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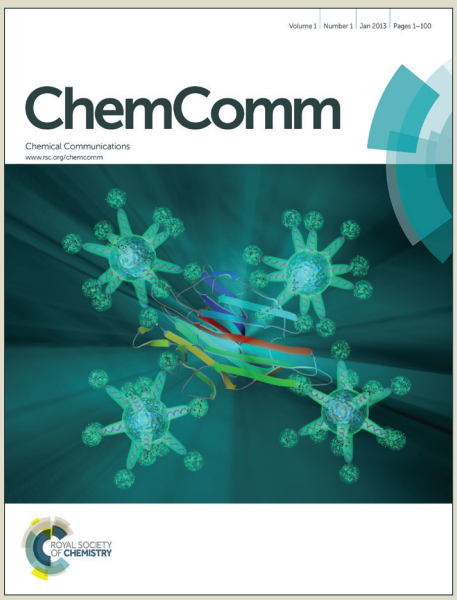

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Glycan reducing end dual isotopic labeling (GREDIL) for mass spectrometry-based quantitative N-glycomics†

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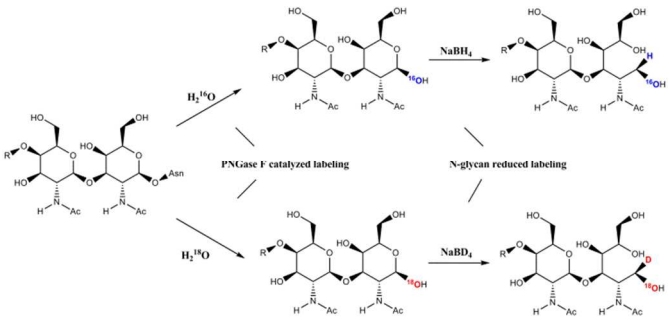
A general and effective enzymatic labeling method, termed glycan reducing end dual isotopic labeling (GREDIL), was developed for mass spectrometry-based quantitative N-glycomics

Protein glycosylation plays vital roles in a wealth of biological processes, including protein conformation, intracellular communication and immune response.[1] Changes in glycosylation are associated with many disorders and diseases.[2] Therefore, quantitative glycomics aiming at studying N-glycan changes in specific physiological and pathological process is important for exploring potential biomarkers in diseases.

Nowadays, mass spectrometry (MS)-based isotopic labeling is considered to be an effective tool in quantitative glycomics.[3] There are three main isotopic labeling glycan quantitation methods,[4] including chemical labeling, metabolic labeling, and enzymatic ^{18}O-labeling. In chemical labeling, stable isotope can be incorporated into glycans via chemical reaction. For example, isotope-coded reagents, such as aminobenzoic acid (2-AA),[5] aminopyridine (PA),[6] aniline[7] and some new isotope compounds,[8] are usually used as the mass tag to label the glycan reducing end based on the reaction at its terminal aldehyde-group. Permethylation labeling based on the chemical derivatization with the reagent of CH_3I /CD_3I, $^{12}CH_3I$ /$^{13}CH_3I$ or $^{13}CH_3I$/CH_2DI, has long been used as mass tags.[9] Although this kind of labeling for glycan quantitation is prevalent, the complex chemical labeling operation, which requires additional steps and reagents, may challenge labeling efficiency and reproducibility. Metabolic labeling is an alternative approach for glycan quantitation, [10] in which amide-^{15}N-glutamine is added as the source of nitrogen for hexosamines in cell culture to incorporate ^{15}N into glycans. However it can be only used for the investigation of cultured cells. In addition, the high cost also limits its application.

Enzymatic ^{18}O-labeling for glycan quantitation has been recently developed as a promising tool for MS-based glycan relative quantification. Compared with chemical labeling and metabolic labeling, enzymatic ^{18}O-labeling is more convenient and efficient: the labeling occurs during the glycans' release by enzyme and only requires the presence of ^{18}O-water; thus, tedious experimental steps, costly reagents, and side reactions are all avoided. A novel strategy of glycan reducing end ^{18}O labeling (GREOL) has been previously developed by us,[11] in which an ^{18}O is incorporated into the glycan reducing end during the release of N-glycans by endo-β-N-acetylglucosaminidase (endoglycosidase) in ^{18}O-water. This approach is easily operated and can quantitatively discriminate structures of isomeric hybrid and complex N-glycans. However, the endoglycosidase hardly cleaving the complex tetraantennary glycans limits its application. Consequently, the possibility of glycan ^{18}O labeling by peptide-N-glycosidase F (PNGase F), which is more comprehensively and extensively used than endoglycosidase for glycan release, has been investigated. However, it is hard to incorporate the ^{18}O into glycans completely by PNGase F due to the reversible deamination reaction of glycosylamine.[11] Although, in later study ^{18}O can be incorporated into glycans by PNGase F catalysis through adjusting the basic pH to the acidity of PNGase F catalytic reaction condition,[12] the labeled ^{18}O atom will slowly exchange with ^{16}O in normal water, and thus greatly reduce the labeling efficiency. In addition, the extra deconvolution steps, which is cumbersome and time-consuming, are required in glycan quantitation to minimize the interference of isotope overlap caused by only 2 Da mass difference between ^{16}O- and ^{18}O-labeled glycans.[12] Thus, it is pressing need to develop an enzymatic ^{18}O-labeling method, which can stably incorporate ^{18}O into glycans by the widely used PNGase F and can produce larger molecular mass difference to avoid isotope overlap and complicated deconvolution steps. Herein, a novel glycan reducing end dual isotopic labeling (GREDIL) by PNGase F catalysis and $NaBH_4$/$NaBD_4$ reduction was developed in this study to fill the gaps of enzymatic ^{18}O-labeling in quantitative glycomics (Scheme 1).



Scheme 1 The strategy of GREDIL for N-glycan relative quantitation.

In the GREDIL strategy, $NaBH_4$ was introduced to treat the N-glycans after they were released by PNGase F, which makes the N-glycan ^{18}O labeling become more stable without exchange with ^{16}O in normal water after the $NaBH_4$ treatment (Fig. S1, ESI†). $NaBH_4$ has very strong reducibility. It can reduce the aldehyde group of the glycan reducing end to hydroxyl so as to interrupt the exchange between ^{18}O and ^{16}O in the process of the conversion between aldehyde group and hemiacetal of the glycan reducing end (Fig. S2a, ESI†). Meanwhile, two hydrogen atoms are introduced into the glycan end by $NaBH_4$ reduction. As shown in Fig. S2b and c (ESI†), N-glycans released from two aliquots of asialofetuin by PNGase F in $H_2{}^{16}O/H_2{}^{18}O$-prepared phosphate buffer, and reduced by $NaBH_4$ treatment were analyzed by MALDI-QIT-TOF MS. The MS results show characteristic 2 Da mass increase after the $^{16}O/^{18}O$-labeled glycan is reduced by $NaBH_4$ because of the two hydrogen atoms introduced by $NaBH_4$ reduction, which indicates the success of glycan being reduced by $NaBH_4$. Meanwhile, there is no signal at the -2 Da ion peak of reduced glycans, indicating the complete reaction and approximately 100% efficiency of the reaction.

The GREDIL not only solves the problem of the instability of N-glycan ^{18}O-labeling but also largely decreases the interference of isotopic clusters overlap. As shown in Fig. 1a, there is characteristic 3 Da mass difference between the dually labeled glycans of the two samples from ovalbumin (various N-glycan types). Enlarged spectra of the peaks at m/z 1544.6 and 1547.6, resulting from singly charged sodium adduct ion of glycan $HexNAc_5Hex_3$, and the enlarged spectra of glycan mixture (1:1 for ^{16}O+H/^{18}O+D-labeled glycans) further demonstrate the existence of pair peaks with 3 Da mass difference (Fig. 1b). Moreover, data derived from the tandem mass spectrometry analysis of this glycan are shown in Fig. 1c with given assignments of key signals. The tri-antenna complex type of this glycan was confirmed by major fragment ions (B_2, $B_{3\alpha}$, $B_{3\beta}$, Y_2, $Y_{3\alpha}$ and $Y_{3\beta}$), and the labeled glycan ends were characterized by the presence of diagnostic ions, such as the ions at m/z 431/434, 449/452, 976/979, 1138/1141, and 1179/1182, which indicate that GREDIL is feasible and stable in obtaining dual isotopic labeled (^{16}O+H/^{18}O+D) N-glycans. In addition, both of the extremely weak isotopic peak (+3 Da) of the ^{16}O+H-labeled glycan and the obvious pair peaks with 1:1 mixture indicate that the overlapping isotopic clusters almost disappeared because of the large molecular mass difference of 3 Da (Fig. 1b). Thus the interference of overlapping isotopic clusters are largely avoided. The proportion of isotopic peak in glycan is significantly lower than that in peptide. Peptides usually need 4 Da mass difference to avoid the overlap of isotopic clusters, while 3 Da mass difference is sufficient for glycans to separate the isotopic peaks (S-1 and Table S1, ESI†), which is related to the small molecular weight and simple element composition of the glycans.

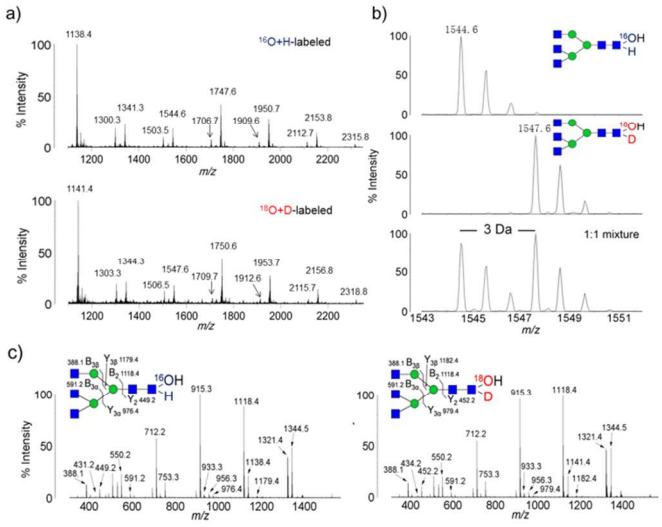


Fig. 1 MALDI-TOF mass spectra of dually labeled glycans from ovalbumin by GREDIL. (a) Spectra of the ^{16}O+H-labeled (top) and ^{18}O+D-labeled (bottom) glycans of ovalbumin. (b) Enlarged spectra of the glycan $HexNAc_5Hex_3$ labeled by ^{16}O+H (top) and ^{18}O+D (middle), and their equal amount mixture (bottom). (c) Tandem mass spectra of the glycan $HexNAc_5Hex_3$ labeled by ^{16}O+H (top) and ^{18}O+D (bottom).

Moreover, the feasibility of GREDIL was also investigated using the other two model glycoproteins, ribonuclease B (high mannose type) and asialofetuin (complex type) (Fig. S3 and Fig. S4, ESI†). N-glycans from rat serum were also labeled and analyzed by GREDIL to test the practicality of GREDIL in biological samples (Fig. S5, ESI†). All of above results show that the GREDIL is feasible and stable.

To evaluate the linearity and reproducibility of GREDIL for relative N-glycan quantitation, N-glycans from ovalbumin, ribonuclease B and asialofetuin were ^{16}O+H-/^{18}O+D-labeled and analyzed by MS with six replicates for each definite ratio (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, v/v). Two isotopic pairs of glycans from each glycoprotein were selected to calculate the ratios and generate dual-logarithm plots between the theoretical and corresponding measured ratios. The results exhibit good linearity within two orders of magnitude range (1:10 to 10:1) with correlation coefficients (R^2) higher than 0.989 (Fig. 2). In addition, quantitative results show high accuracy with the slope of six glycans ranging from 0.6838 to 0.8731 and the Y and X intercept ranging from -0.0918 to 0.1144 (Table S2, ESI†), and indicate the good reproducibility with the overall coefficients of variation (CVs) ranging from 1.35% to 20.53% (Table S2, ESI†). Obviously the glycan pair isotope envelopes with 3 Da gaps cause no effect on the quantitative results.

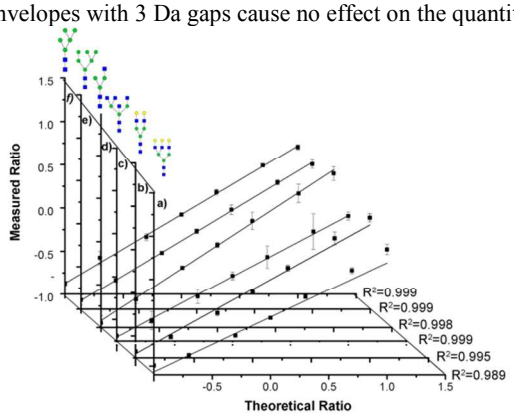


Fig. 2 Dual-logarithm plots between the theoretical and corresponding measured ratios for six glycans from model proteins (n = 6). (a, b) Glycans from asialofetuin. (c, d) Glycans from ovalbumin. (e, f) Glycans from ribonuclease B. All plots show good linearity (R^2> 0.989). Green circle, Man; yellow circle, Gal; blue square, GlcNAc.

To intensively investigate GREDIL for relative N-glycan quantitation in complex biological samples, N-glycans from rat serum were ^{16}O+H-/^{18}O+D-labeled and analyzed by MS with six replicates for each definite ratio (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, v/v). A total of 35 N-glycans were identified in six replicates (Table S3, ESI†). Two isotopic pairs of glycans were selected to calculate the ratios as examples. Results show that the measured ratios for the glycans are in close agreement with the expected ratios, with the overall CVs ranging from 1.92% to 14.35% (Table S4, ESI†), indicating good reproducibility and high accuracy of GREDIL in complex biological samples. In addition, Dual-logarithm plots between the theoretical and corresponding measured ratios for the two glycans exhibit good linearity with correlation coefficients (R^2) higher than 0.992 (Fig. S6, ESI†). Thus, GREDIL is demonstrated to be an effective method not only for relative N-glycan quantitation in model glycoproteins but also for that in complex biological samples with good accuracy, reproducibility and linearity.

As GREDIL showed good performance in relative N-glycan quantitation, it was further applied to the investigation of the changes of N-glycans from pooled normal serum (from nine healthy volunteers) and pooled HCC serum (from nine HCC patients). As a result, a total 25 N-glycans were detected and quantified in three replicated analyses. Quantitative results show good reproducibility with the CVs ranging from 0.49% to 16.29% (Table S5). As the maximal CV was below 20% in this study, a change of more than 20% between HCC samples and healthy controls (HCC/normal <0.83 or >1.20) was distinguishable as a criterion to at least estimate the down- or up-regulation of glycan levels. As shown in Fig. 3, a total of 12 N-glycans changed significantly, in which 7 glycans were up-regulated in HCC sera and 5 glycans were down-regulated in HCC sera. The results show that the glycans with bisecting GlcNAc, sialic acid and core fucosylated types obviously changed in HCC serum and can be potential biomarkers for HCC. This finding is consistent with previously reports.[13]

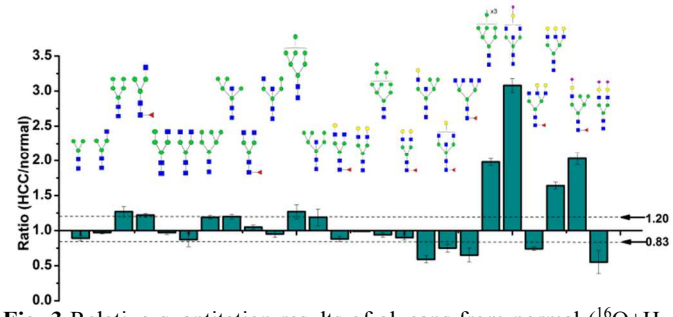


Fig. 3 Relative quantitation results of glycans from normal (^{16}O+H-labeled) and HCC (^{18}O+D-labeled) sera. Ratio (HCC/normal) <0.83 or >1.20 is distinguishable as a criterion to at least estimate the down- or up-regulation of the glycan levels. Red triangle, Fuc; green circle, Man; yellow circle, Gal; blue square, GlcNAc.

In summary, GREDIL, a new strategy for relative glycan quantitation, was developed, in which the ^{16}O+H/^{18}O+D atom are incorporated into the glycan end during the release of glycans by the widely used PNGase F and reduced by $NaBH_4$/$NaBD_4$. The $NaBH_4$/$NaBD_4$-mediated reduction reaction introduces no new chemical group, and just replaces the H atom with the D atom, thereby maintaining the advantage of enzymatic labeling, largely improving the stability of ^{18}O-labeled glycans, and expanding the molecular mass difference to 3 Da between the labeled glycan pairs which greatly reduce the overlap of isotopic clusters. GREDIL allows glycans from two samples to be immediately combined after glycans are released and reduced, avoiding additional steps; hence errors generated from parallel operation are greatly reduced. Furthermore, GREDIL exhibits good linearity and high reproducibility within at least 2 orders of magnitude in dynamic range for relative glycan quantitation, and has also been successfully used for analysis of N-glycan changes in human serum associated with HCC. Overall, GREDIL provides a powerful tool for quantitative glycomics, and has potential application in clinical investigations on the structural and abundance changes of glycans in diseases.

Notes and references

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† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/c000000x/

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