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ARTICLE

PNGase F-Mediated Incorporation of ^{18}O into Glycans for Relative Glycan Quantitation

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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PNGase F-catalyzed glycosylation site ^{18}O-labeling is a widely used method for glycoprotein quantitation owing to its efficiency and simplicity. However, PNGase F-catalyzed glycan ^{18}O-labeling, which offers advantages for glycomics, has not been developed yet. In this study, PNGase F-mediated incorporation of ^{18}O into Glycans during the N-glycans released from glycoproteins by PNGase F was finally realized, naming as PCGOL (PNGase F-catalyzed glycan ^{18}O-labeling), which offers a potential strategy for relative glycan quantitation. This new method showed good linearity and high reproducibility within at least 2 orders of magnitude in dynamic range. Furthermore, PCGOL combined with our previously developed TOSIL method (Tandem ^{18}O stable isotope labeling for N-glycoproteome quantitation) can be used for comprehensive *N*-glycosylation quantification, achieving simultaneous quantification of glycan, glycopeptide and glycoprotein in a single workflow, which was also used to analyze glycosylation changes in immunoglobulin G (IgG) associated with hepatocellular carcinoma in the present work.

Introduction

Glycans participate in a number of important biological processes, such as molecular and cellular recognition, signaling and communication[1]. Quantitative changes in glycosylation abundance and corresponding glycan structures of glycoproteins have been shown to correlate evidently with many disorders and diseases[2]. Therefore, quantitative profiling of glycan variations has been arousing considerable research attention for potential disease biomarker and drug target discovery. Mass spectrometry (MS) based stable isotope labeling is an effective method for quantitative glycomics[3]. Several stable isotope labeling methods have been developed for comparative glycomics, including isotopic incorporation during permethylation[4], reducing ends labeling with heavy and light compouds[5], and metabolic labeling with isotopic amino acid or sugar[6]. However, all these labeling strategies are not without limitations. For example, isotope labeling via permethylation or on the reducing ends requires additional steps and reagents, which may challenge labeling efficiency and reproducibility. The metabolic labeling approaches appear to be limited to the investigation of cells, and the high cost limits their application.

Enzymatic ^{18}O-labeling, a well-established stable isotope labeling strategy, has been widely used in peptide/protein quantitation[7]. Proteases that have high specificities on C-terminal residues, such as trypsin, Glu-C and Lys-C, can stably incorporate two atoms of ^{18}O ($^{18}O_2$) into the newly generated C-terminal carboxyl of peptides during proteolytic digestion, displaying a 4 Da mass shift for peptides. Numerous investigations have utilized proteolytic $^{18}O_2$-labeling for quantitative proteomic analyses[8]. Enzymatic ^{18}O-labeling is undoubtedly the most popular and powerful stable isotopic labeling strategies owing to its efficiency and simplicity: the labeling reaction occurs during enzymatic digestion, and all that is required is the presence of ^{18}O-water. Thereby, costly reagents and laborious steps are avoided, and side reactions are also inexistent. Furthermore, ^{18}O does not cause any chromatographic isotope effects.

However, for relative glycan quantitation, ^{18}O-labeling is still uncommon as compared to its extensive utilization in quantitative peptide/protein analysis. In the previous study, we reported that endoglycosidase universally incorporates ^{18}O into

N-glycan reducing-end during enzymatic release of *N*-glycans in the presence of $H_2{}^{18}O$. Based on this reaction, a novel GREOL strategy[9] was proposed and effectively applied to MS-based relative glycan quantification. However, the drawback of this strategy is that because the innermost GlcNAc still attached to its parent peptide after endoglycosidase hydrolysis, information concerning important core-fucosylation is consequently lost. Moreover, endoglycosidases have remarkable specificities on certain glycan structures and have not yet been widely used as compared to Peptide-*N*-glycosidase F (PNGase F). These shortcomings promote a strong motivation for us to label ^{18}O onto the terminal GlcNAc by the widely used PNGase F.

PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of virtually all subtypes of *N*-glycans from glycoproteins[10]. PNGase F can characteristically introduce an ^{18}O atom into glycosylation site during enzymatic removing of *N*-glycans in $H_2{}^{18}O$, which permits unambiguous assignment of glycosylation site due to a larger mass shift of 2.98 Da[11]. The use of PNGase F is the most extensive and effective method for glycoprotein identification and quantitation[12]. For example, Liu et al.[13] developed an enzymatic $^{18}O_3$-labeling approach, which combined trypsin-mediated peptide $^{18}O_2$-labeling and PNGase F-mediated glycosylation site ^{18}O-labeling, with the unique 6 Da mass difference specifically for glycoprotein quantification. Shakey et al. introduced an integrated strategy allowing for PNGase F-mediated glycosylation site ^{18}O-labeling of hydrazide-enriched glycopeptides, with which they identified and quantitated 224 *N*-glycopeptides from mouse serum[14]. However, PNGase F-catalyzed glycan ^{18}O-labeling has not been implemented so far, not to mention applied to the glycan quantitation.

In the present work, PNGase F-catalyzed complete *N*-glycan ^{18}O-labeling (PCGOL) was realized and successfully used for relative glycan quantification, which showed good linearity and high reproducibility within at least 2 orders of magnitude in dynamic range. Furthermore, by combination of this newly developed PCGOL with our previous developed ^{18}O-labeling for N-glycoproteome quantitation, a novel enzymatic $^{18}O_4$-labeling strategy was developed for comprehensive *N*-glycosylation quantification, achieving simultaneous quantification of glycan, glycopeptide and glycoprotein in a single workflow. Moreover, glycosylation changes in immunoglobulin G (IgG) associated with human hepatocellular carcinoma (HCC) were analyzed with enzymatic $^{18}O_4$-labeling strategy as an example, and quantitative information concerning glycan structure and glycosite of IgG was obtained.

Results and discussion

Realization of PCGOL

We have noticed that there is no reported studies so far to show that PNGase F could catalyze glycan ^{18}O-labeling, although PNGase F has been widely used in N-glycosylation identification and quantitation[15, 16]. We first tried to label the glycans with ^{18}O by PNGase F catalysis at a basic pH condition, and found that at a basic pH condition (alkaline solution, pH 7.5-8.5), ^{18}O cannot be labeled completely onto the glycan reducing end, resulted in an incomplete glycan ^{18}O-labeling by PNGase F (Fig. S1). PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of almost all subtypes of *N*-linked glycans from glycoproteins, and the reaction mechanism of PNGase F involves two steps: first, glycoprotein is hydrolyzed into protein and glycosamine with PNGase F-catalyzed digestion, and then the glycosamine undergoes deamination and forms glycans[17]. We suspected that it is because of this mechanism yielding a basic reaction condition against to the PNGase F-catalyzed N-glycan ^{18}O-labeling. As shown in Fig. S1b (enlarged spectra of the peaks at m/z 1663 and 1665, for sodium adduct ions of $HexNAc_4Hex_5$ glycan), there is still a considerable signal of the -1 Da ion peak [M-1], indicating the existence of glycosamine. It seems that the alkaline condition of pH7.5-8.5 would lead to free basic ammonia after the hydrolysis of glycosamine, and thus inhibit further hydrolysis due to the reversible deamidation process in equilibrium, making an incomplete hydrolysis and label of glycosamine (Fig. S1c).

We believed that an acidity condition should be in favor of the PNGase F-catalyzed N-glycan ^{18}O-labeling, and therefore tried to adjust the basic pH to the acidity for a complete label by the addition of formic acid (1% v/v) to break the balance of hydrolysis after the glycan released by PNGase F. Delightfully, the ^{18}O was completely labeled in the acidic condition as expected (Fig. 1). Enlarged spectra of $HexNAc_4Hex_5$, a sample glycan pair at m/z 1663 and 1665, showed that the original -1 Da ion peak of glycosamine almost disappeared entirely, with no cross-labeling (Fig. 1b). As a consequence, the reaction balance of hydrolysis can be broken and the glycosamine completely hydrolyze after the addition of 1% (v/v) formic acid (Fig. 1c), resulting in an almost 100% labeling efficiency. Furthermore, the structure of $^{16}O/^{18}O$-labeled glycan $HexNAc_4Hex_5$ from asialofetuin can be analyzed by means of tandem mass spectrometry. The major fragmentation of the glycan ions (B_2, B_3, Y_3, Y_4, Y_5) confirmed the di-antenna complex type of $HexNAc_4Hex_5$, which was consistent with N-glycan type of asialofetuin (Fig. 1d). Moreover, the assignments of diagnostic ions especially with 2 Da difference, such as m/z 933/935, 1136/1138, 1298/1300 and 1501/1503, further demonstrated that the ^{18}O was stably labeled onto the reducing end of the glycans.

Thus, PCGOL was finally realized with almost 100% labeling efficiency. According to the importance of PNGase F-mediated glycosylation site ^{18}O-labeling in glycoproteomics research, we believe that the realization of PCGOL has potential application value and broad application prospect in glycomics research.

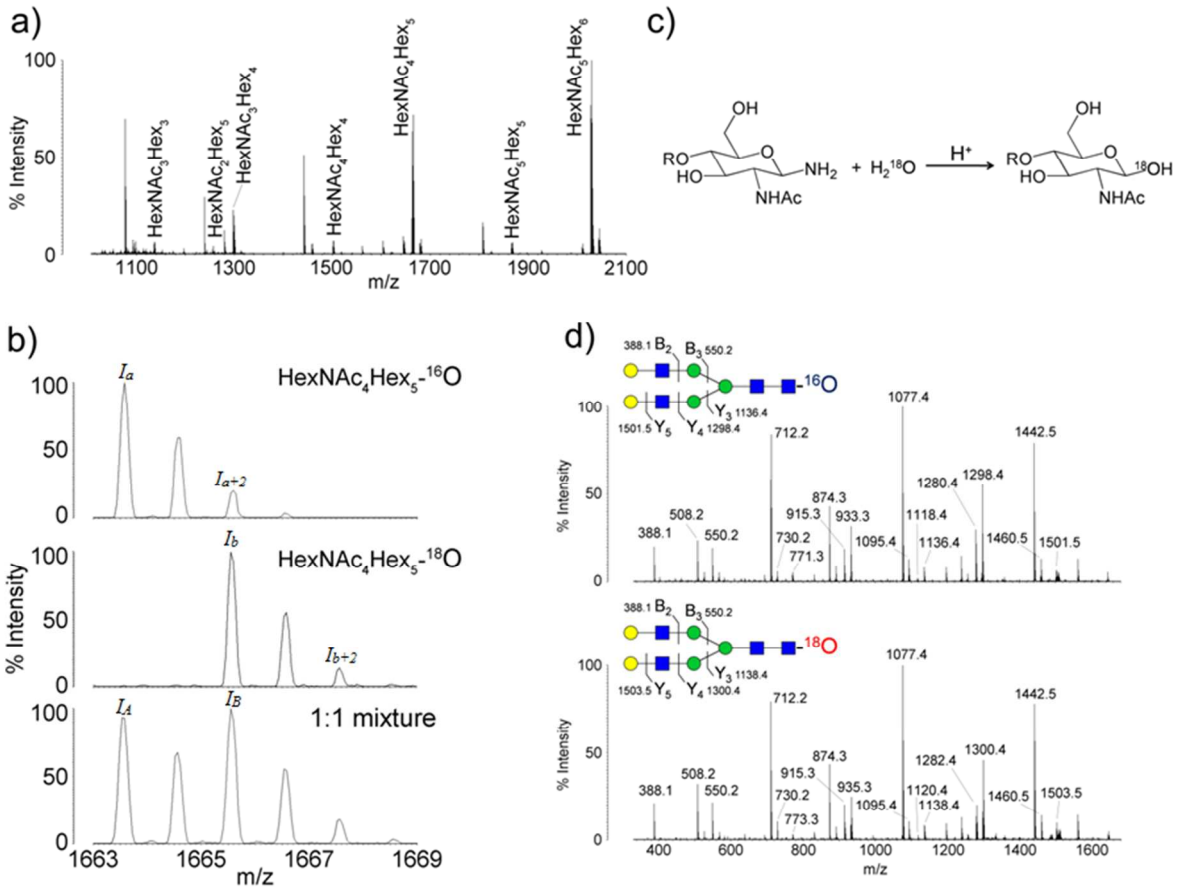

Figure 1. PNGase F-catalyzed N-glycans ^{18}O-labeling (PCGOL). (a) Spectra of $^{16}O/^{18}O$-labeled glycans with equal mixture. (b) Enlarged spectra of the glycan $HexNAc_4Hex_5$ labeled by ^{16}O (top) and ^{18}O (middle), and with equal mixture (bottom). (c) The schematic diagram of glycosamine hydrolysis in acidic condition. (d) Tandem mass spectra of the glycan $HexNAc_4Hex_5$ labeled by ^{16}O (top) and ^{18}O (bottom

PCGOL for Relative Glycan Quantitation

We then applied PCGOL to the relative glycan quantitation. In order to avoid isotope interference, a deconvolution method[9] previously developed was used: both isotope distribution and the partial ^{16}O labeling were taken into consideration (Supplementary Information S-1). The linearity and reproducibility were accessed using ribonuclease B (high mannose type), ovalbumin (variety of N-glycan type), and asialofetuin (complex type) as models. The ^{16}O-labeled and ^{18}O-labeled glycans were mixed in proportions of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10 (v/v) and then analyzed by MS with six replicates for each ratio. Dual-logarithm plots between the theoretical and corresponding measured ratios for six glycans were generated, showing high accuracy with the slope of six glycans ranging from 0.95 to 1.08 and the Y and X intercept ranging from -0.078 to 0.074 (Table S1). Meanwhile, the overall coefficients of variation (CVs) ranging from 3.95% to 19.5% indicate a good reproducibility (Table S1). In addition, as shown in Fig. 2, the quantitative results of six glycans show good linearity within two orders of magnitude range (1:10 to 10:1) with correlation coefficients (R^2) higher than 0.99. Obviously a partial overlap of glycan pair isotope envelopes with 2 Da gaps cause no effect on the quantitative results after deconvolution. The above results demonstrate that PCGOL exhibits good accuracy, reproducibility and linearity with at least two orders of magnitude range, and can be used for relative glycan quantitation. So far, we have creatively established the PCGOL quantitative strategy, providing a powerful tool for N-glycan relative quantitation.

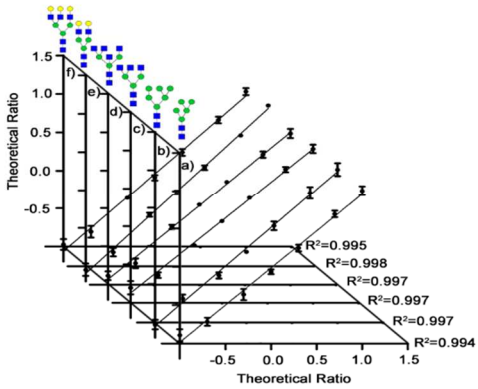

Figure 2. Dual-logarithm plots between the theoretical and corresponding measured ratios for six glycans (n = 6). (a, b) Glycans from ribonuclease B. (d, c) Glycans from ovalbumin. (e, f) Glycans from asialofetuin. All plots show good linearity (R^2> 0.99). Green circle, Man; yellow circle, Gal; blue square, GlcNAc.

Enzymatic four ^{18}O-Labeling Strategy

Base on the above successful PCGOL for quantitation, a new strategy of enzymatic $^{18}O_4$-labeling was eventually developed for the comprehensive N-glycosylation quantitation (Fig. 3a). The glycan reducing end and glycosylation site were labeled with $^{16}O/^{18}O$ during the *N*-glycans released by PNGase F in $H_2{}^{16}O/H_2{}^{18}O$, and the de-glycosylated proteins were digested with immobilized trypsin in $H_2{}^{16}O/H_2{}^{18}O$-prepared buffer for peptide $^{16}O_2/^{18}O_2$-labeling[18]. The $^{16}O/^{18}O$-labeled glycans and peptides were mixed in a 1:1 molar ratio respectively and then analyzed by MALDI-MS. The feasibility and stability of enzymatic $^{18}O_4$-labeling were investigated using asialofetuin as a model glycoprotein. Results show that all N-glycan peaks present peak pairs, except for glycan fragments from loss of reducing ends. The enlarged spectrum of $HexNAc_4Hex_5$ directly indicates the existence of the peak pair (Fig. 3b). Although only 2 Da mass difference is existent between the $^{16}O/^{18}O$-labeled glycans, it do not exhibit serious overlap of glycan pair isotope due to the simple element composition and small molecular weight of glycan.

The peptide mass spectra results also present the obvious peak pairs and show that immobilized trypsin labeling method largely avoid the phenomenon of the ^{16}O back into the trypic peptide ends, making the ^{18}O-labeling completely (Fig. 3c). For examples, the enlarged spectrum of non-glycopeptide TPIVGQPSIPGGPVR presents an obvious peak pair with 4 Da gaps for $^{16}O/^{18}O$-labeling, while the glycopeptide LCPDCPLLAPLNDSR shows a clear peak pair with 6 Da gaps for $^{16}O_3/^{18}O_3$-labeling. No appearance of back-labeled peaks and isotope peak overlap further demonstrate the efficient and reliable of the trypsin labeling method. In proteomics research, LC-ESI-MS is extensively used for the large-scale analysis of peptides. Thus, we further analyzed $^{16}O/^{18}O$- labeled peptides from asialofetuin in a 1:1 molar ratio by LC-ESI-MS (Fig. S2a), and demonstrated peak pairs with equal intensity and rare back-labeling for a great deal of peptides. The enlarged spectra of non-glycopeptide CDSSPDSAEDVR (Fig. S2b) and glycopeptide LCPDCPLLAPL**N**DSR (Fig. S2c), both from asialofetuin, exhibit a peak pair with 4 Da and 6 Da gaps, respectively.

Eventually, the workflow of the enzyme-catalyzed $^{18}O_4$-labeling approach was established (Fig. 4): N-glycans are quantified through PCGOL with 2 Da difference, meanwhile non-glycopeptides and glycopeptides are quantified by our previously developed tandem ^{18}O stable isotope labeling with 4 Da and 6 Da difference. With this strategy, the changes of glycans, glycosylation level and glycoproteins can be identified and quantitated simultaneously and efficiently.

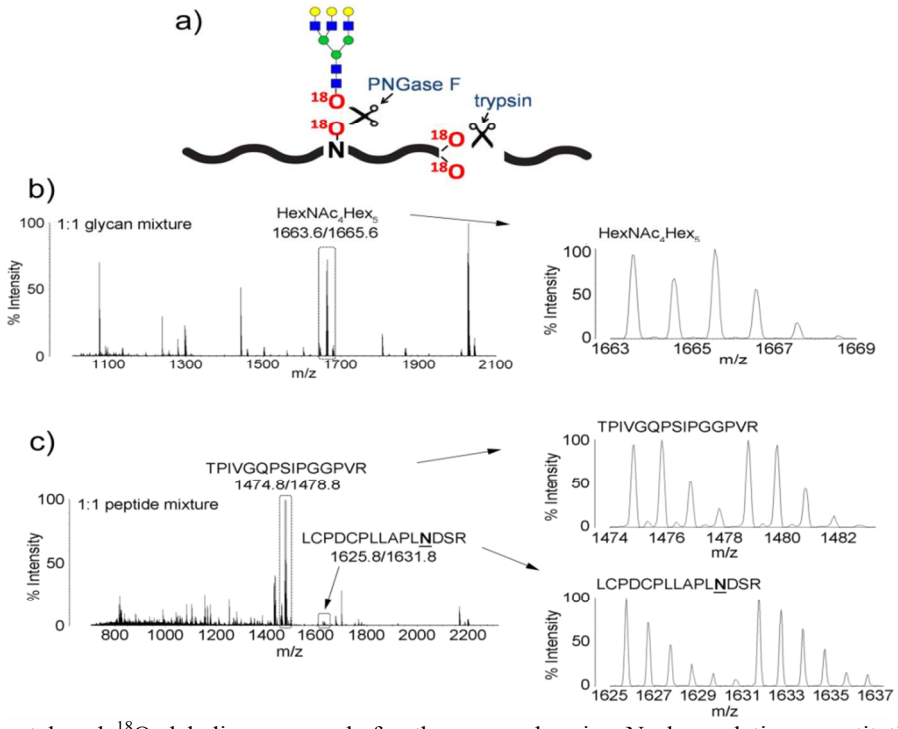

Figure 3. PNGase F-catalyzed $^{18}O_4$-labeling approach for the comprehensive N-glycosylation quantitation simultaneously in glycome and glycoproteome. (a) The diagram of enzymatic $^{18}O_4$-labeling. (b) Spectra of $^{16}O/^{18}O$-labeled glycans with equal mixture (left) and enlarged spectra of $HexNAc_4Hex_5$ (right). (c) Spectra of $^{16}O_2/^{18}O_2$-and $^{16}O_3/^{18}O_3$-labeled peptides (left), and enlarged spectrum of TPIVGQPSIPGGPVR and LCPDCPLLAPLNDSR (right).

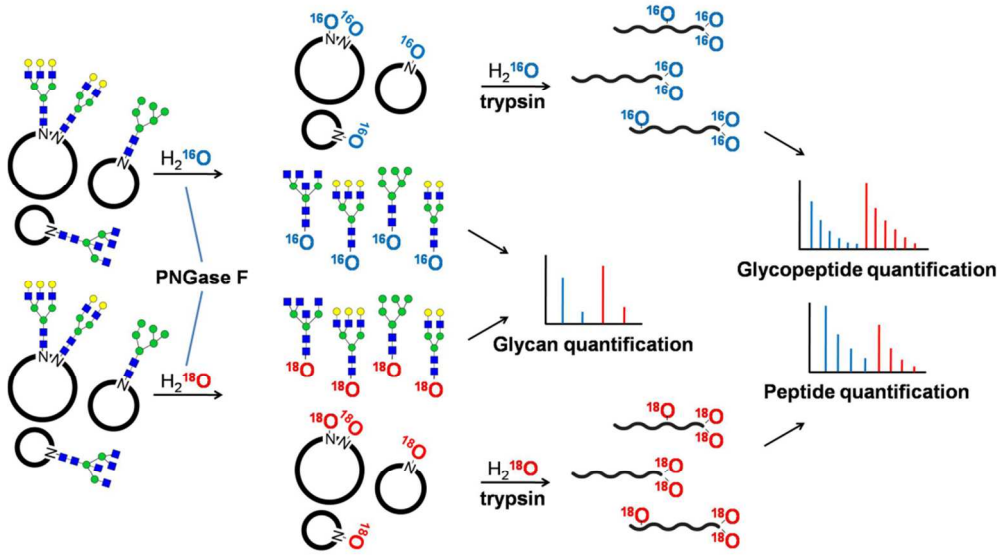

Figure 4. The strategy of enzyme-catalyzed $^{18}O_4$-labeling for N-glycosylation quantitation.

Enzymatic $^{18}O_4$-Labeling Strategy for Quantification of N-glycosylation of IgG from Normal and HCC Sera

We further demonstrated the practicality of enzymatic $^{18}O_4$-labeling strategy in comprehensive quantitation of N-glycosylation in biological samples. Since IgG plays an extremely important role in immune function[19], and the glycosylation variation of IgG is closely related with many pathological processes[20], we specifically quantified N-glycosylation of IgG from normal and HCC (human hepatocellular carcinoma) sera. IgG was effectively purified by SDS-PAGE (Fig. S3), and then, the protein bands of heavy chain and light chain of IgG were cut off and used for enzyme-catalyzed labeling and MS analysis. The labeled glycans were analyzed by MALDI-MS and the labeled peptides were analyzed by LC-ESI-MS (Fig. S4) for six replicates. All the quantitative results have been deducted the effect of isotopic peak overlap. Finally, a total of 16 N-glycans were identified, in which 12 were quantified from both IgG of normal and HCC serum, 3 glycans were only found in the IgG from normal sample and 1 glycan was only found in the IgG from HCC sample (Table S2), with the CVs ranging from 6.1%-19.9%, which showed good reproducibility. The quantitative results showed that the changes of IgG glycans between HCC and normal serum were all more than 20% (HCC/normal<0.83, or >1.20) (Table S2), which showed the distinguishable down- or up-regulation of glycan levels in HCC/normal samples. The HCC/normal ratio ($^{18}O_3/^{16}O_3$) of glycopeptide from IgG_2 and IgG_4 were 0.53 and 0.92 with CVs of 3.9% and 7.3%, respectively (Table S3). No quantitative result of IgG_1 or IgG_3 was obtained probably due to the failures of identification and automatic quantitation of 6 Da peak pairs for their weak or interfered signals of glycopeptides. The ratios of HCC/normal for 6 non-glycopeptides ($^{18}O_2/^{16}O_2$) were between 0.90 and 1.93, with CVs of 6.1%-19.9% (Table S4).

We further compared the quantitative results of glycans, glycopeptides and non-glycopeptides for the degree of glycosylation changes over the overall changes of glycoprotein. As shown in Fig. 5, the changes of all peptides are within 2 folds, and especially except the G2 and N2 the ratios of all other peptides are about 1.0, showing almost no change and implying that there is no evident change on the overall protein and glycosylation level of IgG in HCC. However, compared with peptides, the changes of N-glycans are more significant. A total of 4 glycans change more than 2 folds, in which 3 glycans ($GlcNAc_4Man_3Gal_2$, $GlcNAc_4Man_3Gal_2Fuc_1$, $GlcNAc_5Man_3Gal_1Fuc_1$) are up-regulated and 1 glycan ($GlcNAc_3Man_3Fuc_1$) is down-regulated in IgG of HCC. In total, the N-glycans with integrity chains, large molecular weight and bisecting GlcNAc types are generally increased, while the N-glycans with incomplete chains and less molecular weight are generally decreased. Therefore, our results obviously show that the alternation in glycan structure is more significant than that in either glycosylated-protein or protein levels of IgG from HCC sera, which also support the fact that some glycoproteins produced by cancer cells have altered glycan structures, although the proteins themselves are common, ubiquitous, abundant, and familiar[21].

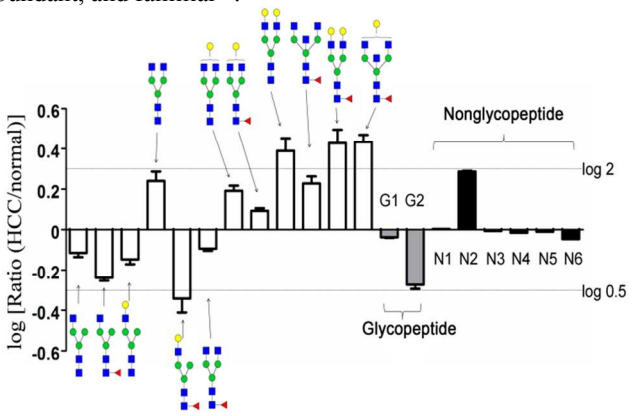

Figure 5. The total N-glycosylation quantitative results of IgG from HCC/normal serum. Red triangle, Fuc; green circle, Man; yellow circle, Gal; blue square, GlcNAc.

It was worth mentioning that in-solution digestion was also used for the IgG glycosylation quantitation analysis, and the quantitative results were the same with that of in-gel digestion as described above. It showed that the two technical routes of in-gel and in-solution digestion were equally effective, and any of them can be used in the $^{18}O_4$–labeling strategy according to actual needs.

Experimental

Materials and Chemicals

$H_2{}^{18}O$ (97%) was purchased from Cambridge Isotope Laboratories (Andover, MA). PNGase F (glycerol free) was purchased from New England Biolabs (Ipswich, MA), and immobilized trypsin beads were purchased from Applied Biosystems (Carlsbad, CA). The 10,000 Da MWCO centrifugal filters were purchased from Millipore (Bedford, MA), and porous graphitized carbon (PGC) columns were purchased from Grace (Columbia, MD). Melon gel IgG spin purification kit was purchased from Pierce (Rockford, IL). Trypsin, ovalbumin, asialofetuin, ribonuclease B, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Release and Labeling of *N*-Glycans

About 200 μg model glycoprotein (ovalbumin, asialofetuin, or ribonuclease B) solution was denatured at 95 °C for 10 min and divided into two identical aliquots. The aliquots were dried through vacuum centrifugation and redissolved in 100 μL $H_2{}^{16}O$/$H_2{}^{18}O$ prepared phosphate buffer (50 mM, pH 7.5), respectively. PNGase F (about 40 U) was then added to the samples, after which the samples were incubated overnight at 37 °C for glycan cleavage and labeling. Afterwards, the deglycosylated proteins were removed by a 10 kDa MWCO centrifugal filter at 4 °C for 30 min, and the purified glycans were further incubated at 37 °C for 2 h by adding 1 μL 1% formic acid (FA) for complete labeling. The treated glycans were desalted on a PGC column, and lyophilized through vacuum centrifugation. The deglycosylated proteins were used for digestion and labeling.

Digestion and Labeling of Deglycosylated Glycoproteins

The deglycosylated proteins were reduced by 10 mM dithiothreitol (DTT) at 56 °C for 30 min, alkylated in dark by 50 mM iodoacetamide (IAA) at room temperature for 40 min, and precipitated with 6 volumes of chilled acetone at -20 °C for 3 h. The precipitates were respectively resuspended in 100 μL 50 mM NH_4HCO_3 buffer, incubated with 2 μg trypsin (1:50 for enzyme/substrate, w/w) at 37 °C for 4 h, and then digested overnight at 37 °C by adding another equal amount of trypsin. The reaction was quenched by heating to 95 °C for 10 min, and the tryptic peptides were filtered by 10 kDa MWCO centrifugal filters as described above.

Then the tryptic peptides were labeled by immobilized trypsin beads[22]. Briefly, the peptides were redissolved in 100 μL $H_2{}^{16}O$/$H_2{}^{18}O$ prepared 20% (v/v) acetonitrile (ACN), respectively. Then, 2 μL of immobilized trypsin bead slurry (20%, v/v) was washed by water and mixed with each peptide sample (1:50 for slurry/sample, v/v). Then the mixtures were incubated in the dark at 37 °C for 24 h to label the peptides. Afterwards, the immobilized trypsin beads were removed using spin column with centrifugation (4,000 *g*) for 1 min, and the labeled samples were dried through vacuum centrifugation.

Purification and SDS-PAGE Separation of IgG from Serum

The serum samples, which were obtained with informed consent from three hepatocellular carcinoma (HCC) patients and three healthy individuals at the Zhongshan Hospital of Fudan University (Shanghai, China), were mixed on equal volume respectively. About 1μl of pooled normal serum samples and HCC serum samples were applied to the isolation of IgG of using Melo^{TM} Gel IgG Spin Purification Kit according to the reference manual. Briefly, the Melton^{TM} gel suspension was transferred to the column and washed twice with purification buffer. The serum was diluted in purification buffer (1/10, v/v) and mixed with the Melton^{TM} gel at room temperature for 10 min. After centrifugation (4000 g) for 1min, purified IgG solution was collected, and desalted by ultrafiltration (14000 g) at 4 °C for 40 min with 10 kDa MWCO centrifugal filter. Then, a total of 20μg purified IgG obtained from different pooled serum samples and 1μl serum were separated by 10% SDS-PAGE. The protein bands were visualized by 0.1% Coomassie blue R-250.

In-gel digestion and labelling

The gel pieces were washed with water and destainer (25% (v/v) ACN, 25 mM NH_4HCO_3) alternately. After dried with 100% ACN, the proteins were reduced with DTT and alkylated with IAA. Then the gel pieces were washed with water and dried with 100% ACN. Then 30 μl of 50 mM phosphate buffer, which was prepared in $H_2{}^{16}O$/ $H_2{}^{18}O$, was added to the dried gel pieces respectively. Enough PNGase F (about 500 U) was added and then incubated at 37 °C for 24 h. Afterwards, glycans were extracted from the gel pieces by two extractions with approximately 100 μl $H_2{}^{16}O$/ $H_2{}^{18}O$ prepared 50% (v/v) ACN and 100 μl 100% ACN, with agitation for 10 min each. The extracts were combined and filtrated (14000 g) at 4 °C for 30 min with 10 kDa MWCO centrifugal filter. Then 100 μl $H_2{}^{16}O$/ $H_2{}^{18}O$ prepared 0.1% (v/v) FA was added to the filtrate respectively, and incubated at room temperature for 2 h. The glycans were desalted on a PGC column and dried through vacuum centrifugation. After enzymatic labeling of glycans, the proteins in gel pieces were digested with trypsin and enzymatic labeled. Briefly the gel pieces were washed with water thoroughly and dried as described above. Then 30 μl of 25 mM NH_4HCO_3 buffer containing 5 μg trypsin was added and 25 mM NH_4HCO_3 buffer was added and incubated in a shaker at 37 °C overnight. After incubation, the peptides were extracted with 100 μl of 50% (v/v) ACN aqueous solution containing 0.1% (v/v) TFA. The extracts were filtered by 10,000 Da MWCO centrifugal filters and dried through vacuum centrifugation. Afterwards, the peptide labeling was performed by immobilized trypsin beads as described above.

MALDI-MS/MS Analysis.

The dried labeled glycans and peptides were dissolved in about 50 μl of 50% (v/v) ACN aqueous solution containing 0.1% (v/v) TFA and a 1-μl aliquot of each dissolved samples was spotted

onto a MALD target. After air drying, the sample spots were overlaid with 1μl of 2,5-dihydroxybenzoicacid matrix (DHB, 50 mg/mL in 100% ethanol) and then analyzed using an Axima MALDI-QIT-TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan) with nitrogen pulsed laser (337 nm). CID was performed at a collision energy of 4 keV using argon as the collision gas. For the model glycoproteins, ^{16}O- and ^{18}O-labeled glycans were directly analyzed by MS and MS^2 to determine their labeling efficiency or pooled at various designated molar ratios (1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1) and then analyzed (six replicates for each ratio) to determine the linearity and reproducibility. For glycans from purified IgG samples, ^{16}O- and ^{18}O-labeled glycans were mixed in a 1:1 molar ratio and then analyzed (six replicates) to determine the quantitative changes. The acquired mass spectra were interpreted manually using GlycoWorkbench, Glycan Mass Spectral Database, and GlycoBase (Version 2; http://glycoba se.nibrt.ie/glycobase.html). For peptides from the model glycoproteins, ^{16}O- and ^{18}O-labeled peptides were mixed in a 1:1 molar ratio and analyzed.

Nano-LC-ESI-MS/MS analysis

The ^{16}O- and ^{18}O-labeled peptides from purified IgG samples were mixed in a 1:1 molar ratio and analyzed by nano-LC-ESI-MS/MS. Briefly, the dried labeled peptides were suspended in 5% (v/v) ACN containing 0.1 % (v/v) FA (phase A), separated by a 15-cm reverse phase column with a gradient of 5%-45% phase B (95% ACN with 0.1% FA) over 60 min. The peptides were analyzed using a LC-20AB system (Shimadzu, Tokyo, Japan) connected to an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with an online nano-electrospray ion source. The peptides were analyzed by MS and data-dependent MS/MS acquisition, selecting the 10 most abundant precursor ions for MS/MS with dynamic exclusion durations of 60 s. The scan range was set from m/z 400 to m/z 2000.

MS/MS spectra were searched against the human International Protein Index IPI database (IPI human v3.45 fasta with 71983 entries) by SEQUEST. The database search results were further analyzed statistically using PeptideProphet. A minimum peptideprophet probability score (P) filter of 0.9 was selected to remove low-probability result and the N-linked glycosylation that did not occur at a consensus N-X-S/T motif (X ≠ P) was also removed. The peptide relative quantification was performed using Mascot Distiller. The non-glycopeptides were analyzed by the built-in $^{16}O_2$-/$^{18}O_2$-labeling quantification module[23], and the deglycosylated peptides were analyzed by the newly-built $^{16}O_3$-/$^{18}O_3$-labeling quantification module.

Conclusions

In this study, we for the first time realized PCGOL with almost 100% labeling efficiency through the optimization of acidity of reaction buffer. With the successful of PCGOL, the glycan ^{18}O-labeling quantitation method was developed, which exhibited good accuracy, reproducibility and linearity with at least two orders of magnitude range. We believe that the new developed PNGase F-catalyzed glycan ^{18}O-labeling quantitation method has potential application value and broad application prospect in quantitative glycomics research.

Moreover, a new strategy of enzymatic $^{18}O_4$-labeling, which combined PCGOL with our previously developed TOSIL method (Tandem ^{18}O stable isotope labeling for N-glycoproteome quantitation), was creatively developed for the comprehensive N-glycsosylation quantitation. Enzymatic $^{18}O_4$-labeling can be used for one-pipeline quantitative analysis of glycan, glycopeptide and non-glycopeptide, and realize comprehensive *N*-glycosylation quantification in a single experiment for both glycomics and glycoproteomics simultaneously, which is impossible heretofore . The new strategy promotes the combination of quantitative glycomics and glycoproteomics, and is potentially powerful for clinical investigations on glycosylation changes in diseases. The further challenge in application of the proposed $^{18}O_4$-labeling approach is to decipher the quantitative results for both glycome and glycoproteome in a synergic manner in any selective physiological or pathological processes.

Acknowledgements

The research was supported by MOST 973 projects (2011CB910600, 2012CB910600, 2013CB911201), 863 project (2012AA020200), National Natural Science Foundation of China (31100590), Shanghai Municipal Natural Science Foundation (11ZR1403000).

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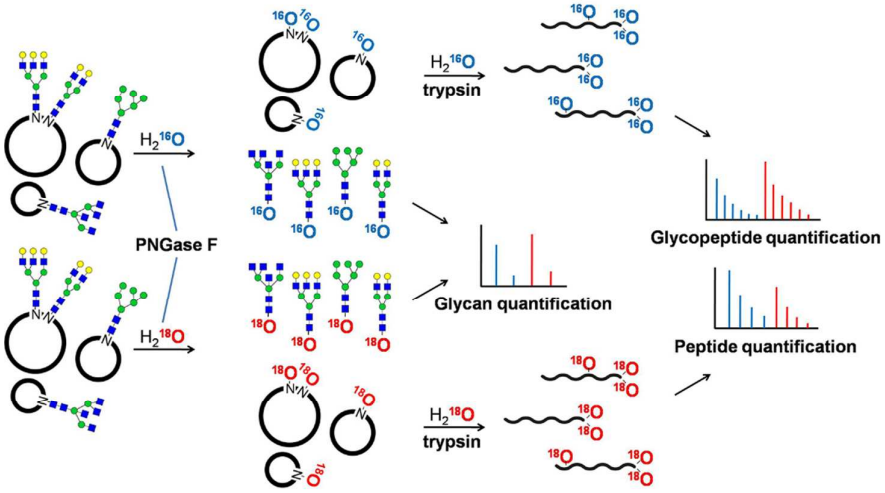
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/



An enzymatic four ^{18}O-labeling strategy based on PNGase F-catalyzed glycan ^{18}O-labeling (PCGOL) developed in this work can be used for simultaneous quantification of glycans, nonglycopeptides and glycopeptides in a single workflow.