the Direction of Ron Orlando)

ABSTRACT

Mass spectrometry (MS) has become a highly informative analytical tool to provide structural and quantitative measures for glycomics. In this research, an MS-based quantitative isobaric labeling approach (QUIBL) was developed to identify and quantify glycans in complex mixtures. This method introduces isobaric labels, $^{13}CH_3$ or $^{12}CH_2D$ in the process of permethylation of glycans. Modified glycans are then mixed and analyzed by a hybrid mass spectrometer. Structural identification of glycans was manually performed based on the molecular weight and fragmentation information obtained by MS and MS^n experiments. Quantitation was calculated for each identified glycan, that is, the ratio of the sum of the intensities of all ^{13}C-labeled isotopomer ions to that of all D-labeled ions. N-linked glycans extracted from fetuin, human blood serum and embryonic stem cells were analyzed. The reproducibility and accuracy of QUIBL were confirmed over 2 orders of magnitude. Then the capability of QUIBL for the relative quantitation of glycans in isomeric mixtures was validated using milk oligosaccharides Lacto-N-fucopentaose I (LNFP I) and Lacto-N-fucopentaose II (LNFP II).

INDEX WORDS: Mass Spectrometry, Glycomics, Quantitation, N-glycans, Isobaric labeling, Isomeric glycans

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DEDICATION

To My Mother: Yuhua Zhang, for your unconditional love throughout my life. Though the surrounding tough living conditions, you sacrificed to provide me a world to grow up and to be an honest person.

To My Husband: Jiang He, for your generous acceptance and firm support. Deep in my heart, you are the one who inspires our belief and hope for the future.

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CHAPTER 1

INTRODUCTION

Mass spectrometry (MS) is currently a widespread analytical tool used for the large scale analysis of large and nonvolatile molecules, such as proteins, peptides and oligosaccharides. Analogous to proteomics, glycomics is the systematic study of the glycans in an organism. It is reported that more than 50% of eukaryotes proteins are glycosylated.[1] Glycans dramatically enhanced the molecular and functional diversity of proteins due to the inherent heterogeneity and structural diversity.[2] Glycoprotein glycans serve important roles in many biological activities. As one critical task, quantitative comparison of glycan expression requires sensitive and efficient strategy. Herein, a MS-based advanced quantitative approach was developed for quantitative glycomics.

Quantitative isobaric labeling (QUIBL) was demonstrated to be a rapid and high-quality identification and quantitation of glycans. N-linked glycans from standard glycoprotein and biological samples were used to perform QUIBL. The general strategy for N-glycan analysis using MS is very mature.[3-5] QUIBL incorporates light and heavy isobaric tags onto glycans for quantitative purpose. Isobaric compounds have the same nominal mass, but their exact masses differ by a tiny number. After labeling, equal amounts of the isobarically labeled N-glycans are combined and analyzed by a hybrid mass spectrometer, linear ion trap and Fourier transform ion cyclotron resonance MS (LTQ-FTICRMS). Structural interpretation of MS and MS/MS spectra

allows identification of individual glycan.[6,7] Relative quantitation is performed by comparing the intensities of the isobaric species for each identified glycan. QUIBL was verified to be a sensitive and efficient quantitative approach to quantify a broad range of glycans from complex samples.

QUIBL is capable of providing the relatively quantify isomeric glycans in mixtures. As known, structural isomers with different arrangements, linkages and branching can be determined by their unique fragment ions observed by MS/MS or MS^n.[8, 9] Quantitation of isobrically labeled unique fragment ions can be used to quantify the relative abundance of precursor isomer in different isomeric mixtures. Milk oligosaccharides Lacto-N-fucopentaose I (LNFP I) and Lacto-N-fucopentaose II (LNFP II) were used to validate QUIBL for quantitation of isomeric glycans.

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CHAPTER 2

LITERATURE REVIEW

Mass spectrometry (MS) has been one of the most important techniques in proteomics and glycomics because of its ability to identify individual peptides, proteins or glycans in a variety of complex biological samples. In this chapter, the basic principles and construction of MS are reviewed, including the ionization methods and common mass analyzers. This will be followed by a review of the role MS plays in proteomics and glycomics.

2.1 MASS SPECTRAMETRY (MS)

Mass spectrometry has gradually developed into a technique with a broad range of applications since its introduction by J. J. Thomson over one hundred years ago.[1] For instance, MS is currently an indispensable tool in the structure analysis of proteins, peptides and oligosaccharides, because this approach provides high sensitivity and high mass accuracy. MS is used to determine the mass of a molecule. Typically, a mass spectrometer consists of three procedures. First, the sample is converted into gas-phase and charged species in the ion source. Second, all ions are introduced into a mass analyzer, which separates ion species based on their mass-to-charge ratio (*m/z*) in vacuum. Last, the ions are detected and results are exported in the form of a mass spectrum. The following sections will discuss the operation principles and characteristics of current ionization approaches and popular mass analyzers.

Ion Source

The first step of MS analysis involves the production of gas-phase ions, which is a requirement imposed by the mass analyzer. Early MS techniques required samples to be volatile due to conventional ionization methods such as chemical ionization (CI) or electron ionization (EI). However, most biological samples are nonvolatile, polar and thermally unstable, which were then not amenable to being analyzed by MS using these ionization techniques. In 1980's, MS gained a breakthrough with the invention of other ionization methods. In particular, matrix-assisted laser desorption/ionization (MALDI)[2, 3] and electrospray ionization (ESI)[4], result in the widespread application of MS in the analysis of nonvolatile macromolecules. These ionization techniques are both soft-ionization techniques, resulting with little or no fragmentation.[5, 6]

MALDI was demonstrated in 1985 by Franz Hillenkamp, Michael Karas and their colleagues.[7] In 1987, Koichi Tanaka and his co-workers developed MALDI to ionize a protein with the proper combination of laser wavelength and matrix.[8] The sample preparation involves the crystallization of the sample in a low molecular weight ultraviolet-absorbing matrix followed by the irradiation with a laser beam to generate gas-phase ions. The function of the matrix is to isolate the sample molecules from each other for lower desorption energy, and to absorb laser light at a wavelength at which the analyte is only weakly absorbing. Absorption of energy from the laser beam causes evaporation and ionization of analytes.[9] Different lasers can be used in MALDI, of which ultraviolet lasers, such as Nitrogen lasers, are the most common. This ionization technique has an advantage that the ionization efficiency isn't affected by the increase

of the mass and size of the molecules,[10] but it is balanced by the disadvantage of the presence of metastable ions formed by ions decomposing during flight. MALDI predominantly produces intact singly charged molecular ions. MALDI MS is reasonably tolerant toward the presence of salts, buffers and other addictives.[11] This method is very sensitive in the range from 100 femtomole to 2 picomole. MALDI is very useful as one of the two widely used ionization methods for biopolymers and organic molecules for MS.

ESI was introduced as an important interface for biological macromolecules solution samples to MS by John Bennett Fenn and coworkers.[12, 13] In ESI, a stream of liquid containing the sample is passed through a capillary. When a high voltage is applied to the capillary, an electrospray is produced. A flow of dry nitrogen is often used to expedite desolvation of the solution and droplet shrinkage. Finally, the analyte molecules are stripped of solvent molecules to form gas phase ions.[14] There are two major models for the production of gas-phase ions: charged residue model (CRM)[15] and ion evaporation model (IEM)[16]. In the CRM model, charged droplets shrink in size because of solvent evaporation followed by fission into smaller highly charged droplets because the repulsive Coulombic forces exceeds the droplet surface tension. After several cycles of evaporation and fission, droplets contain one analyte ion. Gas-phase ions are formed after the remaining solvent molecules evaporate. In IEM model, the evaporation and fission cycles happen as in CRM. But IEM proposes that electric field strength is enough to cause direct ion desorption after multiple cycles of evaporation and fission. ESI generates multiply charged ions, which allows the analysis of very large molecules even if the mass range of the mass analyzer is small. This ionization technique can be coupled with almost all available

mass analyzers. ESI has another virtue that it can be an real-time interface coupling liquid separation techniques, such as HPLC with MS[17, 18]. Nanoelctrospray (nanoESI) utilizes different conditions by significant reduction in the needle tip diameter from 20~150 μm to 2~50 μm and the flow rate from 1000~10 μl/min to 1000~10 nl/min,[19, 20] Nanospray has advantages of high sensitivity at femto-attomole level, better tolerance toward buffer and lower sample requirements compared to low-flow rate ESI.[21, 22] NanoESI MS and MS/MS are significant analytical techniques.

Mass Analyzer

The heart of the mass spectrometer is the mass-to-charge analyzer. Diverse mass analyzers are commercially available and extensively applied into practical research. Versatile research demands can be satisfied with appropriate choice of mass analyzer. Each type of mass analyzer has its own advantages and disadvantages as will be explained in the following sections.

Quadrupole. The quadrupole mass analyzer is a low-cost, compact size instrument. It utilizes a low electric field 2~50 V to accelerate ions from the source into the analyzer which consists of four parallel arranged cylindrical rods. (Fig. 2.1) Opposite pairs of rods are electrically connected. Direct current (DC) and radio frequency (RF) voltages are applied to these two pairs of electrodes and produce an oscillating electric field, in which ions undergo a complex set of three-dimensional-wave motions. Usually the ratio of the RF and DC potentials is kept constant. Only ions having stable trajectories in the oscillating electric fields are capable of passing through the quadrupole to reach the detector. The voltages of RF and DC are scanned while measuring the detector signal to collect a complete mass spectrum. The quadrupole has the upper

mass limit varying from 300 to 4000, mass accuracy of 0.1~1Da and unit mass resolution.[23, 24] According to its features, quadrupole is a portable device and very easy to operate.

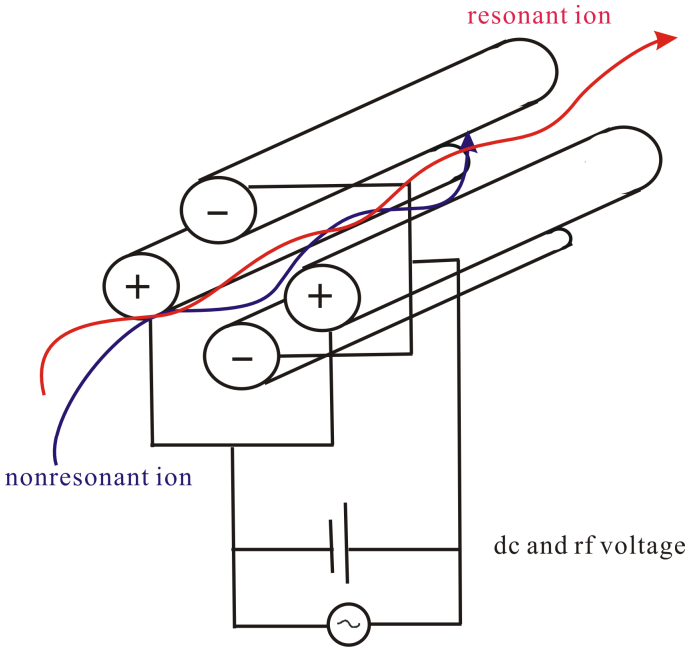

Figure 2.1 A schematic of quadrupole mass analyzer

Time-of-flight (TOF). TOF is the simplest mass spectrometer, which determines an ion's m/z ratio from its flight time through fixed field free drift region. Ions are accelerated through a fixed voltage which ranges from 2 to 25 kV into the region. Since the ions with the same charges gain the same kinetic energy from the acceleration procedure, the lower *m/z* the ion is, the higher velocity the ion has. The signal is measured as a function of the flight time, which is related to *m/z* as the following equation.

$$t = \frac{d}{\sqrt{2U}}\sqrt{\frac{m}{z}}$$

Where t is the time needed to fly the field-free region distance d, U is the strength of the electric field, and t. The flight time of the ion is proportional with the square root of its mass-to-charge

ratio. TOF theoretically has an unlimited mass range, and is able to analyze ions at several hundred thousand *m/z*. Mass accuracy can be in the tens of ppm. Because all ions are transmitted to the detector, TOF has a high sensitivity.[23] MALDI is usually applicable to TOF.[25]

Linear TOF has a very poor resolution of about 500 ΔM/M units as a result of peak broadening due to spread in the initial positions of the ions in the ion source and dispersion in initial ion kinetic energy prior to acceleration.[26] Better resolution performance is obtained by special technical treatments: delayed extraction and reflectron (electrostatic mirror).[27] Delayed extraction means that the acceleration voltage of the electric field used to extract ions out of the source is switched on some hundred nanoseconds to several microseconds after the laser pulse has been operated.[28] Ions are generated in the source region and obtained their initial velocity. For ions of the same *m/z*, those having a higher initial kinetic energy move faster and then are closer to detector than those having less initial energy. After a selected time period, the delayed extraction pulse is applied to compensate for this spread in kinetic energies. Finally, the ions with the same *m/z* are focused as a package before reaching the detector. Using this technique, the resolution of TOF can be 3000~6000 unit.[29] However, this technique obviously only works properly for a limited mass range for a fixed delay setting.[30]

The other approach to improve the resolving power of a TOF is the reflectron, which is located at the end of the flight region. The reflectron is an electrostatic field which reflects the direction of motion of ions towards the detector. The ions with higher initial kinetic energy penetrate deeper into the electrostatic mirror and spend a longer time in the electrostatic mirror. Inversely, ions with lower initial kinetic energies will reach the reflectron later and spend less

time in the electric field. Consequently, ions of identical *m/z* with lower or higher initial kinetic energy will hit the detector surface at the same time.[23] An additional benefit of reflectron is to increase the resolution by increasing the flight-path length in a given length of flight tube. All of the above operation modes lead to a narrowing of the peaks and high resolution about 10^4. One shortcoming of reflectron is the m/z value can be alternated when ions fragment in the flight region, resulting in reduced signal sensitivity.[30]

Quadrupole Ion trap (QIT). QIT is a three dimensional analogue of a quadrupole mass analyzer. QIT consists of two endcap electrodes and a ring electrode (Fig. 2.2). DC and main RF voltages are applied on the ring electrode to form a potential well in the center of the analyzer. One torr of helium is used to reduce ions kinetic energy and to focus ions the center of the trap. The stability diagram provides a straightforward view of ions trajectories inside the three dimensional electric field.[31] The coordinates of the stability diagram are Mathieu parameters "a_z" and "q_z" .[32, 33]

$$a_z = \frac{-8eU}{mr_0^2\omega^2} \quad ; \quad q_z = \frac{4eV}{mr_0^2\omega^2}$$

Where V is the amplitude of RF voltage applied to ring electrode, U is amplitude of DC voltage applied to ring electrode, r_o is the hyperbolic radius of the ring electrode, ω is the angular frequency.

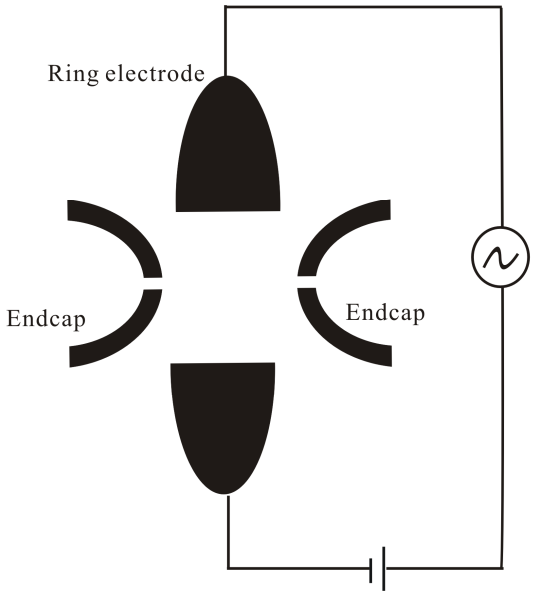

Figure 2.2 A schematic of ion trap mass spectrometer

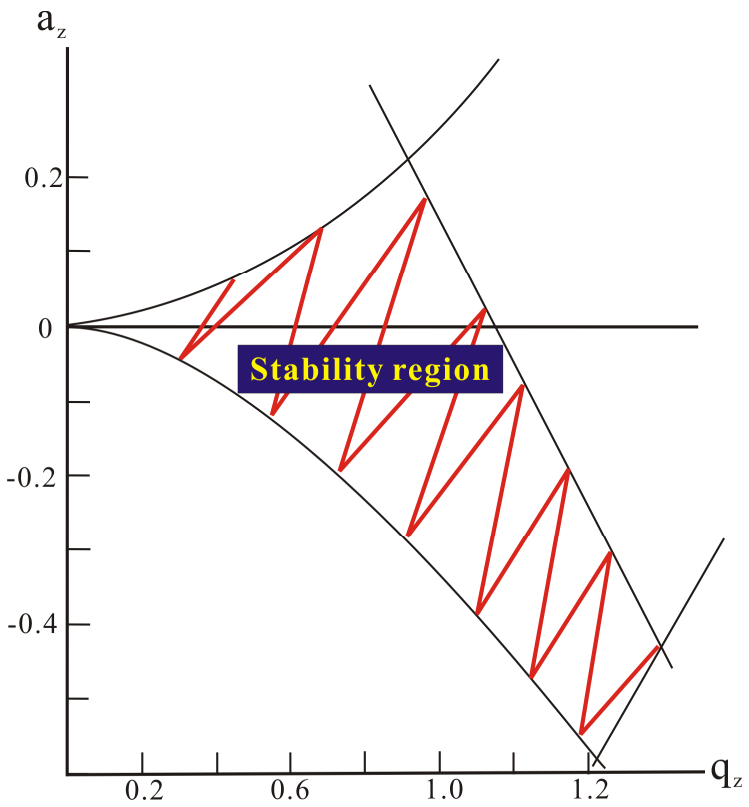

Figure 2.3 Stability diagram for the three-dimensional quadrupole ion trap.

QIT separates ions by selective ejection of ions out of the trapping to the electron multiplier detector.[34] As alternating the amplitude of the DC and main RF fields, ion packets

are ejected out for detection based on their stability trajectories. Additional alternating current (AC) of selected frequencies and amplitudes can be applied on the endcap electrodes which can be used to induce resonance ejection that extends the mass-to-charge range from 3000 to 6000 *m/z*[23] or the resonance excitation that fragment ions. The mass spectrum is obtained by scanning the fields at which ions are ejected from the analyzer. QIT is relatively inexpensive, very sensitive, robust tool for biochemical analysis.

There is another configuration of IT. Linear ion trap (LIT) has a set of quadrupole rods to confine ions radically and two end electrodes to confine ions axially. LIT allows a larger volume chamber, in possession of improved trapping efficiency and increased ion storage capacity. LIT provides higher sensitivity, resolution and mass accuracy at similar mass range than conventional 3-D ion trap.[35, 36]

Fourier transform ion cyclotron resonance (FTICR). FTICR[37] provides ultra high resolution with the usage of a superconducting magnetic field. The ICR cell is placed in the center of the field having great magnetic strength and homogeneity along the vector of the magnetic field. Several analyzer cell designs have been developed. Two general types of analyzer cells are cubic cell and open end cylindrical cell.

The cylindrical ICR cell contains trapping electrodes at two ends, excitation electrodes and detection electrodes. Static electric fields keep the ions trapping along the magnetic field axis. Four electrodes are parallel to each other, and opposite electrodes are connected together as the excitation pair and detection pair. (Fig. 2.4) The moving ions are subject to a Lorenz force and

perform the cyclotron motions in plane which is perpendicular to the vector of the uniform magnetic field. (Fig 2.5).

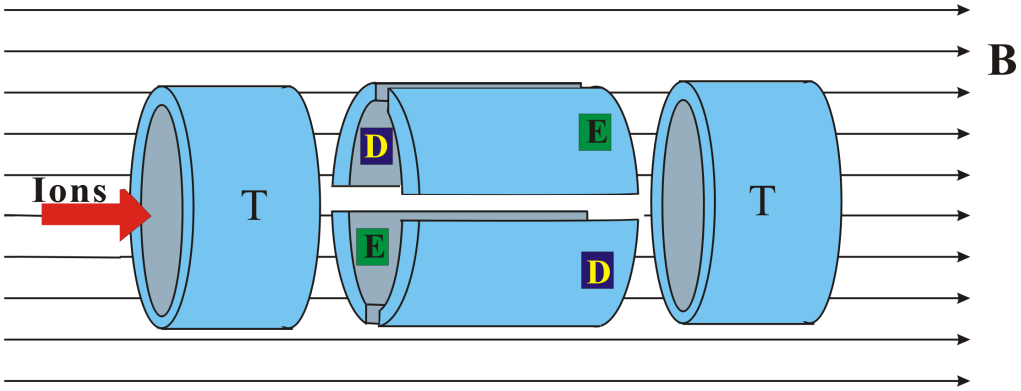

Figure 2.4 Diagram of an cylindrical ion cyclotron resonance cell. E, excitation plates; D, detection rods; T, trapping plates.

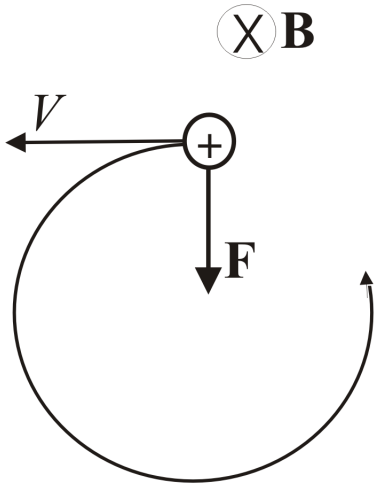

Figure 2.5 Ion cyclotron motion.

The frequency of the ion cyclotron motion is dependent on the ion's mass-to-charge ratio and the magnetic field strength as following equations[38, 39]:

$$\mathrm{F} = qV\mathbf{B} = \frac{mV^2}{r} \quad ; \quad \omega_c = \frac{V}{r} \quad ; \quad \omega_c = \frac{\mathrm{B}}{m/q}$$

Where F is the given force to the ion; m, q, and V are ionic mass, charge, and velocity; B is the spatially uniform magnetic field; ω_c is the angular cyclotron frequency.

By applying an RF voltage to the pair of excitation plates, ions in the cyclotron can be excited nearly simultaneously to larger orbits. Ions at the same frequency as the RF irradiation can absorb energy, which causes an increase in the kinetic energy of the ions. Upon excitation, trapped ions undergo an expansion in the radius of their cyclotron orbit.[38] The ions of the same *m/z* circulating near the cell wall induce an image current to the detection electrodes. The ultra high vacuum of 10^{-9}~10^{-10} Torr is required to eliminate ion-molecule collisions during the detection period, which has a duration of 100 ms ~10 s. The image current alternates at the same frequency as the ion cyclotron frequency. Thereupon, a simultaneous detection of a large frequency range is measured. During the data processing, the spectrum of amplitude as a time function is converted into a amplitude as a function of frequency through application of Fourier transform and then into a mass spectrum based on the relationship between frequency and *m/z*.[23] A feature of this unique ion detection method by image current is that longer analysis time leads to higher mass resolution. The accuracy and precision of the measurement increase with the duration of the acquired signal. These aspects result in the ultra high resolving power up to 10^6. FTICR provides high sensitivity around attomole and high mass accuracy. The drawbacks are the high cost and the maintenance efforts, and low throughput.

MS/MS and MS^n. MS/MS and multiple MS stages (MS^n) are powerful techniques for further significant structural analysis of biopolymers, which can be achieved by tandem-in-space (by coupling more than one mass spectrometer) and tandem-in-time (by performing a sequence of MS events in an ion storage device) spectrometers. Tandem-in-space mass spectrometers generally include triple-quadrupoles (QqQ), quadrupole time-of-flight (Q-TOF), and TOF/TOFs.

Tandem MS can also be accomplished in one mass analyzer over time, such as QIT and FTICR. There are a variety of ways to fragment precursor ions for tandem MS, of which collision induced dissociation (CID) or collision activated dissociation (CAD) is the most popular and can be applied in almost all mass analyzers. FTICR has distinctive features to form fragmentation ions other than CID due to high vacuum system of ICR difficult for gas inlet or release cycle. FTMS utilizes infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD) techniques, which produce different fragment ions spectra from those by CID to provide complementary fragmentation information.

In a MS/MS CID experiment, the precursor ion of interest is isolated and enters the collision cell, or in the case of QIT and FTICR, the precursor ion is isolated and stored in the trapping cell. Inert collision gas, such as Ar, He, or N_2 is used to collide with the precursor ion at low energy (1~100eV range) or high energy (KeV range). The collision converts a part of the translational energy of the ion to internal energy which causes bond breakage and the fragmentation of the precursor ion into smaller fragments. MS^n (*n* is equal to the number of stages of MS) can be performed depending on the setting of mass spectrometers.

2.2 PROTEOMICS

Proteomics is defined as the large-scale study of the proteins being expressed in a cell, tissue, organism, etc.[40, 41] Proteomics has three main tasks which are large-scale identification of proteins and their post-translation modifications, differential protein expression and protein-protein interactions. The rise of proteomics profits from databases created by

whole-genome sequencing projects, and from the rapid development of biological mass spectrometry.[40]

MS is one of the most informative methods for proteomics. Innovations in the ionization methods and analyzers of MS extended its capability to achieve fast, high-throughput and automated protein analysis.[42-44] There are two main MS-based strategies: top-down and bottom-up proteomics.[45] In the top-down approach, intact proteins are analyzed. But in the bottom-up approach (referred to as "shotgun proteomics"), proteins are proteolytic digested into peptides before analyses.

Top-down Proteomics. A well-established top-down strategy is a combination of two dimension gel electrophoresis (2DE) and MS. Another strategy is to introduce intact proteins into mass spectrometer as gas phase ions and to analyze directly protein ions.

2DE was introduced in 1970s[46, 47] to separate proteins by two dimensions. The first dimension separation is by isoelectric points (pI). The sample is loaded onto an immobilized pH gradient (IPG) strip in the separation medium and then separated under an electric field. The principle is that the protein migrates and focuses on their isoelectric point where the net charge of protein is zero. In the second dimension, the proteins are separated by sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) based on molecular weight (M_r). The gel from the first step separation is treated by a buffer containing the SDS detergent. SDS binds to and unfolds the protein, making an approximately uniform mass to charge ratio for each protein. Under the applied voltage, proteins are fractionated. Hence, the migration length of proteins is assumed directly related to the size of the proteins. Smaller proteins move faster through the gel whereas

larger molecules move slower.[48] Protein spots formed by 2DE can be visualized by staining and be relatively quantified by the staining intensity. To identify proteins of interest, the protein spot is excised and degraded in-gel by sequence-specific tryptic digestion. Next, peptides are extracted then analyzed by MS or tandem MS. Practically, MALDI-TOF is usually coupled with 2DE. This protein-expression mapping strategy, 2DE/MS or 2DE/MS/MS-based sequence identification of separated proteins has been applied to catalog a large numbers of proteins in a complex sample.[49-51] However, this approach has shortcomings. 2DE/MS is not truly global and high-throughput method. In 2DE, it is reported that up to 11,000 protein spots resolved, which only represents 25% of total proteins.[52] Low abundance and hydrophobic proteins are difficult to identify.[53, 54] In addition, proteins with extreme pI and molecular weight will not be retained in the gel.[48]

In another top-down approach, intact proteins are analyzed by mass spectrometer. Protein ions are trapped and directly fragmented into peptide ladders. MALDI-MS and ESI-MS have been successfully used to ionize proteins. ESI is preferred, as MALDI MS produces broad peaks and low sensitivity for proteins over 30 kDa.[55] However, it is difficult to produce extensive fragment ions of intact large proteins and to determine molecular weight of typically heterogeneous large proteins. Endeavors were made to improve the dynamic range of fragment ions in MS/MS for proteins over 200 kDa[56], as well as to explore the large scale direct fragmentation of proteins for PTMs detection using ESI FTMS[57]. The high resolution measurement of molecular weight value of intact proteins can be obtained by FTICR MS.[58] This type of top-down experiment has the informatics advantage of complete coverage of an

entire protein sequence and confident assignment of posttranslational modification (PTM) sites. The difference between the measured molecular mass and the theoretical mass by the sequence database straightforwardly indicate the PTM(s) or sequence error. And the relevant difference in masses of the fragment ions shows the location of PTM.[58] The routine application of top-down proteomics still remains challenges for interrogating complex mixtures.

Shotgun Proteomics. Alternative protein profiling strategy is needed for high-throughput study of complex protein mixtures. Hunt exploited the use of a liquid chromatography and tandem mass spectrometry (LC-MS/MS) for complex peptide sample.[59, 60]

The LC-MS/MS method is variously referred to as bottom-up or shotgun proteomics, involving in-solution proteolysis of a complex mixture of proteins followed by peptides separated chromatography before MS/MS analysis. To improve the peak capacity, protein and peptide separation platforms are deployed. Frequently, proteins are purified and separated by protein chromatography or organelle purification. As a variation, proteins can be fractionated by 1D SDS-PAGE prior to digestion.[61] Currently, two-dimensional or multidimensional chromatographic separations of peptide mixtures are popularly used even without gel electrophoresis separation prior to digestion.[62-64] Reverse phase liquid chromatography (RPLC) is the most common used, which separate peptides by hydrophobicity. Besides, strong cation exchange (SCX) chromatography or size exclusion chromatography (SEC) can be orthogonal coupled with RP. SCX separates peptide based on number of basic residues present on the peptides.[65, 66] The separation is performed online, which means HPLC connected directly to the mass spectrometer. The separated peptides elute directly into the mass spectrometer for analysis.

ESI ion trap is commonly used because of its compatibility with online HPLC and automated data acquisition programs. LC-MS/MS methods have higher sensitivity about 1-10 fmol when applying low-flow rate nanoESI.

The capability of shotgun proteomics was successfully proven for global protein identification including low abundance proteins.[67]

Peptide Massfingerprint (PMF) and Peptide Sequencing. There are two main approaches for MS-based protein identification. The first is peptide mass fingerprinting (PMF).[68] MALDI-TOF is usually utilized for PMF to identify a protein. In this method, a list of mass of experimental peptides is generated from mass spectrum of the peptide mixture. The set of masses is then compared with all theoretical peptide masses of each protein encoded in the database. The candidate proteins can be exported in the order of the number of peptide matches, which is accomplished by a scoring algorithm. PMF requires essential purification of target protein, so the technique is generally coupled with proteins separated by gel electrophorises.[44]

The second method is searching CID fragmentation data generated by MS/MS of peptide mixtures against the fragmentation ion database to determine peptide sequence.[40] There are three breakages on the backbone of peptides by the collision. (Fig. 2.6)

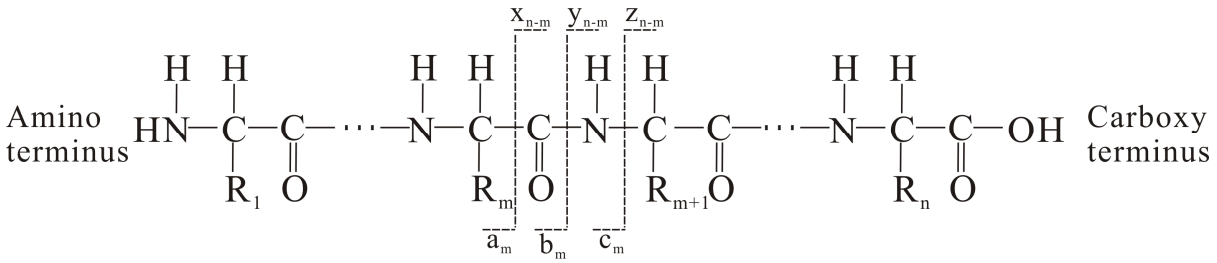

Figure 2.6 Peptide fragmentation in the mass spectrometer.

Ions containing amino-terminal amino acid are denoted by a, b, c; other ions containing carboxy-terminal amino acid are x, y, z ions, respectively. Breakage at the bond between the alpha carbon and the carbonyl carbon gives rise to a- and x-ion series; at the carbonyl carbon and amide nitrogen bond produces b- and y-type ions; and at the amide nitrogen and alpha carbon gives c- and z-type ions. Sequence specific trypsin is the most common enzyme used in proteomics,[48] and peptides produced by proteolysis with trypsin are inclined to form b- and y-ions. The tandem mass spectra are correlated to protein sequence database via computer programs for the large-scale identification of proteins.

Data Analysis. The data collected by a typical proteomic analysis is huge. So it is important to design computer algorithms which automatically search peptide sequences against databases and identify in principle proteins in the mixture.[69, 70] Databases contain all the probable peptide sequences of proteins that can be expressed in the sample from the genome or expressed sequenced tags (EST). The most successful algorithms are Mascot and Sequest.[71-73] In the search, the experimental MS/MS spectra of the peptide are compared with predicted fragmentation spectra of candidate peptides in the database. The similarity or the probability of these candidate peptides is ranked by Mascot or Sequest prior to report the hit list of possible peptides. Based on the list, protein identification is obtained. Moreover, these identifications need to be validated for high confidence acceptances using different algorithms. So a randomized protein data base which is created by inverting each protein sequence contained in the normal database or a totally different random database, is used for the reverse search trial.[74] All identifications against the random database are incorrect which provides an estimate of false

positive. An estimate probability threshold would be set to control the desired false positive rate for confident assignments.[75, 76] At present, innovative strategies are needed to improve the sensitivity and accuracy of the peptide identification, to discriminate the protein isoforms, to and to identify the covalent modification of peptides.[70]

In conclusion, the advancement of MS-based proteomics relies on the fast progress in the MS instrumentation, and available automation tools for daunting amount of data analysis.

2.3 GLYCOMICS

With the deep development of genomics and proteomics, the field of glycomics has gained more and more attention of researchers. Glycomics emerges at the end of the 20th century, which is analogous to genomics and proteomics. Its target is the glycome – the whole set of glycans synthesized in a single or defined biological source. The study of glycomics comprises the characterization of glycans and the functional analysis of the role of glycans in the biological events.

Glycans usually exist as forms of glycoconjugates located on cell surfaces and in extracellular materials,[77] and also as free glycans found in bodily fluids. It is estimated that, more than 50% of mammalian and plant proteins are glycosylated. [78, 79] Glycosylation, as a ubiquitous post-translational modification (PTM), significantly enhance the molecular and functional diversity of proteins.[80] Protein glycosylation occurs in the endoplasmic reticulum (ER) and Golgi compartments of the cell and involves membrane-bound glycosyltransferases and glycosidases, most of which are sensitive to the surrounding environment. Therefore, glycans vary in different cell types and cell physiological states, with different expression level.

Glycans are implicated in a variety of biological processes, such as cell growth and development, cell-cell recognition, intracellular signaling and cell-matrix interaction.[81-84] Compared to nucleic acids and proteins, the study of glycans is the most challenging owing to the complexity of glycan structures and diversity of *in vivo* glycosylation. First, as post-genomic products, the structures of glycans are not predictable. The biosynthesis of glycans is nontemplate-driven, which is regulated by certain glycotransferases, glycosidase and carbohydrate enzymes encoded from a small portion of genes. Second, the enormous structural heterogeneity is as a result of several aspects: constituent carbohydrate isomers, like glucose (Glc), galactose (Gal), mannose (Man); distinct arrangements of the same components; multiply linkage points; α or β anomeric linkage configurations; and a wide range of branches. For an instance, the chemical structures of an oligosaccharide composed by six monosaccharides could theoretically be more than 1×10^{12}. Third, attachments of different glycan chains into the protein which has several glycosylation sites with various susceptibilities to glycosylation would lead to a considerable number of glycoforms. These inherent features render the lagging phase of glycomics behind the genomics and proteomics. And it is necessary to release glycan motifs to screen the glycome of specific biological source.

Functional glycomics emerges as a bridge correlating glycomics with biomedicine and biology field. It includes the analyses of glycan structures and investigation of the functional role of glycans. Furthermore, the in-depth and high-throughput development of glycomics necessarily encompasses pertinent researches in genomics, proteomics, transcriptomics strategies as well as bioinformatics.[85]

Glycosylation alteration has been identified in some systemic genetic disease like congenital disorder of glycosylation (CDG) syndrome, caused by genetic defects affecting the activity of specific glycotransferases in biosynthesis pathways of glycans.[86] Also, aberrant glycosylation has been implicated in many types of cancer, where many carbohydrate epitopes are involved in all steps of tumor progression, from tumor cell proliferation and tumor cell adhesion to metastasis.[81, 87-89] With the awareness of the different expression levels of glycosylation in diseases, extensive biomedical and biological applications have been opened up for glycomics.[90] Biomarker discovery for diagnosis and prognosis, new glycan therapeutics have become the frontier in glycomics.[91-93]

Glycosylation

Glycans are produced via specific *in vivo* biosynthesis pathways in the presence of enzymes, which gives rise to limited possible glycan structures and facilitates the interpretation of glycan populations.[94] Protein-bounded glycans are classified into five groups: 1. N-glycans, which are attached to the amide nitrogen of Asn in the sequon –Asn-X-Ser/Thr, where X can be any amino acid except proline. In some rare cases, N-glycosylation can occur even when the serine or threonine residue is replaced with a cysteine as Asn-X-Cys.[95-97] 2. O-glycans which are attached to a hydroxyl group of Ser/Thr. They are less branched than N-glycans. There is no consensus amino acid sequence for O-glycosylation sites. 3. Glycophosphatidylinositol (GPI) anchors, which link lipids to the carboxyl terminus of proteins and serve to anchor these proteins to cell membranes. 4 Glycosaminoglycans (GAGs), long unbranched polysaccharides containing a repeating disaccharide unit. GAGs link to the hydroxy oxygen of serine; 5. C-mannosylation. A

mannose sugar attaches to the carbon of tryptophan residues in Thrombospondin repeats. N-glycosylation is very important, which determine and influence protein folding, stability, trafficking and localization. It happens to nearly all proteins that travel through the endoplasmic reticulum (ER)-Golgi conduit.

The US Consortium for Functional Glycomics (CFG) is a large research organization to understand the role of carbohydrate–protein interactions at the cell surface in cell-cell communication. It has announced a common nomenclature for convenient annotation of N- and O-glycans. Generally, monosaccharide residues include N-acetylgalactosamine (GalNAc); glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc) and N-acetylneuraminic acid (Neu5Ac, sialic acids). Glycans are often represented by their composition. The description Man5GlcNAc2Fuc1 indicates a glycans consisting of five mannoses, two N-acetylglucosamines and one fucose. Topology or symbolic representations indicate detailed linkage information. N-glycans consist of a common trimannosyl-chitobiose core. Each mannose residue on the non-reducing termini can be extended. N-glycans are classified into three types based on compositions of other branches: high-mannose, complex and hybrid. (Fig. 2.7) The high-mannose glycan is mainly constituted by polymannosyl residues in all antennae. The complex type has the special Gal(β1-4)GlcNAc.

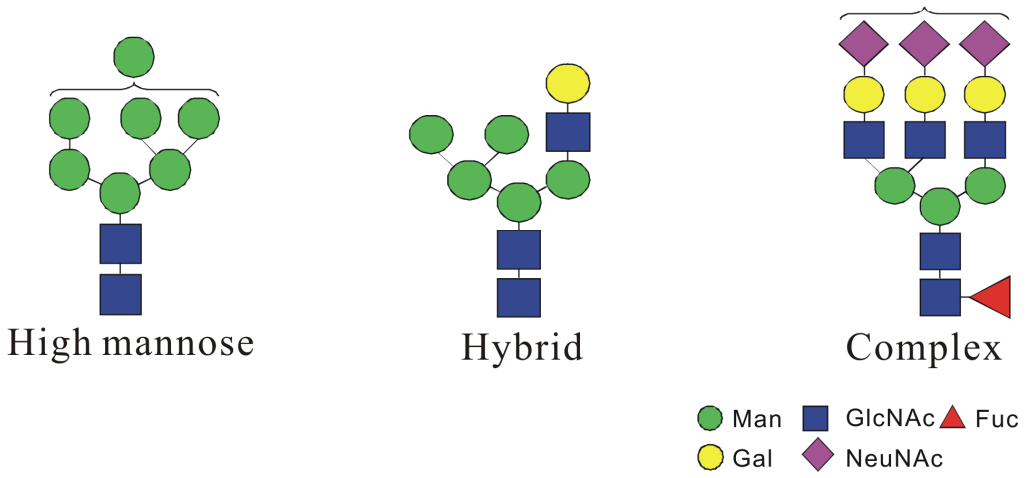

Figure 2.7 Three classifications of N-glycans.

In contrast, O-glycans are less branched than N-glycans and are commonly biantennary structures. They can be either short or extended chains. Components of O-glycans are more variable with the core GalNAc linked to peptides.

Analytical Mass Spectrometric Strategy for Glycomics

In recent decades, the study of glycomics undergoes rapid development as a result of advances in analytical techniques. Most of the research has focused on structural glycomics, systematic repertoire of glycans in the sample, which is prerequisite to fully understanding the functional glycomics.[5, 98] Glycan structures are complicated due to the diversity of the anomeric configurations, interglycosidic linkages and variety of branching attached to the glycosylation sites. Though the analytical approach may vary for individual research report, Mass spectrometric analysis of glycomics contains five clear steps to get target glycans for further analysis: (a) release of the intact glycans using enzymatic or chemical methods, (b) appropriate derivatization to improve detection sensitivity and resolution, (c) separation and characterization by high performance liquid chromatography, (d) high sensitive fingerprint and

characterization by mass spectrometry and (e) data interpretation by individual calculation or by automated programs. This fundamental strategy has been extensively applied to screening glycan repertoire isolated from a great many biological samples.

Release of glycans. There are two existing methods to release glycans from proteins for glycan analysis: chemical and enzymatic release. Intact N-glycans cab be released by hydrazinolysis, however, this approach is not widely used because it leads to the destruction of the peptide backbone and has the potential to degrade carbohydrates. O-linked oligosaccharides are typically released chemically from the protein backbone by a β-elimination reaction.[99, 100] It is preferred to obtain N-glycans from glycoproteins using a wide variety of endoglycosidases, exoglycosidases and glycoamidases, but few effective enzymes are accessible for release of O-glycans. The most widely used enzyme for releasing intact N-glycans is Peptide N-glycosidase F (PNGase F). It directly cleaves amide linkages to yield an aspartyl acid residue and a glycosylamine, which rapidly undergoes hydrolysis to produce the native glycan. However, there are exceptions. Glycans containing core fucose α 1→3 linked to the terminal GlcNAc residue or only one GlcNAc linked to peptide will not be released by PNGase F. In these situations, PNGase A works well. Other endoglycosidases specifically cleave N-glycan, such as Endo H, which is specific for high-mannose and hybrid types, and releases N-glycan within the chitobiosyl core leaving one GlcNAc attached to the protein or peptide.[101] Exoglycosidases, such as β-galactosidase, neuraminidase, α- or β-mannosidase and α-fucosidase, can be used for structural analysis of oligosaccharides by sequential treatment.

Derivatization of glycans. Glycans are usually handled by derivatization to improve sensitivity. These derivatives enhance the hydrophobicity of glycans and help to increase the signal strength and provide more structural information in tandem mass spectrometry analysis.[102] There are two categories. One is reductive amination tagging the reducing ends with chromophore or fluorophore groups.[103] Commonly used fluorescent tags include 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB), 2-aminopyridine (2-AP) and 2- aminoacridone (2-AMAC), as well as other amide compounds.[104, 105] These tags allow for chromatographic detectability, and are mainly contact regions to improve chromatographic purification, and help formation of reducing-end fragment ions in MS and MS/MS.[103] The other is protection of functional groups (the hydroxyl group and amide group), permethylation and peracetylation. Permethylation is the most widely used derivatization method used for mass spectrometric analysis of oligosaccharides.[106, 107] It is preferred than peracetylation to take place active hydrogen atoms, because of smaller mass increase and a greater volatility.

Characterization of glycans by high performance liquid chromatography and MS^n. HPLC allows preliminary separation, identification and quantification for complex glycans. Methods have been explored for both underivertized and derivertized glycans. Researchers can identify carbohydrates by comparing the retention times of samples with those of standards. A three-dimensional (3-D) mapping technique easily separated pyridylaminated (PA) labeled neutral and sialyl N-linked oligosaccharides using three columns.[108] Versatile chromatographic methods have been explored to study oligosaccharide profiling, including normal phase chromatography for glycan mixtures,[109] hydrophilic chromatography on amino-silica column

even for α- or β-anomer separation,[110, 111] reverse phase chromatography with octadecylsilane (ODS or C18) column,[112] or with a graphitized carbon column (GCC) to get rapid sugar mapping by LC/MS,[113, 114] high-performance anion-exchange chromatography using pulsed amperometric detection (HAPEC-PAD) with the advantage of not requiring derivatization.[115] However, HAPEC cannot couple with MS due to the high pH and high concentration of salts.

Rapid and remarkable renovation of MS has been experienced with the increasing demands for proteomics and glycomics. MALDI and ESI are capable to be combined with different analyzers for different levels of characterization of underivertized or derivertized glycans[116, 117] Tandem mass spectrometry is significant to achieve directly or indirectly structural information of the composition, sequence, branching and interglycosidic linkages of glycans.[118] Furthermore, MS^n tree is explored for the identification of structural isomers.[119, 120] Extensive research has been done to study the fragmentation mechanisms for glycans. The nomenclature used was developed by Domon and Costello. (Fig. 2.8) Fragmentation ions containing the non-reducing terminus are labeled with capital letters from the beginning, as A, B, C; those containing reducing end are labeled with letters from the end, as X, Y, Z; subscripts indicate the position of the cleavage corresponding to each end;

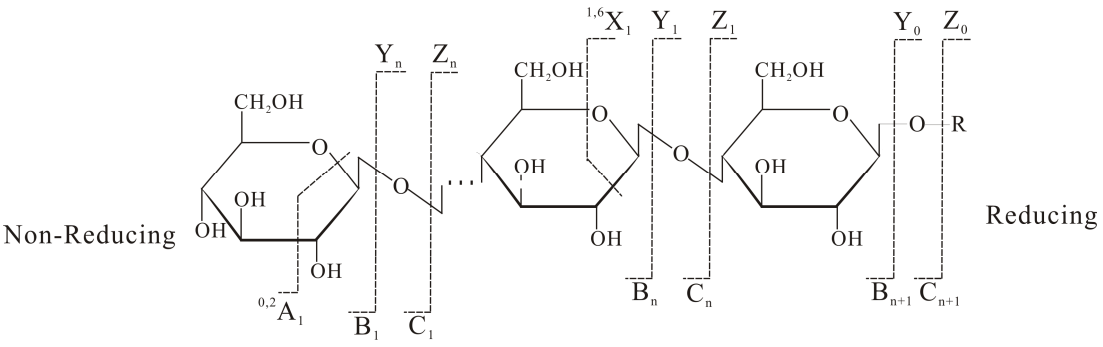

Fig 2.8 Fragmentation mechanism of N-glycans

superscripts mean the bond positions of cleavages in cross-ring fragmentation. Permethylation is usually operated which enhances the hydrophobicity of glycans, stabilizes labile sialic acid residues, and favors the formation of preferential fragmentation ions containing reducing end.[121-124] Then all glycan profiles can be achieved in the positive ion scan mode. In MS spectra, sodium-adducted precursor ions are dominant because of salt background. Permethylation offers easily predictable fragmentation ions. There are two kinds: glycosidic cleavage which breaks the bond between two sugars; cross-ring cleavages which breaks two bonds on the same sugar providing branching and linkage information.[116] Manual structural assignment of glycans according to individual MS^n spectra requires enormous efforts and time. High-throughput congruent sequencing strategies of glycans have been brought forward, involving establishment of ion fragments database and automatic algorithm for convenient identification of glycan structures.[118, 125, 126]

MALDI-TOF is suited for glycan mass mapping. The most common matrix for glycans is 2, 5-dihydroxybenzoic acid (DHB), and other matrixes were tested.[121] A facile method directly uses MALDI-TOF without chromatography to identify sites of glycosylation by comparing MALDI mass spectra of mixture of peptide/glycopeptide before and after enzymatic digestion.[127] Conventional MALDI reflectron TOF can investigate the structural characteristics of glycans by in-source decay (ISD) or post-source decay (PSD), where the metastable decompositions occur in the source region or in the drift tube are observed without or with a stepping reflectron mirror. However, the structural information is very limited for inefficient formation if cross-ring fragmentation ions. Advanced MALDI tandem TOF/TOF has been applied to obtain detailed

structures by producing abundant A- and X-type ions of glycans. Fragmentation patterns of linear and branched types of permethylated glycans were explored.[128-130] ESI-Q-TOF tandem MS/MS was demonstrated for structural analysis too.[131] Moreover, ultrahigh resolution mass analyzer FTICR coupled with MALDI or ESI was utilized to analyze oligosaccharides.[132] CID is not an efficient method as IRMPD for ICR cell to produce MS/MS ions, because it is time-consuming to control factors of the sustained off-resonance irradiation (SORI) and it elongates the duty cycle to remove the collision gas prior to detection. Therefore, CID is externally implemented by other mass analyzer, such as quadrupole and ion trap before FTICR. IRMPD is efficient to gain more extensive product ions compared to CID,[133] as fragmentation ions trapped in the path of the laser can absorb energy and dissociate. ECD provides complementary structural information by frequent C- and Z-type ions.[134] And ESI-quadruple ion trap (LCQ or LTQ) is superior with high resolution and multiple MS^n (n>2) event for tiny structural details indeterminable by MS/MS. Permethylated glycans which preferentially form sodium adducted ions in MS were utilized to demonstrate unambiguous characterization.[120, 122-124] Combined quadrupole and ion trap is a powerful tool which overcomes the low mass cut-off in ion trap MS/MS. This spectrometer was used to study glycan structures and protein glycosylation at high sensitivity.[135]

The combination of HPLC with MS emerges as a new platform thus to provide a fast, high-throughput and sensitive technique for analysis of complex glycan mixtures. HPLC adds one dimension by the separation of glycans, including α or β anomers. Several LC/MS methods were reported for separation of native or reducing end labeled glycans.[136, 137] When characterizing oligosaccharides by MS, reductive labeling is not necessary but permethylation

can be preferentially performed to improve MS^n performance. Online RPLC with ion trap was verified an effective approach. During the HPLC run sodium additive in the mobile phase assists the stable formation of doubly charged ions. They separated branched glycans and differentiated α and β anomers by semiautomated data dependent MS^2 and MS^3 scans.[112] Porous graphitized carbon (PGC) stands out as a promising material with superior resolution for excellent separation of analytes from highly polar to hydrophobic. It was tested for separation of attractive permethylated glycans by ESI-QoTOF.[138]

2.3.3 Quantitative Glycomics

Quantitative profiling of differential glycan patterns is very essential for functional glycomics, such as discovery of cancer biomarkers and drug evaluation. Many researchers found glycosylation changes by comparing the glycan repertoire found in a normal and diseased or treated tissue or body fluids.[139-142] Relative quantitative analysis aims to compare glycans from different states of the organism to evaluate glycosylation changes pertinent to a biological state. Instead of the study of oligosaccharides from specific glycoproteins in the sample, current rapid and general determination of the broad glycan profile for each biological sample is an essential prerequisite to find biomarkers and new insights about the role of an enzyme in a specific cell, organ or tissue.[143] It is equally significant to detect glycan biomarker as relative protein biomarker. Quantitative glycomics comprises the structural illustration and relative quantification of the glycan mixtures. This is a difficult and challenging task.

Analogously to proteomics, there are two types of strategies: label-free approach or stable isotopic labeling approach[144, 145]. Several chromatographic methods were compared for the

quantitation of oligosaccharides released from immunoglobulin G (IgG).[146] MS certainly exerts its predominant analytical capability in quantitative glycomics. MALDI-MS can allow linear concentration-respond relationship to a certain extent which truly reflected more accurate amount of individual oligosaccharides.[147] Selection of matrices and the predominant ion type affected the linear signal response and peak intensity, which was ideally linearly related to the corresponding oligosaccharide. However, difference in the ionization efficiencies of MS is the primary reason that different sample amounts cannot be directly related for quantitative purpose. This strategy applied a known concentration reference compound as the internal or external standard to improve the precision of quantitation, and also compared the integration of the peak areas or relative abundances to quantify components in the oligosaccharides mixture.

It requires different samples to be treated in exactly the same fashion. The isotopic labeled specie is the best internal standard to the individual analyte giving unambiguous measurements. The strategy through the *in vitro* chemical incorporation of 'light' and 'heavy' isotopic tags into oligosaccharide has been implemented in practical multiple ways for quantitative glycomics. Samples are treated with the identical process to make relative quantitation feasible. Stable isotopes such as ^{13}C, ^{15}N or ^{2}H are used. For O-glycans, deuterium was introduced in the β-elimination release procedure using sodium tetradeuterioborate, and the ratio of deuterated to undeuterated species of the same oligosaccharide was obtained to quantify expression levels of glycosylation.[148] For released glycans, isotopic reducing agents or permethylation agents were used to modify oligosaccharides. The mixture of equal amount of d0- and d4-PA oligosaccharides were determined by GCC-LC/ESI MS determined on the basis

of the analyte/internal standard ion-pair intensity ratio.[149] To integrate benefits of permethylation, isotopic labeling and MS analysis, it turns out a powerful pathway for quantitative glycomics. ^{13}C and ^{12}C pair labeled N-glycans from various mixtures of standard glycoproteins or from human milk were mixed at different ratios and analyzed by MALDI-TOF and ESI-FTMS.[150] A part of another diverse analysis of N-glycans from Drosophila wild-type and mutant embryos was accomplished using ^{13}C or ^{12}C labeling.[151] Novotny's group developed a similar labeling method, C-GlycoMAP by MALDI-TOF-TOF, utilizing either methylation or deuteriomethylation. They further demonstrated it with biological differential samples, N-glycans from human blood serum from healthy individual and a breast cancer patient in one run and O-glycans derived from normal and cancer cell extracts.[152] A novel *in vivo* cell culture labeling strategy, isotopic detection of aminosugars with glutamine (IDAWG) was developed for glycomics,[153] in which cells are cultured in Gln-free media with the introduction of glutamine with a ^{15}N labeled side chain (amide-15N-Gln). Then all aminosugars are labeled with ^{15}N. Differentially labeled cells were combined at the beginning of analytical procedures. IDAWG was demonstrated to be a sensitive method for comparative glycomics.

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CHAPTER 3

QUANTITATION BY ISOBARIC LABELLING: APPLICATIONS TO GLYCOMICS[1]

[1] Cheng, L.;† Atwood, J.;† Alvarez-Manilla, G.;† Warren, N.; York, W.; Orlando, R., *Journal of Proteome Research* **2008,** 7, 367–374. †These authors contributed equally to this work.

ABSTRACT

The study of glycosylation patterns (glycomics) in biological samples is an emerging field that can provide key insights into cell development and pathology. A current challenge in the field of glycomics is to determine how to quantify changes in glycan expression between different cells, tissues, or biological fluids. Here we describe a novel strategy, Quantitation by Isobaric Labeling (QUIBL), to facilitate comparative glycomics. Permethylation of a glycan with $^{13}CH_3I$ or $^{12}CH_2DI$ generates a pair of isobaric derivatives, which have the same nominal mass. However, each methylation site introduces a mass difference of 0.002922 Da. As glycans have multiple methylation sites, the total mass difference for the isobaric pair allows separation and quantitation at a resolution of ~30,000 $m/\Delta m$. N-linked oligosaccharides from a standard glycoprotein and human serum were used to demonstrate that QUIBL facilitates relative quantitation over a linear dynamic range of two orders of magnitude and permits the relative quantitation of isomeric glycans. We applied QUIBL to quantitate glycomic changes associated with the differentiation of murine embryonic stem cells to embryoid bodies.

INTRODUCTION

Glycosylation is one of the most common post-translational protein modifications in eukaryotic systems [154, 155]. It has been estimated that 60-90% of all mammalian proteins are glycosylated at some point during their existence[154] and virtually all membrane and secreted proteins are glycosylated[155]. Glycoprotein glycans often play crucial roles in physiological events such as intracellular trafficking, cell-cell recognition[156-158]; signal transduction[159], inflammation[160], tumorigenesis, along with cell development and differentiation[161-165]. The repertoire of glycans expressed by an organism depends on multiple factors such as the species, developmental stage, tissue, and is affected by both the genetic and physiological state of the cells. Given the important physiological roles of protein glycosylation, numerous research groups have devoted significant effort to the characterization of specific glycan structures, the identification of proteins that express each glycan, and the detailed study of how these structures change, e.g., as cells differentiate or as tumor cells progress. All of these efforts have given rise to the emerging field of glycoproteomics[166], which has undergone rapid advances due to the recent development of sensitive analytical techniques, such as mass spectrometry, molecular microarrays and real time PCR[167-169], combined with computational and bioinformatic tools to analyze large sets of data generated by these techniques[169-171].

The quantitative comparison of glycan expression requires highly sensitive methods that can distinguish and identify individual glycans with subtly different structures. Mass spectrometry [172] fulfills these requirements while providing a rapid and reliable method to analyze complex mixtures, and therefore has been widely used in glycomic studies. Several reports have shown

that it is often possible to detect glycans released from glycoproteins using MS techniques without derivatization [173-175]. However, derivatization of oligosaccharides by permethylation is often performed before MS analysis because the addition of methyl groups to an oligosaccharide stabilizes the sialic acid residues and leads to more uniform ionization by converting highly polar -OH and -COO^- groups into non-polar, chemically homogeneous derivatives. Furthermore, the hydrophobic nature of methylated glycans facilitates their separation from salts and other impurities that may affect the MS analysis [176, 177]. Finally, the fragmentation of methylated glycans is more predictable than that of their native counterparts, leading to accurate structural assignments when MS/MS analyses are performed [176, 178-182].

One limitation of using MS for the quantitation of biomolecules is that the ionization efficiencies of distinct molecular species can differ significantly, depending on such factors as the analyte's molecular mass, proton/cation affinity, surface activity, the presence of compounds which compete with or interfere with the ionization of the analyte, etc. In addition, the instrument's response can vary over time, so that the direct comparison of two or more spectra yields a qualitative, rather than quantitative, indication of the glycan content of the two analyte samples. To compensate for these factors, quantitative measurements are typically performed by adding an internal standard and measuring the analyte's response relative to this standard. An ideal internal standard would have chemical properties that are nearly identical to those of the analyte, and thus the optimal internal standard for each analyte is an isotopomer of the analyte itself [183, 184]. For example, a sample could be mixed with an internal standard consisting of an isotopically labeled (^{13}C, D, ^{15}N, etc.) form of the analyte followed by MS analysis. The mass analyzer resolves the

isotopomers permitting their relative abundances to be determined by comparing the intensity of ions from the analyte to the intensity of ions from the isotopically labeled standard. In comparative studies of complex samples where isotopically labeled standards are not available for all species to be analyzed, an isotopic labeling approach where one of the samples is modified with a "light" tag while the other is derivatized with a "heavy" tag can be used [184]. Numerous isotopic labeling procedures have been established for the study of protein mixtures and these are widely used in high throughput proteomic studies [183, 184].

Isotopic labeling can be used for comparative glycomics, making it possible to determine relative changes in the abundances of specific oligosaccharide structures in complex glycoprotein mixtures obtained from biological samples. Initial progress in this area entailed permethylation using heavy/light methyl iodide [$^{13}CH_3I$ vs. $^{12}CH_3I$] prior to MS analysis [185, 186]. An important limitation of these isotopic labeling approaches however, is that the mass difference (Δm) between the heavy and light forms of each glycan is variable and can be very large, as Δm is proportional to the number of methylation sites on the glycan[185]. This variability can confound the analysis of complex mixtures, as it can be difficult to match the differentially labeled forms of the same chemical species. Critically, this approach cannot be used to quantify the structurally distinct isomeric glycans that are often encountered in glycomic analyses. In other words, this method provides a measure of the total abundance of the collection of isomers at a particular mass, rather than the abundances of individual species in that collection.

Here, we introduce a novel strategy for quantitative glycomics that we call Quantitation by Isobaric Labeling (QUIBL), which is based on labeling with $^{13}CH_3I$ or $^{12}CH_2DI$ to generate

isobaric pairs of per-*O*-methylated glycans. We describe the successful application of this method to quantify N-linked oligosaccharides released from a standard glycoprotein and from a complex mixture of glycoproteins, i.e., human serum. The results demonstrate that QUIBL facilitates relative quantitation over a linear dynamic range of two orders of magnitude and permits the relative quantitation of isomeric glycans. These results led us to apply QUIBL to quantitatively determine the changes in the glycome associated with the transition of murine embryonic stem cells to embryoid bodies. This success predicts that the QUIBL will be useful for glycomic studies and that this labeling approach may be adapted to other types of "-omic" investigation.

EXPERIMENTAL

Materials

Bovine fetuin and human blood serum were purchased from Sigma. 99% $^{13}CH_3I$ and 98% CH_2DI were purchased from Cambridge Isotopes Inc (Andover, MA). Acetonitrile for chromatography was purchased from Fischer Scientific. Aurum serum protein mini kit for albumin and IgG depletion was purchased from BIORAD.

Cell culture and embryoid body differentiation

Murine embryonic stem cells (ES) were cultured as previously described [187]. The ES cell culture media was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Commonwealth Serum Laboratories), 1mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1000U/ml recombinant murine leukemia inhibitory factor (LIF) (ESGRO, Chemicon International). The ES cells were cultured at 37°C under 10% CO_2. ES cells were

differentiated into embryoid bodies as previously described [188]. ES were first harvested by trypsinization then seeded into 10cm bacteriological dishes at a density of 1×10^5 cells/ml, in 10ml of ES medium lacking LIF. EBs were harvested daily, the media was changed every 2 days, and the cultures were split one into two at day 4. For the glycan analysis, 1×10^7 ESCs and 1×10^7 EBs were collected by trypsinization, placed into a 15 ml conical tube, and pelleted at 1,000g. The cells were washed 3 times in ice cold phosphate buffered saline (PBS) followed by centrifugation at 1000 g after each wash. All supernatant was removed from the tube and the cell pellets were stored at – 80 ºC until analysis.

ES and EB cell lysis and delipidation

The ES and EB cell lysis was performed by adding 2 mL of water to each cell pellet, placing them into an ice bath, and sonicating for 40 seconds (in four pulses of 10 seconds each) using a probe sonicator at an intensity of 15 watts. Lipids were then extracted from the cells using a modification of the procedure by Svenerholm and Fredman [189]. Chloroform and methanol were then added to a final proportion of 4:8:3 (chloroform:methanol:water). The resulting mixture was incubated 2 hours at -20 ºC and then water was added to modify the chloroform:methanol:water proportion to 4:8:5.6. The mixture was then centrifuged at 5000 g to separate the three phases. The lower (chloroform rich) and upper (aqueous) phases were carefully removed with a Pasteur pipette and the intermediate layer (protein rich) was added to 1 mL of acetone and centrifuged at 5000 g. The acetone supernatant was removed and the delipidated protein pellet was washed once more with cold acetone, suspended in 2 ml of water, and sonicated as described above. The protein mixture was then lyophilized to dryness.

Human serum albumin and IgG depletion

Albumin and IgG were removed from 100 µl of human serum (Sigma) per the manufacturers' recommendations by passage through a spin column containing Affi-Gel Blue and Affi-Gel protein A.

Protein digestion and N-linked glycan release

Enzymatic protein digestion and N-linked glycan release was carried out as previously described with minor modifications [190]. For the bovine fetuin (100 µg) and the human serum glycoproteins, disulfide bond reduction was first performed by the addition of 40mM dithiothreitol (DTT) in 50mM ammonium bicarbonate and incubation at 55ºC for 1h. Carboxyamidomethylation was then performed by addition of 100 mM iodoacetamide (IDA) in 50 mM ammonium bicarbonate and incubation for 1h at room temperature in the dark. Samples were then digested overnight at 37ºC with 2 µg TPCK-treated trypsin in 50 mM ammonium bicarbonate buffer. The trypsin was then removed by filtration through a 30 kDa MW cutoff filter (Millipore, Billerica, MA) and eluent was collected.

The ES and EB protein pellets were solubilized by the addition of 1 mL of 50 mM Tris and 2 M Urea, pH 8.5 followed by sonication. The proteins were reduced with 25 mM DTT for 45 min at 50 ºC and then carbamidomethylated with 90mM iodoacetamide over1 hr at room temperature in the dark. Proteolytic digestion was performed overnight at 37°C in the presence of 100 µg of TPCK-treated trypsin. The resulting mixture of peptides and glycopeptides was desalted using a Sephadex G-15 column (1 X 50 cm), eluted isocratically with 20 mM ammonium bicarbonate. The desalted peptides/glycopeptides were frozen and lyophilized to dryness.

The N-linked glycans from fetuin, serum, ES, and EB cells were then released by overnight incubation with Peptide: N-Glycosidase F (PNGase F, New England BioLab, 1000 U for serum and fetuin and 3000 U for ES and EB) at 37°C.

Glycan isolation

Glycans were separated from peptides by reverse phase liquid chromatography. PNGase F digests were loaded onto a C18-Sep-Pak (Waters Corp.) which had been pre-equilibrate in 3% acetic acid and the glycans were eluted from the column by the addition of 4mL of 3% acetic acid. The fetuin, serum, ES and EB glycans were each divided into two equal aliquots. All of the glycan samples were frozen and lyophilized to dryness.

Glycan permethylation

Dried glycans (30 μg aliquots) were permethylated as described previously[185]. Glycans were suspended in DMSO (0.1mL) and NaOH (20 mg in 0.1 mL of dry DMSO) was added. After strong mixing, 0.1 mL of $^{13}CH_3I$ or $^{12}CH_2DI$ was added. After 10 minutes incubation in a bath sonicator, 1 mL of water was added, and the excess of methyl iodide was removed by bubbling with a stream of N_2. One mL of methylene chloride was added with vigorous mixing, and after phase separation the upper aqueous layer was removed and discarded. The organic phase was then extracted three times with water. Methylene chloride was evaporated under a stream of N_2, and the methylated glycans were dissolved in 25-50 μL of 50% methanol. .

Preparation of glycans for MS analysis

The permethylated glycan samples were dissolved in 50 % MeOH and 1mM NaOH for analysis by tandem mass spectrometry. The $^{13}CH_3$ and $^{12}CH_2D$ labeled glycans from fetuin were first analyzed

independently. To determine the dynamic range for QUIBL, the following mixtures of permethylated fetuin glycans were prepared: 10:1, 8:3, 1:1, 3:8, and 1:10 for the $^{13}CH_3$ to CH_2D. Each mixture was analyzed independently, in triplicate. The $^{13}CH_3$ and CH_2D labeled serum glycans were mixed at a ratio of 1:1.66 ($^{13}CH_3$:CH_2D). Quantitation of the permethylated glycan mixtures from ESCs and EBs were normalized to the Man_5 structure.

MS analysis of the permethylated glycans

The glycans were analyzed on a hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT, Thermo Scientific). Each glycan mixture was infused into the LTQ-FT at a flow rate of 0.3 μl/min and electrosprayed through a 15 μm pulled silica capillary (New Objective, Woburn, MA) at 1.9 kV. MS^n experiments in the LTQ were carried out in positive ion and profile mode using a normalized collision energy of 29%, activation Q of 0.25, and activation time of 30 ms. Glycan precursor ions were isolated for MS^n using a isolation width of 3.0 *m/z*. FTICR experiments were carried out by first isolating the precursor or fragment ion in the LTQ with a isolation width of 10 *m/z* then performing FTICR at 100,000 resolution. Quantitation was performed by separately adding the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled ion intensities over all isotopomers for each glycan.

RESULTS AND DISCUSSION

Principle of Quantitation by Isobaric Labeling (QUIBL).

QUIBL involves the use of $^{13}CH_3I$ or $^{12}CH_2DI$ to generate isobaric pairs of per-*O*-methylated glycans. Two or more compounds are considered to be isobaric if they possess the same nominal mass (i.e., total number of protons and neutrons) but have different elemental or isotopic

compositions.[191] The exact masses of $^{13}CH_3I$ and $^{12}CH_2DI$ differ by 0.002922 Da, and thus isobaric analyte pairs containing a single label are difficult to resolve using current mass spectrometers. However, glycans which contain multiple methylation sites (*i.e.,* –OH and NH_2 groups) are multiply labeled, increasing the Δm between differentially labeled analytes and allowing them to be separated at a resolution of ~30,000 $m/\Delta m$. As the number of methylation sites increases, the mass difference for a pair of differentially labeled isobaric species and the total mass of the glycan also increase in parallel (Table 3.1). Hence, the resolution ($m/\Delta m$) needed to resolve a pair of isobarically labeled glycans is practically independent of the glycan's molecular mass.

The QUIBL method consists of six steps (Fig. 3.1). (i) Two samples containing the same glycans in different proportions are permethylated with either $^{13}CH_3I$ or $^{12}CH_2DI$. (ii) The permethylated samples are mixed (in equal ratios) and analyzed using a hybrid tandem mass spectrometer (such as an ion trap-Fourier transform ion cyclotron resonance mass spectrometer (FTICR) or an ion trap-orbitrap) capable of both low-resolution and high-resolution mass analysis. [190] Nominal analyte masses are determined at low resolution using the ion trap, which is unable to resolve differentially labeled quasimolecular ions that are otherwise identical. (iv). Quasimolecular ions are analyzed (using the FTICR or orbitrap) at high resolution to distinguish ions originating from the $^{13}CH_3$ and $^{12}CH_2D$ labeled glycans. Direct comparison of quasimolecular ion abundances in MS mode (without fragmentation) provides a measure of the abundance ratio for each glycan that is not a component of an isomeric mixture. Analysis of such mixtures, which contain glycans having the same elemental composition but different chemical structures, requires tandem MS. (v)

The structures of quasimolecular precursor ions are identified by MS^n in the low resolution mass analyzer. At this stage, differentially labeled fragment ion pairs (which are otherwise identical) appear at the same nominal mass and thus the ion selection process does not discriminate between the isobaric labels. (vi) The resulting fragment ions are analyzed at high resolution and the abundance ratio for each isomer is determined by comparing ion abundances in a differentially labeled ion pair that is diagnostic for that particular isomer.

Standard glycan analysis using QUIBL

Two glycans purified from bovine fetuin were used as standards to demonstrate the principles of the QUIBL method. The FTICR spectra of the triantennary glycan from fetuin permethylated with $^{13}CH_3I$ or $^{12}CH_2DI$ are shown in Figures 2a and 2b, respectively. Each isotopic quasimolecular ion in the spectrum of the $^{12}CH_2D$ labeled glycan is shifted in its mass-to-charge ratio (*m/z*) units by 0.05 compared to its $^{13}CH_3$ labeled counterpart, in good agreement with the shift predicted for the presence of 50 methyl groups on a triply charged ion ([0.0029 × 50]/3 = 0.05). It is noteworthy that the distribution of isotopic ion abundances depends on the label, as isotope ions at masses lower than the predicted monositopic mass have a higher abundance in the spectrum of the $^{12}CH_2D$ labeled glycan than in spectrum of the $^{13}CH_3$ labeled glycan. This is due to the lower isotopic enrichment in $^{12}CH_2DI$, which contains 98% D, than in $^{13}CH_3I$, which is 99% ^{13}C. For some traditional isotopic labeling procedures, the use of incompletely labeled reagents results in overlapping isotopic peaks, i.e., the ion produced by the under incorporated "heavy" species appears at an *m/z* value that is indistinguishable from an ion produced by the "light" species [185, 186, 192, 193]. In the QUIBL experiment, incompletely labeled ions are still resolved (Fig.

3.2c). Replacing one of the many ^{13}C atoms with a ^{12}C atom or replacing one of the many D (or ^{2}H) atoms with an ^{1}H atom decreases the analyte's mass by approximately 1 Da, however, the resulting ion is detected in the appropriate ($^{13}CH_3$-labeled or $^{12}CH_2D$-labeled) ion series because it still contains a large number of isotopic labels. This greatly simplifies quantitation, which is accomplished by summing the ion abundances for the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled series and comparing these two values. The average ratio obtained by applying this method to a standard 1:1 mixture of differentially labeled, triantennary fetuin glycan was 0.92 ± 0.09 (Fig. 3.2c).

The linearity of response obtained by QUIBL was evaluated by FTICR analysis of five standard mixtures prepared by combining fetuin glycans labeled with $^{13}CH_3$ and $^{12}CH_2D$ in ratios ranging from 10:1 to 1:10 (Supplemental Fig. 3.1, 3.2). The analysis of two triantennary fetuin glycans (performed in triplicate) is shown in Supplemental Figure 1. These results indicate that quantitation using the QUIBL approach is linear over two orders of magnitude. The accuracy of the QUIBL method, as with other isotopic labeling methods increases as the ratio of two labeled species approaches one. This is illustrated in Supplemental Figure 3.2, which shows the high-resolution MS spectra of one of the fetuin glycans from the labeled mixtures. At $^{13}CH_3$ to CH_2D ratios of 1:1, 8:3 and 3:8, all isotopomer signals, including those due to under isotopic incorporation, are clearly visible and contribute to the accuracy and reproducibility of the ratio measurements. For these mixtures, the maximum error was below 17%, which is comparable to other quantitation methods utilizing isotopic labeling. However, as the ratio is increased to 10:1 or decreased to 1:10, the low abundance peaks become more difficult to discern, and the standard deviations and errors associated with the ratio measurements becomes larger.

Application of QUIBL to human serum glycans

Serum glycomics is emerging as a potentially valuable method for the discovery and characterization of biomarkers for human diseases [194-196]. To date, quantitative serum glycomics has been performed using isotopic labels that cause large mass shifts [185, 186]. These approaches have numerous drawbacks, including the doubling of sample complexity and the inability to quantitate individual isoforms. We therefore evaluated the QUIBL approach for its ability to quantitate glycans released from human serum. Serum glycans permethylated with either $^{13}CH_3I$ or $^{12}CH_2DI$ were mixed in a 1:1.6 ratio and analyzed in triplicate using an LTQ-FT (Fig. 3.3). MS was first performed using the low resolution LTQ to determine the nominal masses of the glycans (Fig. 3.3a). Individual glycans were identified through multiple rounds of collision induced dissociation (MS^n) (Fig. 3.3c, 3.3e) and quantified by analysis of the fragment ions using the FTICR (Fig. 3.3b, 3.3d, 3.3f). For each glycan, the QUIBL method generates pairs of differentially labeled ions having the same nominal mass. This confers three distinct advantages to the QUIBL method compared to traditional isotopic labeling procedures. The first is an increase in ion abundance during low-resolution MS and MS^n, as both of the ions of a differentially labeled pair are detected at the same *m/z*. This factor reduces the amount of material needed for the glycan identification stage of the analysis. The second advantage is that glycans that are normally resolved by MS due to molecular weight differences are still resolved during QUIBL analysis. This is not true of traditional labeling, as the large mass shifts that are introduced by these methods often cause the light form of one glycan to have a mass that is very close to the mass of the heavy form of a completely different glycan. The resulting spectral overlap interferes with

both identification and quantitation of the glycans. The third advantage is that the QUIBL method is not susceptible to errors arising from differences in detection efficiency that would occur if the differential labeling resulted in a large mass difference. The QUIBL approach accurately quantitated a broad range of glycan structures in a complex mixture in a single experiment (Table 3.2). These results demonstrate that QUIBL does not depend on glycan composition, size, or ionization efficiency, and is capable of accurately quantitating glycans of both low and high abundance. The maximum error in the calculated glycan ratios for the differentially labeled samples was 18.3% with an average error of 4.8%.

Perhaps the most promising aspect of QUIBL is that it allows simultaneous quantitation of glycans that have the same molecular mass (*i.e.*, isomers). That is, if a fragment ion unique to each of the isomers is observed by MS^n, the ratio of differentially labeled forms of each isomer can be measured by high-resolution analysis of the fragment ions (Fig. 3.3d, 3.3f). This capability was demonstrated by the selection and fragmentation of the $[M+2Na]^{2+}$ ion (*m/z* 1061.1) of the serum glycan $Man_3GlcNAc_4Gal_2$(Fig 3.3c). CID of this precursor ion generated a collection of fragments that included a singly charged ion (*m/z* 1628.55), which was analyzed by high resolution FTICR (Fig. 3.3d). The isobaric labeling of this fragment ion was present at the same 1:1.6 ratio as observed for the intact precursor ion in Figure 3b. The *m/z* 1628.73 fragment ion (Fig. 3.3c) was subjected to CID for MS^3 analysis (Fig. 3.3e). Selection and FTICR analysis of the resulting MS^3 fragment at *m/z* 1158.36 (Fig. 3.3f) gave the same ratio, demonstrating that accurate quantitation can be performed using fragment ions originating from multiple MS/MS events.

These results suggest that QUIBL can be used for the accurate quantitation of glycans that are present as low abundance components of isomeric mixtures.

Application of QUIBL for quantifying glycome changes during early embryogenesis.

Mammalian pluripotent embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocyst-stage embryos. When cultured over extensive periods of time under appropriate conditions, ESCs retain many of the characteristics associated with pluripotent cells of the ICM, including the capacity to generate the three embryonic germ lineages (ectoderm, endoderm and mesoderm) and the extraembryonic tissues that support development. In murine ESCs, Leukemia inhibitory factor (LIF) stimulates the renewal of mouse ESCs and suppresses their differentiation. Removal of LIF from the media promotes the differentiation of ESCs into spheroid colonies called Embryoid Bodies (EBs) [197], which recapitulate certain aspects of early embryogenesis such as the appearance of lineage-specific regions of differentiation [198]. The pluripotency of ESCs provides the basis for developing a wide variety of somatic and extraembryonic tissue cultures [172, 199] with potential therapeutic applications in the treatment of diseases and injuries.

We applied QUIBL to compare N-linked glycan expression levels in murine ESCs and Embryoid Bodies (EBs). To quantify the changes in glycan expression that accompany differentiation of ESCs into EBs, we isolated N-linked glycans from 10^7 cells of each type. The ESC glycans, labeled with $^{12}CH_2DI$, were mixed with the EB glycans labeled with $^{13}CH_3I$ and the mixture was analyzed as described in Figure 3.1. In total, 29 distinct glycans, ranging from high mannose to complex triantennary forms, were characterized and quantitated (supplemental table

1). This demonstrated the potential of QUIBL analysis to accurately quantitate a diverse population of glycans, as shown by an average relative standard deviation below 19% for the entire dataset.

Changes in the expression levels of several cell surface glycan markers, including SSEA1 (stage specific embryonic antigen 1, also known as Lewis X) and the Forssman antigen (FA), are associated with the differentiation of murine ESCs to EBs. Both of these markers are preferentially expressed in ESCs [200, 201]. During early development of the mouse embryo, the Lewis X antigen is expressed as part of embrioglycan, an O-linked proteoglycan [202, 203] that disappears during development. We observed that differentiation of ESC into EBs was accompanied by a greater than three-fold decrease in the expression of two di-fucosylated (Lewis X type) N-linked glycans (Table 3.3) whose structures were confirmed by MS^n analysis (Supplemental figure 3.3). Thus, our results are consistent with previous reports describing a decrease in the expression of Lewis X when ES cells differentiate into EBs [45, 46]. We also observed a twofold decrease in the expression of several other complex fucosylated N-linked glycans. Notably, our results indicate that the developmental regulation of Lewis X epitope is expressed in N-linked glycans and not restricted to the polylactosamine O-linked structures of embryoglycan.

CONCLUSION

Herein we have introduced a novel strategy, based on the use of isobaric labeling, for quantitative/comparative glycomics. The QUIBL method was successfully used to analyze N-linked oligosaccharides released from a standard glycoprotein and from human serum. Isobaric labeling was also used to identify changes in the glycoproteome associated with the transition of

mouse embryonic stem cells to embryoid bodies. In this case, we were able to observe that N-linked glycans containing the Lewis X structure were more abundant in the ES cells than EB. There are numerous advantages of the QUIBL approach, many of which result from the isobaric ions appearing at the same nominal mass to charge ratio. This characteristic leads to increased ion intensity as ions from both samples are not distributed between isotopic species having different m/z values. The small mass difference between these isobars allows the two species to be simultaneously selected for MS^n analysis, permitting the relative quantitation of isomeric glycans, as was used to determine the increased expression of Lewis X glycans discussed above. Although the focus of this presentation is on glycoprotein glycans, this strategy is directly applicable to oligosaccharides from other sources, such as glycolipids. The concept of isobaric labeling is expected to be applicable to other types of "omics" analyses with other derivatizing agents. Lastly, we anticipate that isobaric labeling will also provide a manner to method allowing the absolute quantification of these molecules.

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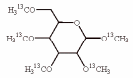
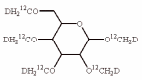
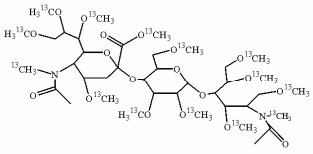
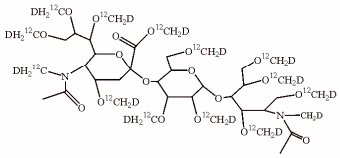
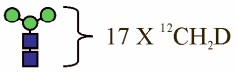
Table 3.1

Table 1 Principle of quantitative isobaric labeling for glycomics

Labeling Agent	$^{13}CH_3$	$^{12}CH_2D$	Mass Differance	Resolving Power
Monoisotopic mass	16.0268	16.0297	0.0029	~5,500
Permethylated Hexose				
Monoisotopic mass	255.1587	255.1732	0.0145	~17,500
Permethylated O-linked glycan NeuAcHexHexNac				
Monoisotopic mass	886.5206	886.5592	0.0386	~23,000
Permethylated N-linked glycan core $Man_3GlcNac_2$	 17 X $^{13}CH_3$	 17 X $^{12}CH_2D$		
Monoisotopic mass	1165.6517	1165.6981	0.0464	~25,100

Resolving power indicates the resolution required to resolve the two molecular species; calculated as $\frac{m}{\Delta m}$

N-acetylneuraminic acid (NeuAc), Hexose (Hex), N-acetylglucosame (HexNac)

● indicates mannose, ■ indicates N-acetylglucosamine

Table 3.2

Table 2 Quantitation of human serum glycans by QUIBL

m/z	Charge state	Glycan structure	Observed ratio[a] (Mean ± SD)	Expected[b] ratio	% Error
812.9	$[M+2Na]^{2+}$		0.6 ± 0.02	0.6	0
916.5	$[M+2Na]^{2+}$		0.61 ± 0.01	0.6	1.6
1019.3	$[M+2Na]^{2+}$		0.60 ± 0.04	0.6	0
1123.6	$[M+2Na]^{2+}$		0.69 ± 0.01	0.6	15
1227.2	$[M+2Na]^{2+}$		0.65 ± 0.03	0.6	8.3
818.4	$[M+2Na]^{2+}$		0.58 ± 0.04	0.6	3.3
853.9	$[M+2Na]^{2+}$		0.57 ± 0.01	0.6	5
942.1	$[M+2Na]^{2+}$		0.63 ± 0.02	0.6	5
957.5	$[M+2Na]^{2+}$		0.51 ± 0.02	0.6	15
978.2	$[M+2Na]^{2+}$		0.68 ± 0.04	0.6	13.3
1045.5	$[M+2Na]^{2+}$		0.60 ± 0.03	0.6	0
1061.1	$[M+2Na]^{2+}$		0.62 ± 0.01	0.6	3.3
1081.6	$[M+2Na]^{2+}$		0.65 ± 0.02	0.6	8.3
1103.6	$[M+2Na]^{2+}$		0.62 ± 0.03	0.6	3.3
1149.1	$[M+2Na]^{2+}$		0.60 ± 0.04	0.6	0
1169.7	$[M+2Na]^{2+}$		0.58 ± 0.03	0.6	3.3
1185.1	$[M+2Na]^{2+}$		0.58 ± 0	0.6	3.3
1244.2	$[M+2Na]^{2+}$		0.49 ± 0	0.6	18.3
1273.2	$[M+2Na]^{2+}$		0.58 ± 0.02	0.6	3.3
1289.2	$[M+2Na]^{2+}$		0.60 ± 0.01	0.6	0
1377.3	$[M+2Na]^{2+}$		0.62 ± 0.02	0.6	3.3
1427.8	$[M+2Na]^{2+}$		0.52 ± 0	0.6	13.3
1471.8	$[M+2Na]^{2+}$		0.50 ± 0.04	0.6	16.6

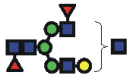
[a]Observed ratios were calculated as shown in figure 2.

[b]Expected ratios were determined from the known amounts of each glycan in the mixture.

● indicates mannose, ■ indicates N-acetylglucosamine, ◆ indicates N-acetylneuraminic acid, ▲ indicates fucose, ○ indicates galactose

Table 3.3

Table 3 Differentially expressed N-linked glycans from murine ES and EB cells

m/z	Charge state	Glycan structure	EB(^{13}C)/ES(D) Run 1	EB(^{13}C)/ES(D) Run 2	EB(^{13}C)/ES(D) Run 3	EB(^{13}C)/ES(D) %SDEV
1066.57	$[M+2Na]^{2+}$		-0.84	-1.30	-1.21	24.12
1170.13	$[M+2Na]^{2+}$		-0.94	-1.27	-1.18	17.11
1273.68	$[M+2Na]^{2+}$		-1.12	-0.94	-0.80	16.15
1258.68	$[M+2Na]^{2+}$		-0.91	-1.15	-1.00	12.35
1362.24	$[M+2Na]^{2+}$		-1.61	-1.86	-1.49	18.91

Expression ratios calculated as Log_2 for glycans from murine embroid bodies (EB) and embryonic stem cells(ES). %SDEV calculated as (SDEV/Average Expression Ratio X 100%).

● indicates mannose, ■ indicates N-acetylglucosamine, ▲ indicates fucose, ○ indicates galactose

Figure 3.1: Flow chart for quantitative glycan analysis using isobaric labeling. Glycans from two biological samples are permethylated in either $^{13}CH_3I$ or $^{12}CH_2DI$ and mixed together prior to analysis. At low mass resolution, the two labeled species appear at the same *m/z* value thereby increasing their abundance and decreasing sample complexity. Analysis of the glycans by high resolution MS separates the differentially labeled glycan precursor ions permitting their relative quantitation by comparing the peak intensities from the $^{13}CH_3$ to the $^{12}CH_2D$ labeled glycans. Structural information on the glycan is provided by low resolution MS^n, which does not alter the ratio of isobaric labels. High resolution analysis of the MS^n fragment ions permits the isomeric glycans to be quantified.

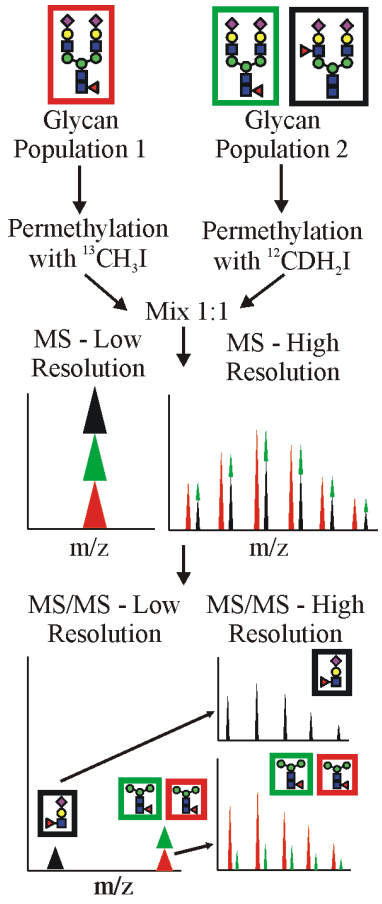


Figure 3.2: FTICR spectra of the triantennary glycan permethylated with (a) $^{13}CH_3I$ and (b) $^{12}CH_2DI$. (c) FTICR spectrum of a 1:1 mixture of the $^{13}CH_3$ and $^{12}CH_2D$ labeled fetuin glycan.

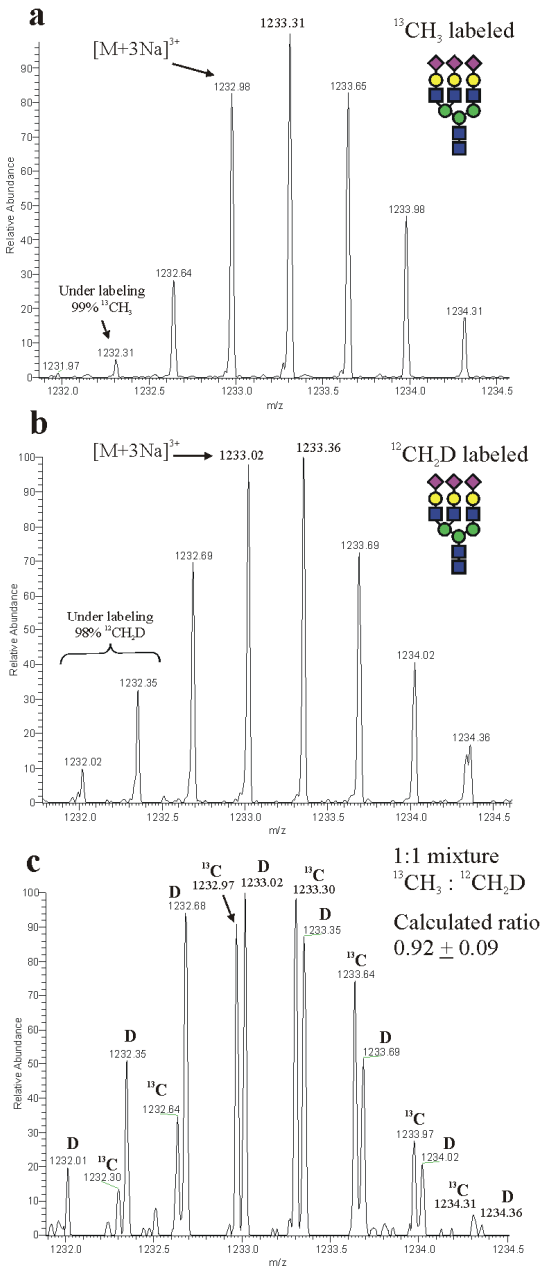
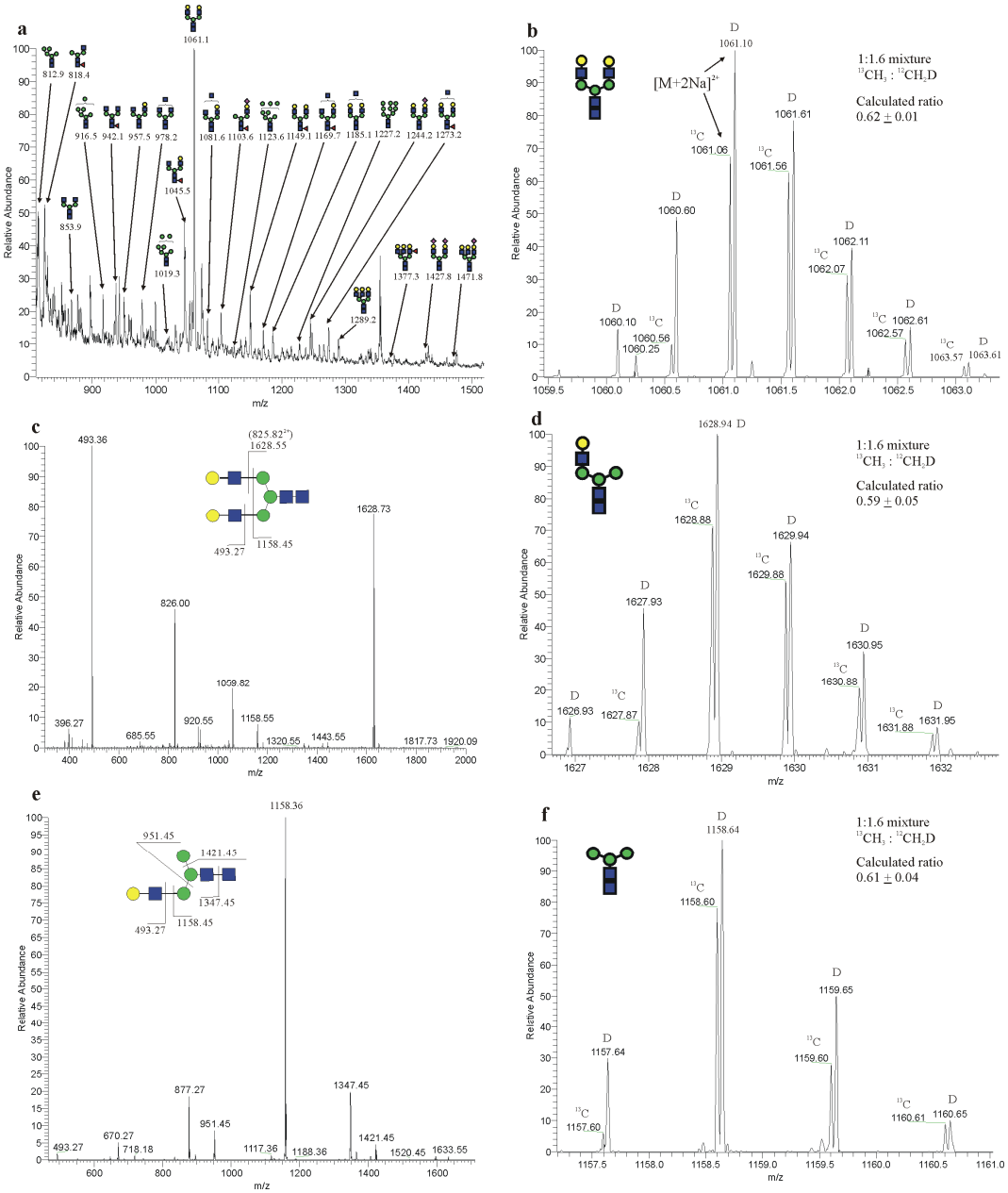


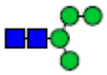
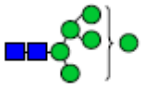
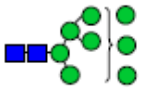
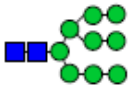
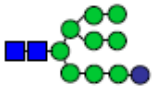
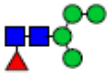
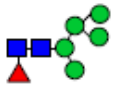
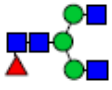
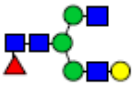
Figure 3.3: Quantitation of human serum glycans by QUIBL. Glycans from human serum were permethylated in either $^{13}CH_3I$ or $^{12}CH_2DI$. The two labeled glycan samples were mixed together at a ratio of 1:1.6 and analyzed using an LTQ-FT in triplicate. (a) Ion trap MS spectrum of the serum glycan mixture. The differentially labeled glycan precursor ions appear at the same nominal m/z. (b) FTICR spectrum of a biantennary serum glycan. The calculated expression ration 0.62 corresponds well with the expected ratio for quantitation of the glycan precursor ion. (c) The biantennary complex glycan was subjected to MS^2 in the ion trap and the most abundant ion at 1628.73 *m/z* was analyzed by FTICR (d).FTICR spectrum of the glycan fragment ion at 1628.73 *m/z*. (e) MS^3 spectrum resulting from collision induced dissociation of MS^2 fragment ion 1628.73 *m/z*. (f) FTICR spectrum of the MS^3 fragment ion at 1158.36 *m/z*.

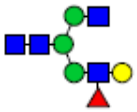
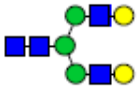
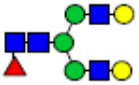
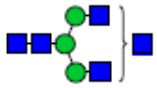
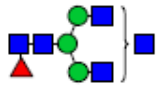
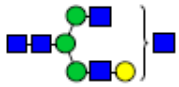
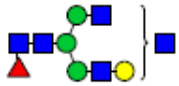
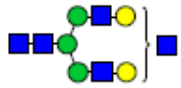
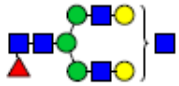
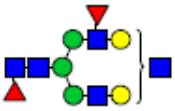
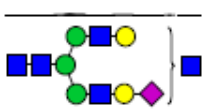
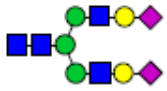
Figure 3.3


a
b
c
d
e
f
Relative Abundance
m/z
[M+2Na]2+
1:1.6 mixture
13CH3 : 13CH2D
Calculated ratio
0.62 ± 0.01
0.59 ± 0.05
0.61 ± 0.04

Supplement Table 3.1

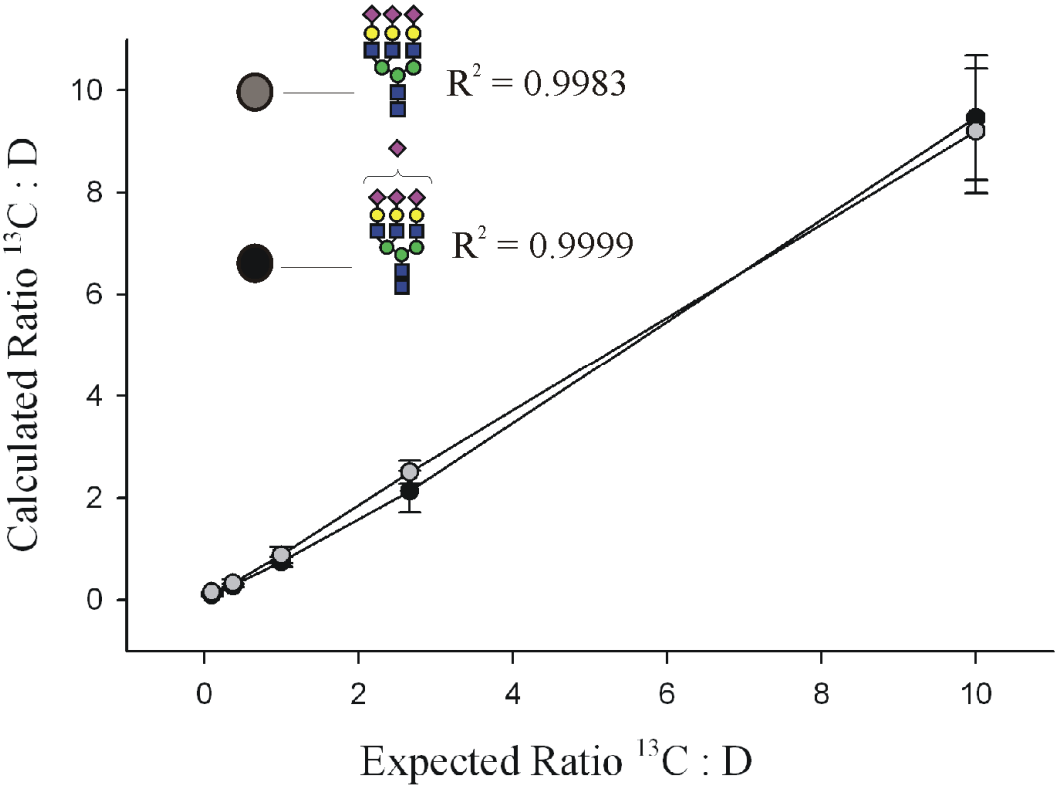
Quantitation of N-linked glycans from murine embryonic stem cells and embroid bodies

m/z	Charge state	Glycan structure	EB(^{13}C)/ES(D) Run 1	EB(^{13}C)/ES(D) Run 2	EB(^{13}C)/ES(D) Run 3	EB(^{13}C)/ES(D) %SDEV
1188.65	$[M+1Na]^{1+}$		0.18	-0.32	-0.04	24.96
1395.75	$[M+1Na]^{1+}$		0.60	0.17	0.10	27.23
812.93, 1602.86	$[M+2Na]^{2+}$, $[M+1Na]^{1+}$		0.16	-0.17	0.01	16.48
916.48, 1809.97	$[M+2Na]^{2+}$, $[M+1Na]^{1+}$		-0.56	-1.03	-0.80	23.61
1020.04	$[M+2Na]^{2+}$		-0.27	-0.74	-0.44	23.99
756.73, 1123.59	$[M+3Na]^{3+}$, $[M+2Na]^{2+}$		-1.54	-1.32	-1.12	21.35
825.76, 1227.15	$[M+3Na]^{3+}$, $[M+2Na]^{2+}$		-0.47	-0.93	-0.41	28.12
1330.71	$[M+2Na]^{2+}$		-1.29	-1.53	-1.16	18.77
1365.75	$[M+1Na]^{1+}$		0.93	0.37	0.50	29.42
1572.85	$[M+1Na]^{1+}$		0.19	0.08	0.25	8.58
1779.96	$[M+1Na]^{1+}$		-0.32	-0.64	-0.34	17.80
1005.03, 1987.07	$[M+2Na]^{2+}$, $[M+1Na]^{1+}$		0.08	-0.07	0.32	19.69
942.51	$[M+2Na]^{2+}$		-0.06	0.05	-0.18	11.60
957.51	$[M+2Na]^{2+}$		0.15	0.51	-0.06	29.03
1046.06	$[M+2Na]^{2+}$		-0.38	-0.64	-0.36	15.75

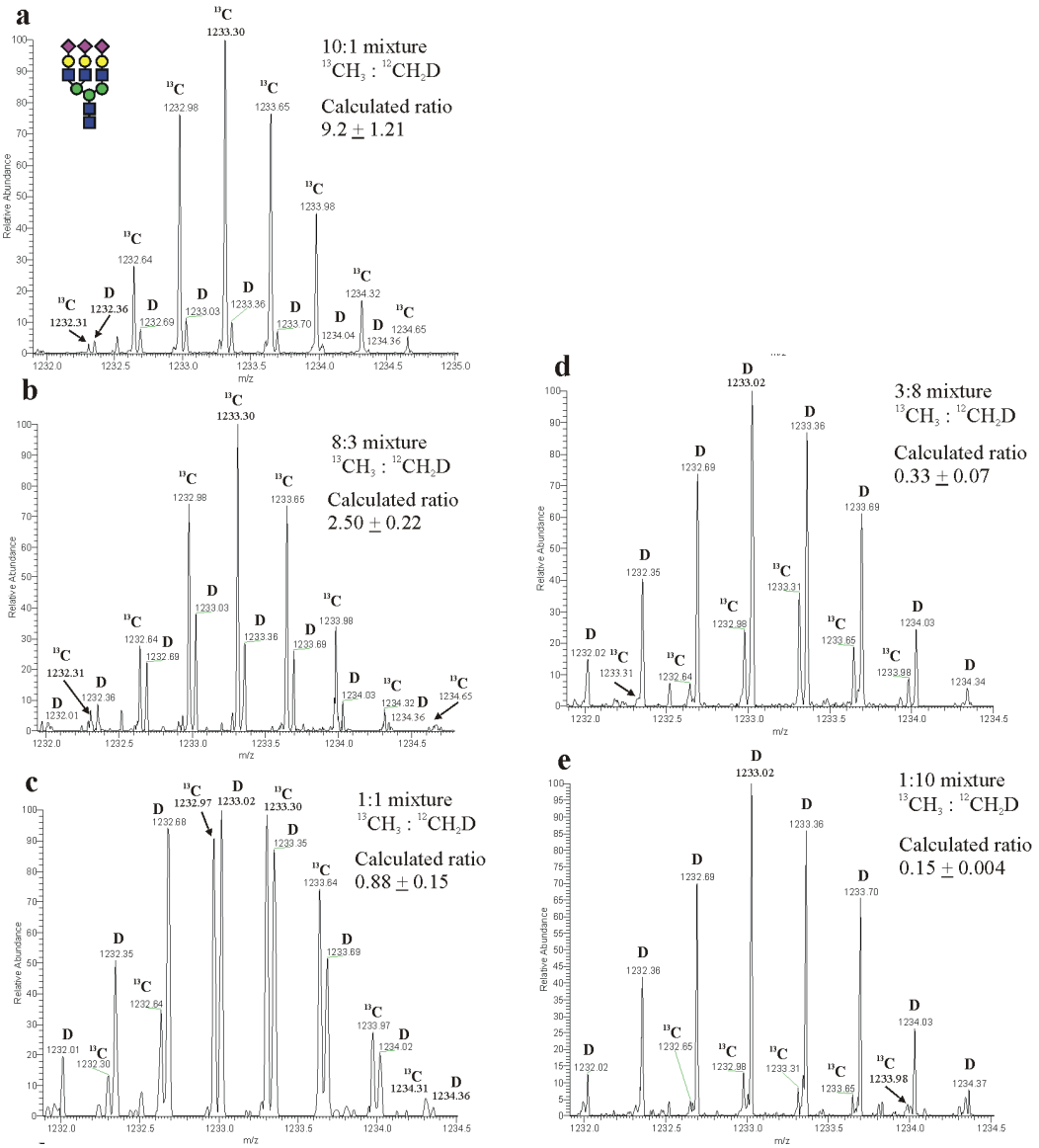
1046.06	$[M+2Na]^{2+}$		-0.38	-0.64	-0.36	15.75
1061.07	$[M+2Na]^{2+}$		-0.74	-1.27	-0.77	29.72
1149.62	$[M+2Na]^{2+}$		-0.57	-0.99	-0.69	21.67
978.02	$[M+2Na]^{2+}$		-0.90	-0.95	-0.95	2.77
1066.57	$[M+2Na]^{2+}$		-0.84	-1.30	-1.21	24.12
1081.58	$[M+2Na]^{2+}$		-0.86	-1.00	-1.00	7.90
1170.13	$[M+2Na]^{2+}$		-0.94	-1.27	-1.18	17.11
1258.68	$[M+2Na]^{2+}$		-0.91	-1.15	-1.00	12.35
1185.13	$[M+2Na]^{2+}$		0.37	0.41	0.39	2.00
1273.68	$[M+2Na]^{2+}$		-1.12	-0.94	-0.80	16.15
1362.24	$[M+2Na]^{2+}$		-1.61	-1.86	-1.49	18.91
1264.67	$[M+2Na]^{2+}$		-0.68	-0.89	-0.50	19.11
1368.23	$[M+2Na]^{2+}$		-0.84	-1.18	-1.09	17.90
1427.26	$[M+2Na]^{2+}$		0.29	0.14	-0.03	15.80

All expression ratios calculated as Log_2; %SDEV calculated as the relative percent standard deviation taken from the average expression ratio.

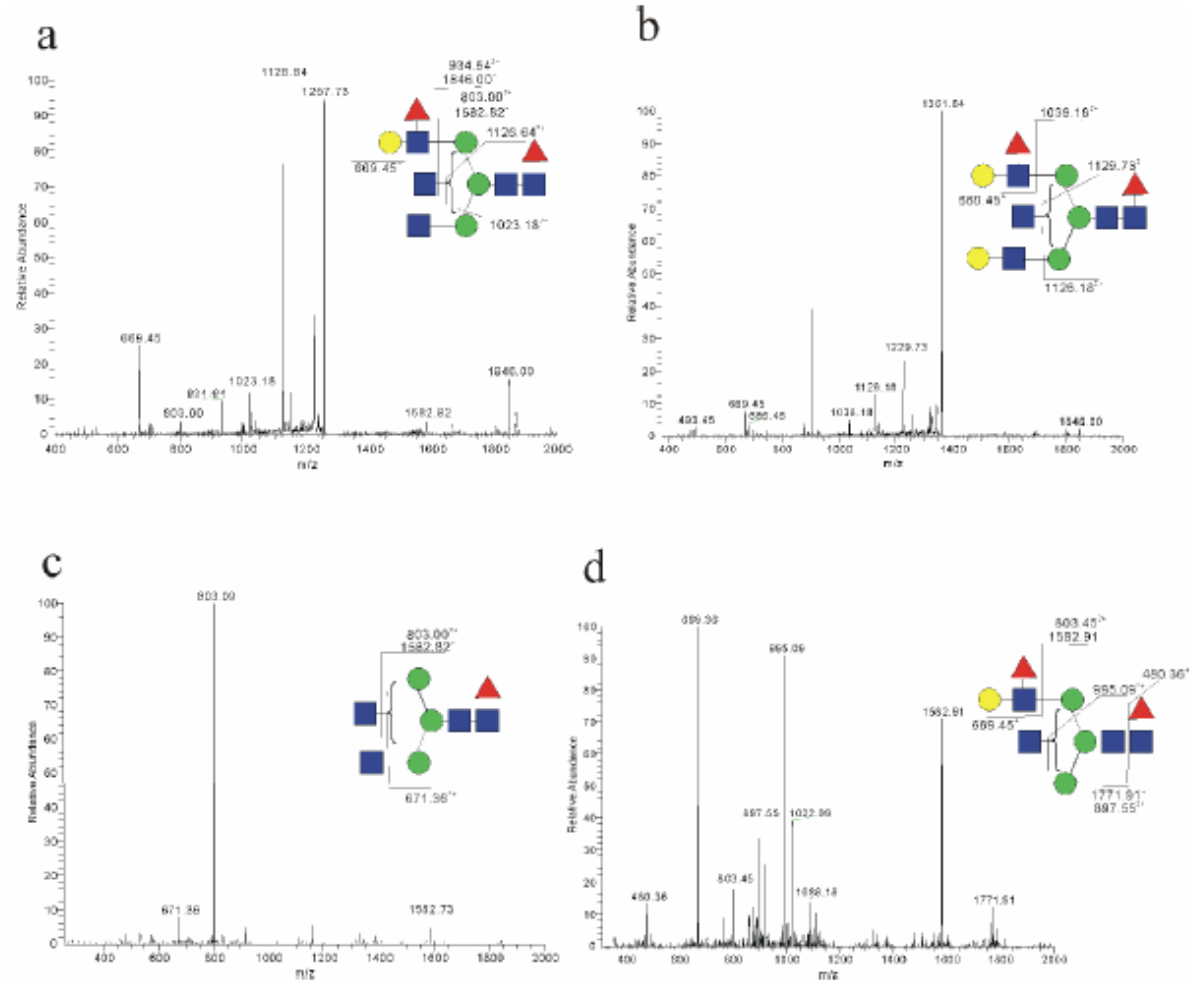
Supplement Figure 3.1: Correlation between calculated and expected ratios for quantitation of two fetuin glycans by QUIBL. In each experiment the $^{13}CH_3$ and $^{12}CH_2D$ labeled glycans were mixed together at the ratios 10:1, 8:3, 1:1, 3:8, and 1:10 ($^{13}CH_3$: $^{12}CH_2D$) and analyzed by FTICR. The calculated expression ratios were determined by comparing the sum of the peak intensities for all isotopes between $^{13}CH_3$ and $^{12}CH_2D$ labeled precursor ions for each glycan. For both glycans a linear correlation was observed between the calculated and the expected ratios with a minimum R^2 of 0.9983.



Supplement Figure 3.2: QUIBL analysis of a differently labeled fetuin glycan mixed at five different ratios. Two fetuin glycan mixtures were permethylated in either $^{13}CH_3$ or $^{12}CH_2D$. The two differentially labeled glycan mixtures were then mixed together at the ratios 10:1, 8:3,1:1, 3:8, and 1:10 ($^{13}CH_3$: $^{12}CH_2D$) and analyzed by FTICR (a,b,c,d,e). Accurate quantitation was achieved at all ratios over two orders of magnitude.



Supplement Figure 3.3: MS^n analysis of two di-fucosylated (Lewis X type) N-linked glycans from ES and EB cells. (a, b) MS^2 of the two Lewis X type N-linked glycans. (c) MS^3 of the fragment ion at 1846.00 m/z from MS^2 of the glycan in shown in (a). (d) MS^3 of the fragment ion at 1126.18 m/z from MS^2 of the glycan in shown in (b).



CHAPTER 4

QUANTITATION BY ISOBARIC LABELLING for ISOMERIC GLYCANS ANALYSIS[1]

[1] Cheng, L; Atwood, J.; Alvarez-Manilla, G.; York, W.; Orlando, R.,
To be submitted.

SUMMARY

A novel method is introduced for the relative quantitation of individual glycans present in isomeric mixtures using quantitation by isobaric labeling and sequential mass spectrometry (MS^n) on an ion cyclotron resonance-Fourier transformation MS (LTQ-FTICR MS). In this approach, glycans in one sample are permethylated with $^{13}CH_3I$ while the other sample is permethylated with $^{12}CH_2DI$. These two reagents have the same nominal mass but differ in their exact masses by 0.002922 Da. Since glycans contain multiple sites of methylation, the mass difference between the two multiply-labeled species allows them to be separated with a resolution of ~30,000 m/Δm. A previously un-tested use of this labeling strategy is its ability to simultaneously quantitate individual glycans present in a mixture with other glycans of the same molecular mass (*i.e.*, isomers), provided that a fragment ion unique to each of the isomers is observed by MS^n. The ratio of differentially labeled forms of each isomer can be measured by high-resolution analysis of the fragment ions. Same aliquots of isomeric glycans Lacto-N-fucopentaose I (LNFP I) and Lacto-N-fucopentaose II (LNFP II) were labeled with either ^{13}C or D methyl iodide. Four labeled samples were mixed in known proportion and analyzed by LTQ-FTICR MS. The results demonstrated that QUIBL has the capability of relative quantitation of isomeric glycans in mixtures.

INTRODUCTION

Of mammalian and plant proteins, more than half are glycosylated.[204] Glycosylation is a ubiquitous post-translational modification. Glycoprotein glycans are involved in many biological processes, such as cell–cell recognition, immune response, cell–extracellular matrix interactions, tissue development, host–pathogen recognition, and so on.[205, 206] Glycan moieties have a great diversity of composition residences, linkage and branching (as *glycoforms*). Structural glycan isomers increase the heterogeneity of glycoproteins. Abnormal glycosylation happens under differentiate states, such as loss of nearly an entire class or subclass of glycans, or more subtle, such as change of only a few isomers within a subclass.[207]

Mass spectrometry (MS) is widely used to characterize glycoprotein-linked glycans with high sensitivity, wide dynamic range and minute sample amount requirement.[208] Research has focused on separation and characterization of oligosaccharide isomers using liquid chromatography or electrophoresis coupled with mass spectrometry.[209-211] Sequential mass spectrometry (MS^n) has been utilized to develop a comprehensive carbohydrate sequencing strategy to characterize carbohydrate structures. The interpretation of structures of isomers is completed by the comparison of spectra at different stages of sequential molecular fragmentation.[212, 213]

A limitation of MS for quantitative application in biomolecules is ionization efficiencies which can differ significantly for distinct samples. The process of ionization depends on many factors, such as the analyte's molecular mass, proton/cation affinity, surface activity, etc. Another limitation is the instrument error which can vary over time. Hence, quantitation of

different samples cannot be directly related to the comparison of the spectra. A typical way of relative quantitative analysis is adding internal standard and measures the analyte's response to this standard. Isotopic labeled form of the analyte is an ideal internal standard, because of approximately identical chemical properties to those of the analyte. For complex sample including many species, there is no one ideal internal standard for all analytes. Instead, one of the samples is derivatized with a "light" tag while the other is modified with a "heavy" tag.[214-217] However, no reports has been related to the quantitation of isomeric glycans using mass spectrometry, because isomers have the same mass which wouldn't be discriminate by MS.

In this report, we investigate the capability of a new labeling approach to achieve the relative quantitation of structural isomeric glycans in mixtures using LTQ-FTICR MS. The principle of quantitative isobaric labeling (QUIBL) has just been reported and its application into quantitation of glycans has been demonstrated. Here, we use QUIBL to quantitate two fucosylated isomers. The results lead us to the promising research to determine quantitatively changes of isomeric glycans which are related to diseases.

MATERIAL and METHODS

Material

Isomeric glycans Lacto-N-fucopentaose I (LNFP I) and Lacto-N-fucopentaose II (LNFP II) were purchased from V-labs Inc. (Covington, LA). 99% $^{13}CH_3I$ was purchase from Sigma. 98% $^{12}CH_2DI$ was purchased from Cambridge Isotopes Inc (Andover, MA).

Glycan permethylation Dried glycans (40 μg aliquots) were permethylated as described in Alvarez-Manilla et al.[150] Glycans were suspended in DMSO (0.1mL) and NaOH (20 mg in 0.1

mL of dry DMSO) was added. After strong mixing, 0.1 mL of $^{13}CH_3I$ or $^{12}CH_2DI$ was added. After 10 minutes of incubation in a bath sonicator 1 mL water was added. The excess of methyl iodide was removed by bubbling with a stream of N_2. One mL of methylene chloride was added with vigorous mixing. After phase separation the upper aqueous layer was removed and discarded. The organic phase was then extracted three times with water. Methylene chloride was evaporated under a stream of N2, and the methylated glycans were dissolved in 25-50 μL of 50% methanol.

Preparation of glycans for MS analysis The permethylated glycans were suspended in 50 % MeOH and 1mM NaOH for analysis by tandem mass spectrometry. The $^{13}CH_3I$ labeled LNFPI and LNFPII were combined at 1:1 ratio (MixC). The $^{12}CH_2DI$ labeled LNFP I and LNFP II were combined at 5 different ratios (MixD1~5). Then MixC was combined at 5 different ratios with MixDn (n=1,2,3,4,5) to obtain total 25 mixtures.

Glycan analysis by MS The isomeric glycan mixtures were analyzed on a hybrid linear ion trap Fourier transform mass spectrometer (Thermo Scientific). Each glycan mixture was infused into the LTQ-FT at a flow rate of 0.5 μl/min and electrosprayed through a 15 μm pulled silica capillary at 1.9 kV. MS^n experiments were carried out in positive ion and profile mode using a normalized collision energy of 35%, activation Q of 0.25, and activation time of 30 ms. Glycan precursor ions were isolated for MS^n using an isolation width of 2 m/z. FTMS was performed at 200,000. Quantitation was performed by comparing the sum of the peak intensities of all $^{13}CH_3$-labeled isotopomer ions to that of $^{12}CH_2D$-labeled for each glycan or each fragment ion.

RESULTS and DISCUSSION

QUIBL involves the use of $^{13}CH_3I$ or $^{12}CH_2DI$ to generate nearly isobaric pairs of permethylated glycans. Isobaric compounds possess the same nominal mass (i.e., total number of protons and neutrons) but different elemental compositions. The exact masses difference unit of $^{13}CH_3I$ and $^{12}CH_2DI$ is 0.002922Da. The fact is that glycans contain multiple methylation sites (i.e., –OH and NH_2 groups). Hence, the differentially labeled glycans have a proper Δm and can be separated at a resolution of ~30,000 m/Δm. In addition, the practical mass difference of permethylated glycans from biological specimen is less than 1Da. As the Δm between differentially labeled glycans increases with the number of permethylation sites, i.e. the size of the glycans, the mass difference between isobaric species also increases (m).[218] Thus, the resolution required to resolve these isobarically labeled oligosaccharides is practically independent of the glycan's molecular weight. Usually, structural isomers are defined through a certain pathway of sequential mass spectrometry until anomalous ions at one stage give clue to their identities.[8] These anomalous ions are considered as characteristic ions here.

The mass spectrometric strategy of QUIBL for isomeric mixtures is described as follows (Fig. 4.1): (a) Two identical glycan populations containing isomeric glycans are permethylated with either $^{13}CH_3I$ or $^{12}CH_2DI$. (b) The permethylated glycans from each population are mixed at1:1 ratio and analyzed by a hybrid tandem mass spectrometer (such as an ion trap-FTMS or an ion trap-Orbitrap) which is capable of both low-resolution and high-resolution mass analysis. (c) Nominal masses are defined using the ion trap, the resolution of which is not enough to discriminate quasimolecular ions of glycans that are labeled with $^{13}CH_3$ and $^{12}CH_2D$. (d)

Quasimolecular ions originating from the $^{13}CH_3$ and $^{12}CH_2D$ labeled glycans can be resolved by high resolution mass analyzers (using the FTMS or Orbitrap). The abundances of the differentially labeled quasimolecular ions can be directly compared in MS mode to quantitate glycans. When isomeric glycans are present, the quantitation involves the total abundance of each isomer. Thereby, tandem MS is performed. (e) LTQ stepwise total ion mapping is performed to acquire the MS^2 of all ions from start to the end of spectrum, which is of help to interpret glycan components by MS/MS spectra. When the presence of the isomers is ascertained, MS^n is carried out for each individual isomeric glycan of interest. (f) Precursor ions can be isolated in a narrow window width using LTQ and fragmented by CID. At this stage, identical glycan fragment ions containing $^{13}CH_3$ and $^{12}CH_2D$ groups appear at the same nominal mass. If characteristic fragment ions belonging to an isomer appear in this stage, high resolution analysis can be followed up. If not, more MS event is needed until the character fragment ions show up. (g) Isobarically labeled characteristic fragment ions can be distinguished at high resolution and the quantitation of the fragment ions is performed in the same manner as in the MS mode. Through this procedure, the relative quantitation of individual isomeric glycan in two populations can be obtained.

The capability of QUIBL to quantitate glycans has been demonstrated using N-glycans from standard glycoprotein Fetuin, as well as those from human serum.[218] A pair of structural isomers are chosen as standard isomeric glycans, Lacto-N-fucopentaose I (LNFP I), Fucα1-2Galβ1-3GlcNacβ1-3Galβ1-4Glc and Lacto-N-fucopentaose II (LNFP II) Galβ1-3(Fucα1-4)GlcNacβ1-3Galβ1-4Glc, respectively. To identify characteristic fragment ions

from each glycan, $^{13}CH_3$ permethylated LNFP I and LNFP II was subjected to LTQ MS/MS (Fig. 4.2). The precursor ion of isobarically labeled LNFP I and LNFP II in LTQ was at *m/z* 1116.91. From the LTQ MS/MS spectra, the ion at *m/z* 669.55 was common to both isomers. MS/MS analysis identified the fragment ion at *m/z* 439.2 as being unique to LNFP I, and the fragment ion at *m/z* 907.5 as unique to LNFP II. Other potential diagnostic ions appear at *m/z* 700.55 (LNFP I) and *m/z* 876.64 (LNFP II) can also be used due to their higher abundance at each MS/MS spectrum than the other.

To verify that the quantitation of fragmentation ions is equally associated with the quantitation of precursor glycans, we mixed $^{13}CH_3$ labeled LNFP I to $^{12}CH_2DI$ labeled LNFP I to $^{13}CH_3$ labeled LNFP II to $^{12}CH_2DI$ labeled LNFP II at 1:1:1:1 ratio, followed by LTQ FT analysis in triplet. As shown in Fig. 4.3, the similarity in the ratios of isobaric labeled species between the precursor ions and common fragmentation ions, and unique or diagnostic fragment ions demonstrates that the MS/MS process does not alter the ratio of the isobaric labels. This ability suggests that it is possible to use quantitation of fragment ions for the quantitation of individual isomeric glycan precursor ions. Quantitation is accomplished by summing the ion intensities for the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled isotopic series and comparing these two values. This method is more accurate than the quantitation by comparing the abundance of monoisotopic peak ions. It is because the content of isotopic agents we used is not 100%. $^{12}CH_2DI$ contains 98% D, and the $^{13}CH_3I$ reagent is 99% ^{13}C. And this is also for the sharper drop of the intensity of uncompleted labeled isotope ion peaks which masses were lower than the

predicted monoisotopic in the spectrum of the $^{13}CH_3$ labeled analyte than in spectrum of the $^{12}CH_2D$ labeled analyte.

Relative quantitation of both isomeric glycans is simultaneously acquired. This is illustrated by the FTMS spectra of four isomer mixtures of $^{13}CH_3$ labeled LNFP I and $^{12}CH_2DI$ labeled LNFP I and $^{13}CH_3$ labeled LNFP II and $^{12}CH_2DI$ labeled LNFP II, in which the expected ratio of $^{13}CH_3$ labeled to $^{12}CH_2D$ labeled LNFPI are 12:1, 8:3, 2:5 and 3:20; while, for LNFPII the isobaric ratios are 12:5, 8:1, 2:1 and 3:4. Analyses of FTMS spectra of unique fragment ions of LNFPI and LNFPII are shown in Figure 4.4 and Figure 4.5. And also diagnostic fragmentation ions spectra are in supplement materials (Supplement fig. 4.1 and fig. 4.2). Even at ratio around 12:1, the two isobarically labeled species could be discerned.

The linearity of response obtained by QUIBL was evaluated by FTMS analysis of twenty five standard mixtures containing $^{13}CH_3$ to $^{12}CH_2D$ labeled LNFPI as well as $^{13}CH_3$ to $^{12}CH_2D$ LNFPII in ratios ranging from 12:1 to 3:20 (Fig. 4.6). These results indicate that quantitation using the QUIBL approach is linear greater than two orders of magnitude. The accuracy of the quantitation of QUIBL becomes better when the expected ratio of isobaric labeling components approaches one. We also evaluate the dynamic range of the quantitation using unique fragmentation ions with using diagnostic fragmentation ions (supplement fig. 4.3 and fig. 4.4). The diagnostic fragment ions selected for this study are not truly specific for each glycan. For example, the ion used for LNFPII quantitation at 876.6 *m/z* is actually produced by both glycans, albeit at a much lower extent in LNFPI to a much lower extent. Then the selection of a truly

unique fragment ion is expected to alleviate this problem, and ameliorate the accuracy of the quantitation.

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Figure 4.1 Flow chart for quantitative glycan analysis using isobaric labeling for the isomaric glycan mixture analysis.

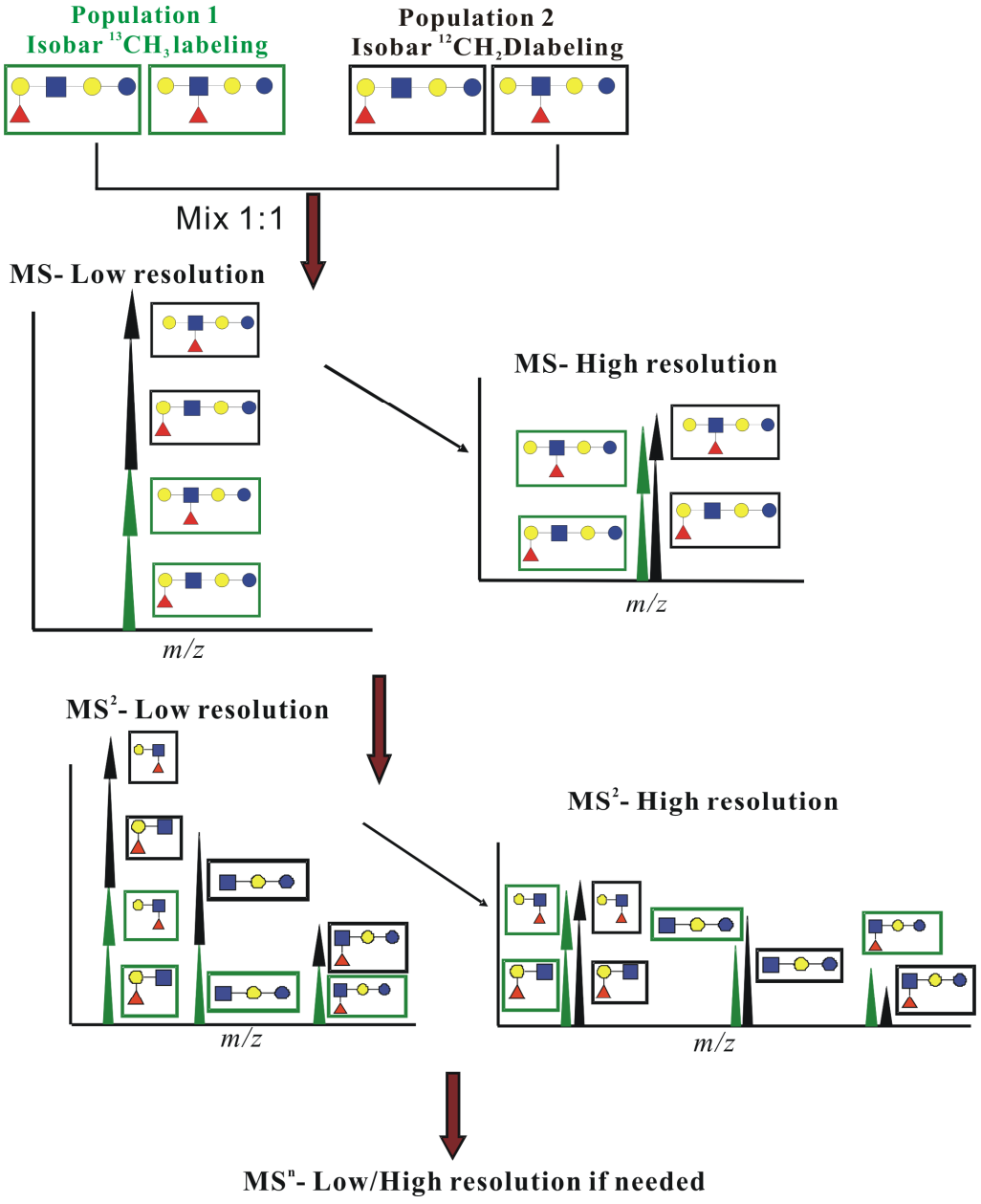


Figure 4.2. LTQ MS/MS spectra of the permethylated (a) LNFPI and (b) LNFPII with $^{13}CH_3I$.

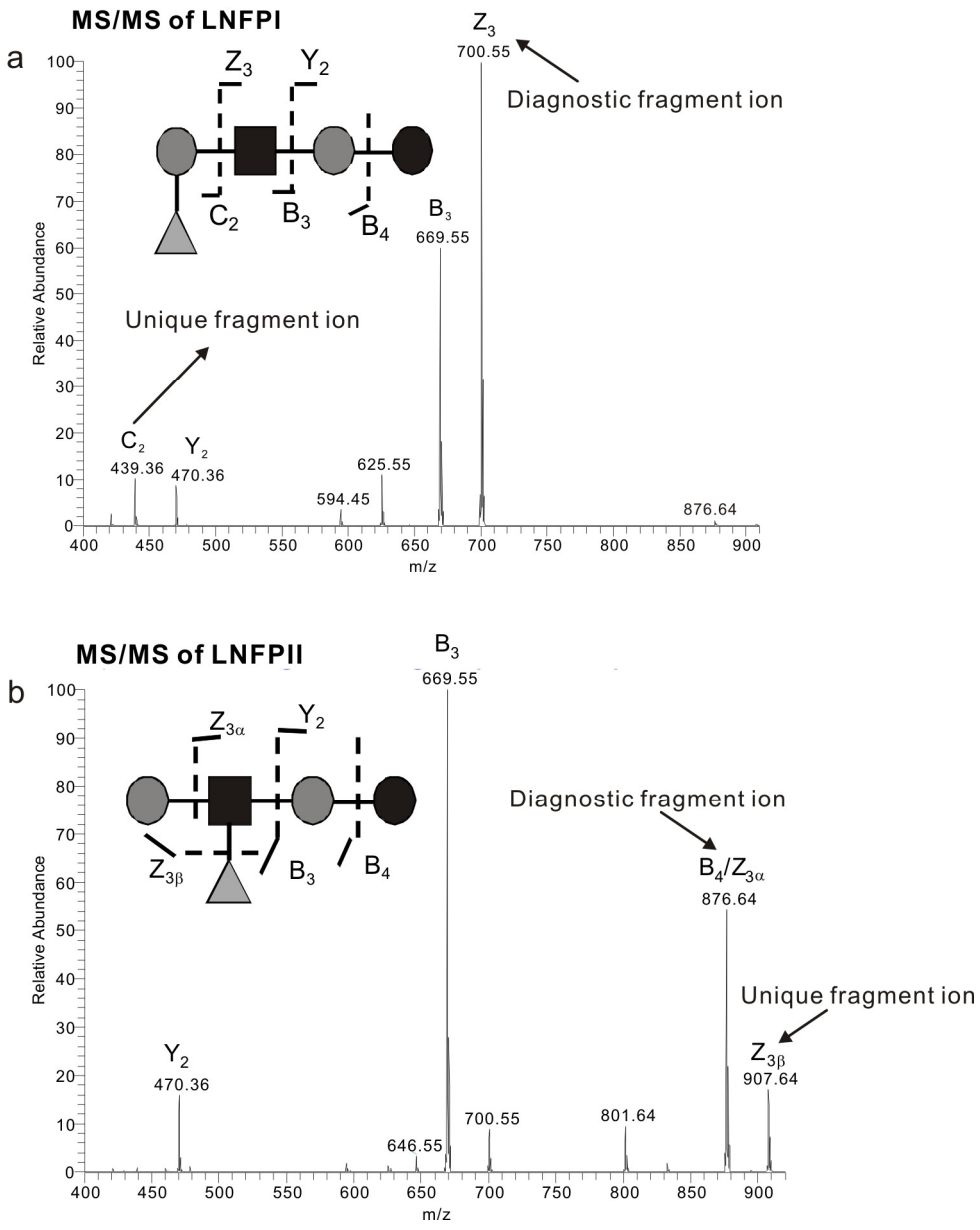


Figure 4.3 FTICR spectra of (a) precursor ion at *m/z* 1116; (b) common fragment ion at *m/z* 669.5; (c) unique fragment ion for LNFPI at *m/z* 439.4; (d) diagnostic fragment ion for LNFPII at *m/z* 876.6. when analyzing the 1:1 mixture of LNFPI and LNFPII permethylated respectively with $^{13}CH_3I$ and $^{12}CH_2DI$.

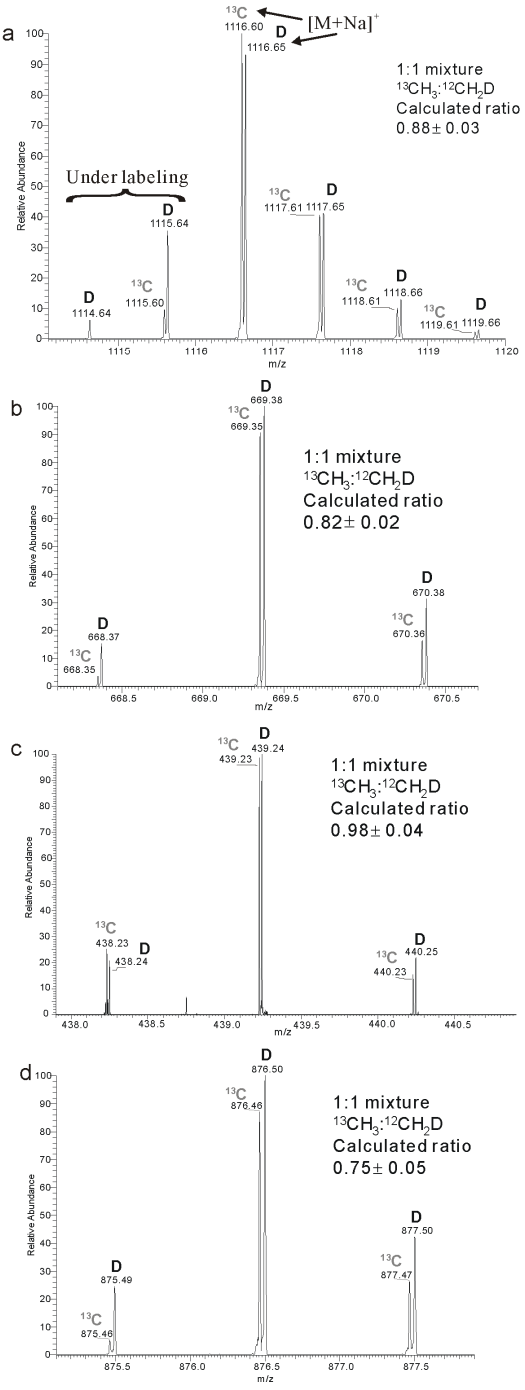


Figure 4.4 FTMS spectra of unique fragment ions of LNFPI from four isomer mixtures of $^{13}CH_3$ labeled LNFP I and $^{12}CH_2DI$ labeled LNFP I and $^{13}CH_3$ labeled LNFP II and $^{12}CH_2DI$ labeled LNFP II, in which the expected ratio of $^{13}CH_3$ labeled to $^{12}CH_2D$ labeled LNFPI are (a)12:1; (b) 8:3; (c) 2:5 and (d) 3:20.

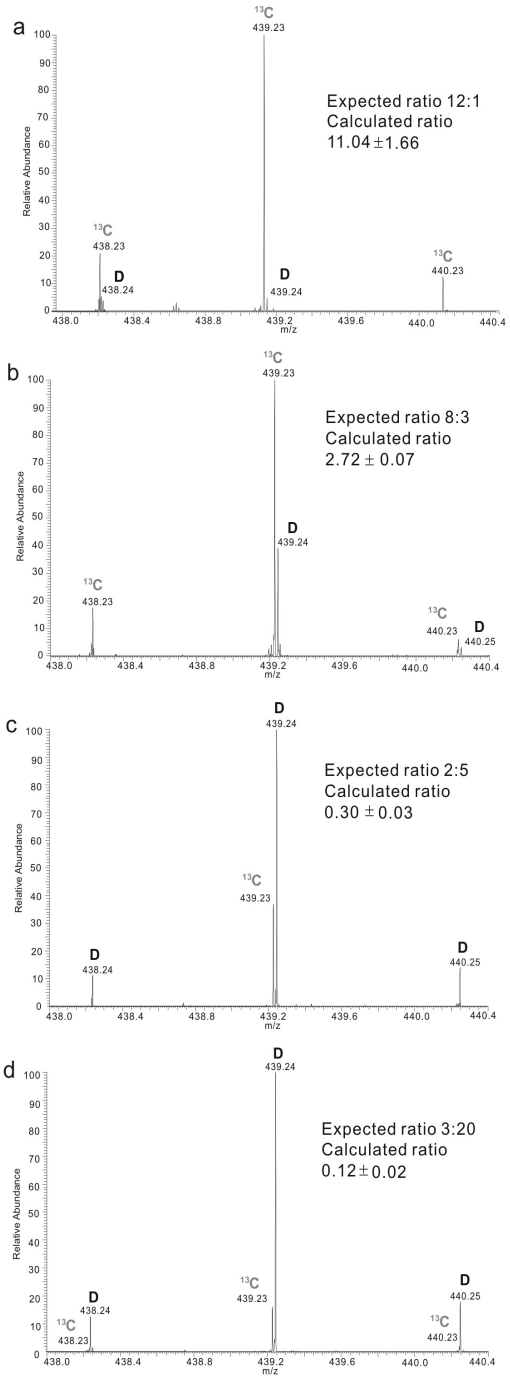


Figure 4.5 FTMS spectra of unique fragment ions of LNFPII from four isomer mixtures of $^{13}CH_3$ labeled LNFP I and $^{12}CH_2DI$ labeled LNFP I and $^{13}CH_3$ labeled LNFP II and $^{12}CH_2DI$ labeled LNFP II, in which the expected ratio of $^{13}CH_3$ labeled to $^{12}CH_2D$ labeled LNFPI are (a)12:5; (b) 8:1; (c) 2:1 and (d) 3:4.

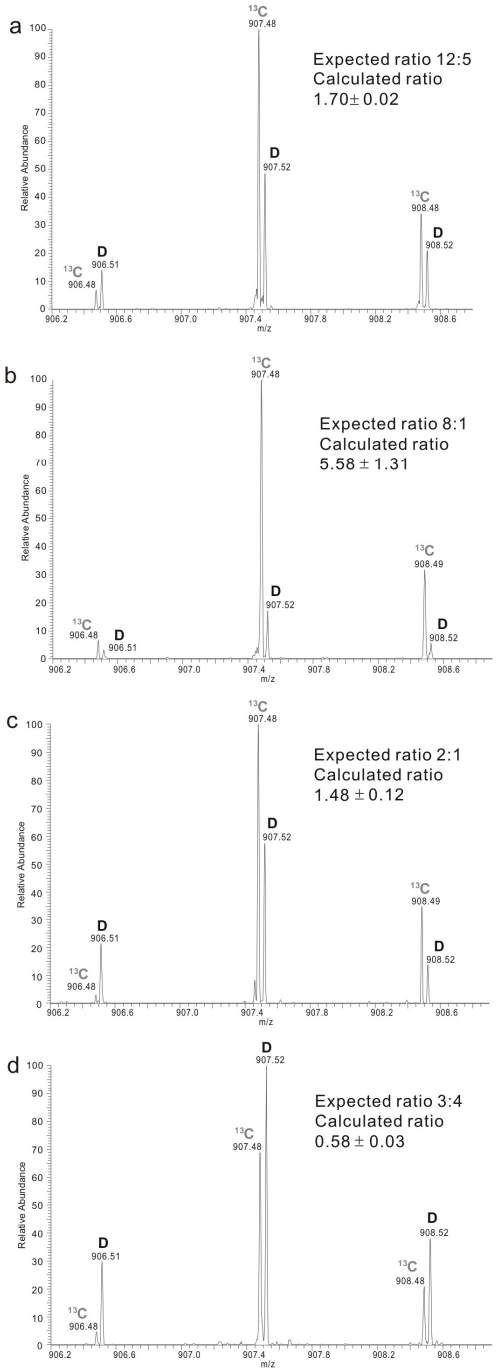
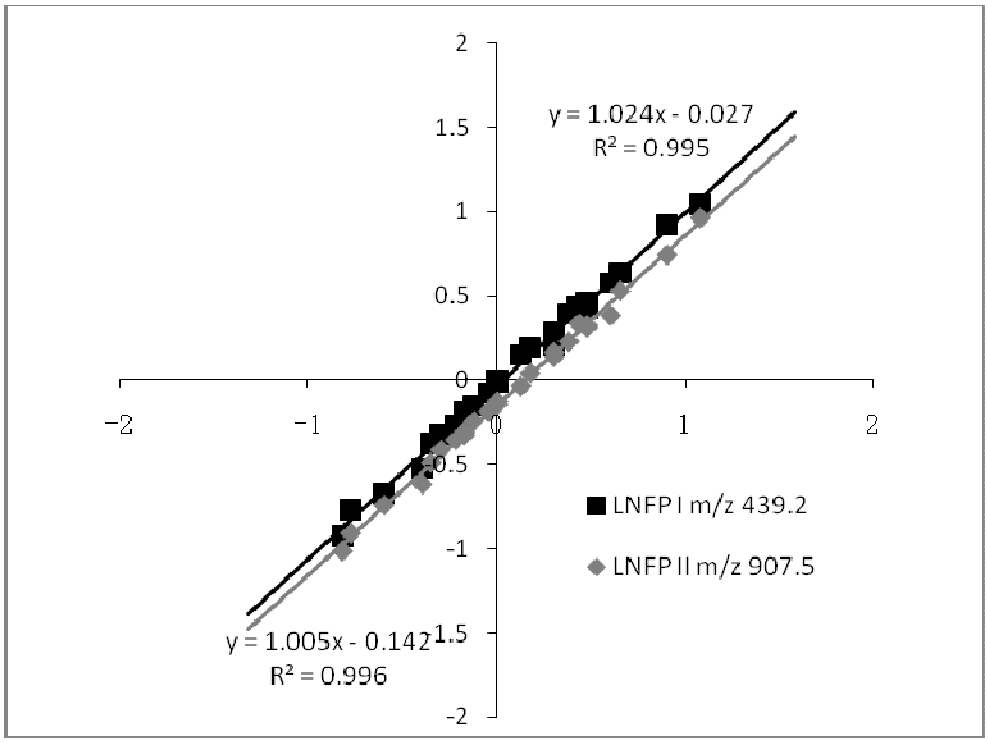
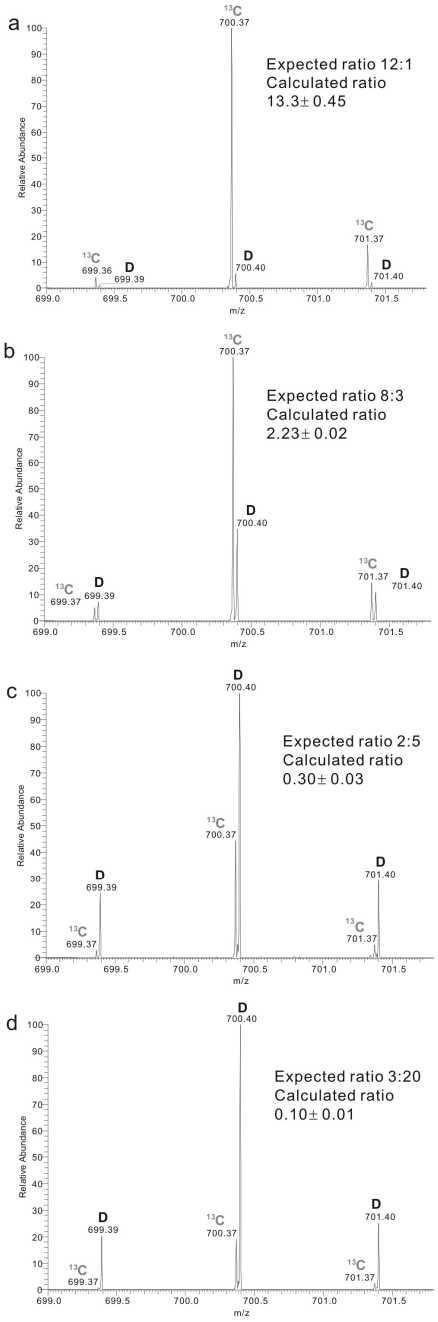


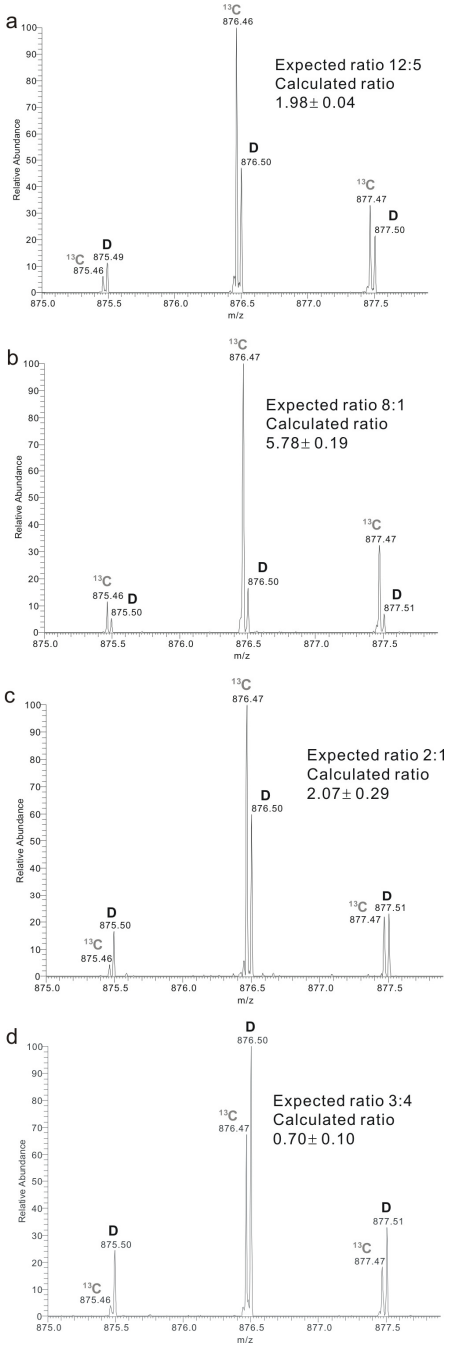
Figure 4.6 Correlation between calculated and expected ratios for quantitation of isomeric glycan mixtures by QUIBL by unique fragment ions.



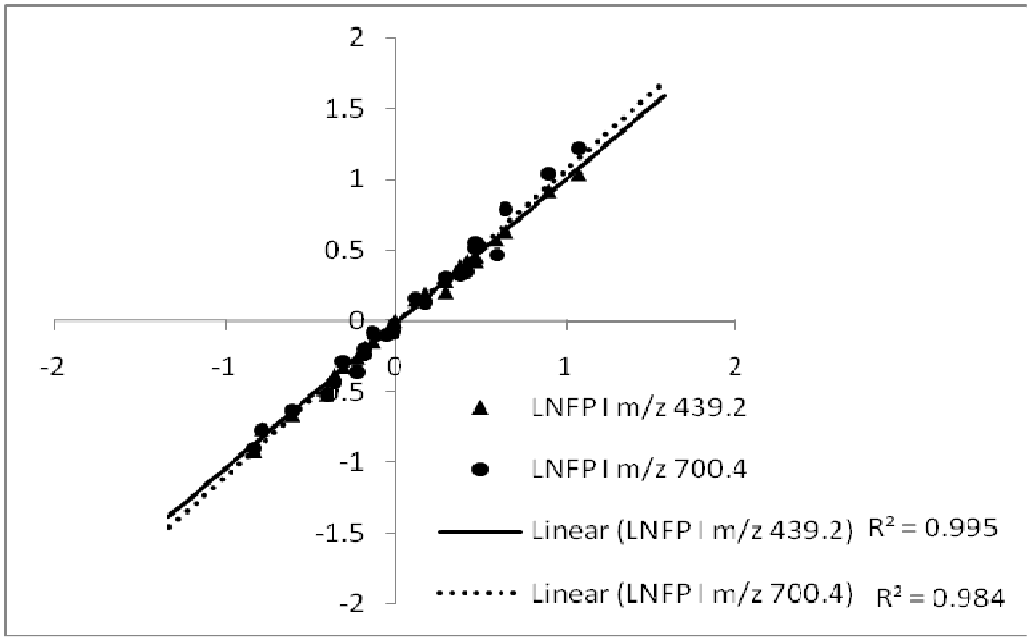
Supplement figure 4.1. FTMS spectra of diagnostic fragment ions of LNFPI from four isomer mixtures of $^{13}CH_3$ labeled LNFP I and $^{12}CH_2DI$ labeled LNFP I and $^{13}CH_3$ labeled LNFP II and $^{12}CH_2DI$ labeled LNFP II, in which the expected ratio of $^{13}CH_3$ labeled to $^{12}CH_2D$ labeled LNFPI are (a)12:1; (b) 8:3; (c) 2:5 and (d) 3:20.



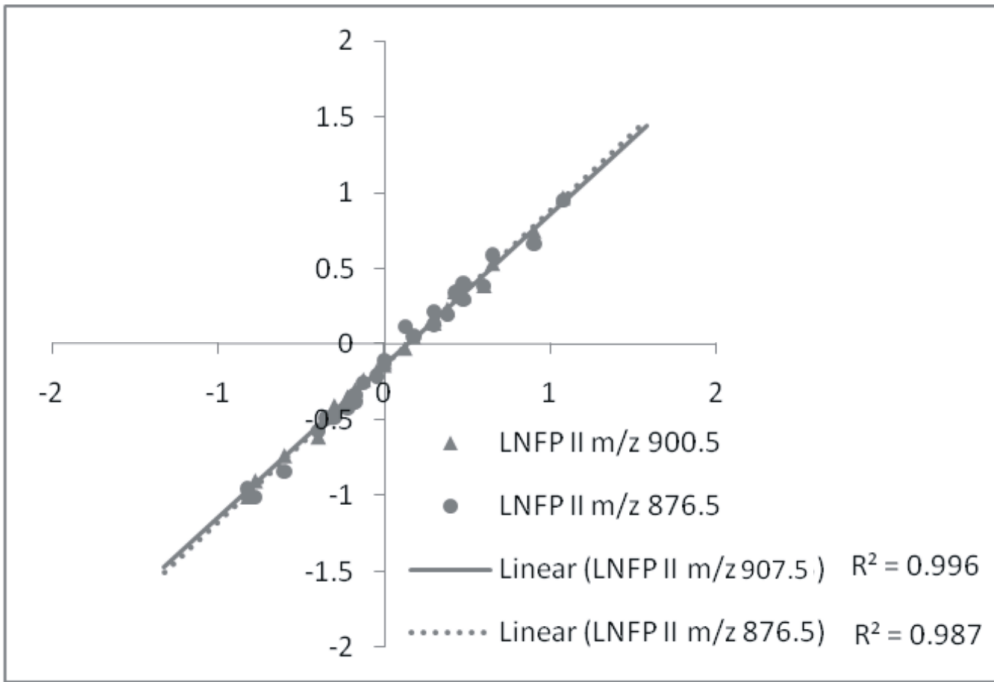
Supplement figure 4.2. FTMS spectra of diagnostic fragment ions of LNFPII from four isomer mixtures of $^{13}CH_3$ labeled LNFP I and $^{12}CH_2DI$ labeled LNFP I and $^{13}CH_3$ labeled LNFP II and $^{12}CH_2DI$ labeled LNFP II, in which the expected ratio of $^{13}CH_3$ labeled to $^{12}CH_2D$ labeled LNFPI are (a)12:5; (b) 8:1; (c) 2:1 and (d) 3:4.



Supplement figure 4.3 Correlation between calculated and expected ratios for quantitation of LNFPI by QUIBL by unique fragment ions at *m/z* 439.2 or by diagnostic fragment ion at *m/z* 700.4.



Supplement figure 4.4 Correlation between calculated and expected ratios for quantitation of LNFPII by QUIBL by unique fragment ions at *m/z* 907.5 or by diagnostic fragment ion at *m/z* 876.5.



CHAPTER 5

CONCLUSION

The overall purpose of this work was to develop a MS-based methodology, quantitative isobaric labeling (QUIBL) for quantitative glycomics. As well as rapid identification of the whole glycan profiles in the samples, QUIBL is able to quantify the relative amount of individual glycans expressed in two differential samples.

Chapter 3

The principle of QUIBL was brought up with the core idea involving permethylation of glycans with $^{13}CH_3I$ or $^{12}CH_2DI$ and the discrimination of isobaric labeled species by the use of high resolution mass analyzer FTICR. First, QUIBL was proven by using the glycoprotein Fetuin. N-glycans of Fetuin were release using PNGase F and extracted by C18 SepPak cartridge using 5% acetic acid buffer. After permethylation, $^{13}CH_3$ labeled N-glycans was mixed with $^{12}CH_2D$ labeled N-glycans at five different ratios. The mixtures were respectively analyzed by LTQ-FTICR. The quantitation was performed by separately adding the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled ion intensity over all isotopomers for the glycan. Two typical glycans were quantified. The results demonstrate QUIBL is an efficient method for quantitative glycomics. Next, QUIBL was verified to be able to quantify N-glycans from human serum in a single experiment. QUIBL works independently on glycan composition, size, or ionization efficiency,

and is capable of accurately quantitating glycans of both low and high abundance. Last, QUBIL was used to study the glycome changes during early embryogenesis.

Chapter 4

The most promising aspect of QUIBL is that it allows simultaneous quantitation of glycans that have the same molecular mass (*i.e.*, isomers). If a fragment ion unique to each of the isomers is observed by MS^n, the ratio of differentially labeled forms of each isomer can be measured by high-resolution analysis of the isobaric labeled fragment ions. This capability was demonstrated by analyzing the mixtures of $^{13}CH_3I$-labled LNFPI and LNFPII with $^{12}CH_2DI$-labeled LNFPI and LNFPII. At first, these four labeled isomers were mixed at 1:1:1:1 ratio. QUIBL was operated by the selection and fragmentation of the ion (*m/z* 1116.6). CID of this precursor ion generated a collection of fragments that included unique fragment ions for LNFPI (*m/z* 439.2) and for LNFPII (*m/z* 907.5). Unique fragment ions were analyzed by high resolution FTICR for the quantitation, which was consistent with the theoretic ratio of precursor isomers. This experiment demonstrated that accurate quantitation can be performed using fragment ions originating from MS/MS events, or multiple MS/MS events if further fragment ions needed. More mixtures at different ratios were analyzed to evaluate the dynamic range of QUIBL for the quantitation of glycans that are present as low abundance components of isomeric mixtures.