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Glycan Analysis by Isobaric Aldehyde Reactive Tags and Mass Spectrometry

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Abstract

Glycans play significant roles in physiological and pathological processes. Therefore, quantitative analysis of glycans from normal and disease specimens can provide insight into disease onset and progression. Relative glycan quantification usually requires modification of the glycans with either chromogenic or fluorogenic tags for optical measurement or isotopic tags for mass spectrometric analysis. Due to rapid advances in mass spectrometry (MS) instruments in resolution, sensitivity and speed, MS-based methods have become increasingly popular for glycan analysis in the past decade. However, current isotopic tags for glycan labeling are mostly mass-shift tags generating mass differences in precursor ions for quantification, which can complicate mass spectra. In this study, we report the synthesis and characterization of isobaric aldehyde reactive tags (iARTs) for glycan quantification using tandem MS. We applied iARTs to the relative identification and quantification of glycans of gp120, a glycoprotein from human immunodeficiency virus. The results show that iARTs provide strong signals for glycan identification. Although we only show the synthesis and characterization of two iARTs reagents, iARTs can be readily expanded to six-plex tags for quantitative analysis of six samples concurrently.

Glycosylation is considered one of the most significant protein modifications ^{1–4}. Quantitative analysis of glycoproteins and glycans has been recognized as the key to detect changes in glycoproteins and their associated glycans for the understanding of glycoprotein functions in biological pathways and disease development ^{5,6}. Tremendous effort has been devoted to the development of technologies for the high throughput analysis of glycoproteins and glycosylation sites ⁵. Glycoprotein quantification has been accurately and effectively performed by labeling of its amino groups using isobaric tags for relative and absolute quantitation (iTRAQ) ⁷. A reliable quantitative method similar to the iTRAQ for glycan analysis has yet to be developed.

Several methods have been used for glycan analysis ⁸, including anion-exchange chromatography ⁹, normal-phase or reverse-phase chromatography ^{10,11}, electrophoresis ^{12,13}, and mass spectrometry (MS) ^{14,15}. Glycans are traditionally quantified by fluorescence in which glycans are first labeled by a fluorophore tag (e.g. aminobenzoic acid or 2-aminobenzamide acid) at their reducing ends ^{16,17}. The labeled glycans are then

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separated by liquid chromatography and further detected by fluorescence spectroscopy¹⁸. In those methods, glycan quantification relies on glycan labeling and chromatographic resolution since adjacent peaks could interfere with each other. As a result, some glycans may not be precisely characterized. Recently, MS has become a powerful tool for the qualitative analysis of glycans^{19–21}. Yet, it lacks the necessary accuracy for quantitative analysis without glycan labeling because the signal intensities of specimens depend on many factors such as sample deposit uniformity²², matrix crystallization quality²³, instrumental response²⁴, ionization efficiency²⁵, and other variations during sample preparation. Permethylation of glycan hydroxyl groups is often used to boost MS detection sensitivity of glycans by increasing hydrophobicity of glycans and glycan stability in MS analysis^{26,27}. Isotopic labeling of glycans with isotopic methyl iodide can be implemented during permethylation to determine the relative abundance of glycans from different samples^{28–31}. However, the glycan permethylation must be complete in order to maintain the mass resolution of different glycans for their quantitation accuracy³², which is often difficult to achieve.

Most isotopic tags for glycan labeling are based on mass-shift that introduces a mass difference on the same glycans from different samples. They are usually limited to pairwise measurement as they increase the complexity of MS spectra. On the other hand, isobaric tags can easily achieve concurrent quantification of multiple samples (> 2) in a single run, which should improve both the throughput and sensitivity of glycan analysis. However, despite the popularity of isobaric tags for peptide labeling, there are few reports on glycan labeling. A recent attempt using carbonyl-reactive tandem mass tags (glyco-TMT, an isobaric tag for peptides) showed little promise as TMT-labeled glycans cannot generate signature reporter ions strong enough for accurate quantification upon MS fragmentation.³² This may be caused by the fact that glycosidic bonds are relatively easier to break apart than the amide bond linking the reporter and the balancer of TMT tags in collision cells. To solve this problem, we decided to develop isobaric aldehyde reactive tags for glycan labeling based on Deuterium isobaric Amine-Reactive Tags (DiART)^{33,34} that contain much stronger reporter ions due to their unique chemical structures³⁵.

Herein, we report the preparation of isobaric Aldehyde Reactive Tags (iARTs) for glycan labeling and analysis of iARTs-labeled glycans by tandem MS. Glycans were labeled with iARTs by reductive amination at their reducing ends. We found that the iARTs labeling not only allowed us to quantify glycans, but also increased glycan ionization efficiency by enhancing their hydrophobicity. The modifying tags at the single reducing end of each native glycan generated low-mass reporter ions for quantifying glycans from different samples during MS/MS analysis without complicating the MS spectrum. In conjugation with glycoprotein immobilization for glycan extraction (GIG)³⁶, we applied this method for the quantitative analysis of glycans from gp120 glycoprotein in human immunodeficiency virus (HIV).

EXPERIMENTAL PROCEDURES

Materials and Reagents

Concentrated denaturing buffer consists of 400 mM Dithiothreitol (DTT) and 5% sodium dodecyl sulfate (SDS) (New England BioLabs; Ipswich, MA). Peptide-N-glycosidase F (PNGase F) was from New England BioLabs. Both snap-cap spin-column and AminoLink resin were from Pierce (Thermo Scientific; Rockford, IL). μ -Focus MALDI plate (Hudson Surface Technology; Fort Lee, NJ) was amounted to Shimadzu adapter for glycan identification by AXIMA Resonance MALDI-QIT-TOF MS (Shimadzu Corporation; Columbia, MD). HPLC-grade solvents, including high-performance liquid chromatography (HPLC) grade water, acetonitrile (99.9%), methanol (99.9%), ethanol (99.5%) and glacial

acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Human immunodeficiency virus type 1 gp120/SU protein (His Tag) (gp120) was purchased from Sino Biological Inc (Beijing, China). All reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard oligosaccharides, maltotetraose (DP4, 689.57 Da, mono-Na⁺), maltopentose (DP5, 851.26 Da, mono-Na⁺), maltohexanose (DP6, 1013.31 Da, mono-Na⁺), and maltoheptaose (DP7, 1175.37 Da, mono-Na⁺) were dissolved in HPLC grade water to 10 mM mixture.

Synthesis of aldehyde reactive tags (iARTs (114 or 115))

N-hydroxysuccinimide ester (16 mg), monotritylethylenediamine (19 mg, Sigma), and triisopropylamine (17 μ L) were added to 250 μ L dichloromethane (DCM). After incubation for 2 h, the reaction mixture was washed with saturated sodium bicarbonate solution (750 μ L) twice and water (750 μ L) three times. The organic layer was then dried by anhydrous sodium sulfate, and the solvent was removed by Rotavapor (Buchi, Switzerland) to produce 25 mg white solid. A mixture of 10% trifluoroacetic acid, 2% triisopropylsilane in DCM was added and left at room temperature for 2 h. The solvent was removed by Rotavapor. The residue was resuspended in 500 μ L water and washed with ether (750 μ L) three times. The aqueous layer was lyophilized to provide 12 mg iARTs containing a primary amine group for glycan labeling.

N-glycans isolation from HIV gp120

Human immunodeficiency virus (HIV) glycoprotein gp120 (100 μ g; Sino Biological Inc.; Beijing, China) was dissolved in 90 μ L of pH 10.0 buffer consisting of 40 mM sodium citrate and 20 mM sodium carbonate (Sigma-Aldrich). Glycan preparation via glycoprotein immobilization for glycan extraction (GIG) follows protocol described previously³⁶. gp120 (100 μ g) was conjugated to Aminolink resin and N-glycans were released using 2 μ L of PNGase F in the presence of 1 $\times$ G7 reaction buffer. The N-glycans were purified using Carbograph columns (Extract-Clean SPE Carbo 150 mg; Grace Division Discovery Science; Deerfield, IL) and eluted in 0.1% TFA 80% acetonitrile/water (800 μ L). Glycan Carbograph purification followed steps described previously³⁷.

N-glycan labeling

The dried DPs or gp120 glycans were re-suspended in 100 μ L of solution mixture consisting of dimethyl sulfoxide (DMSO) and acetic acid (AA) (70:30, vol). After complete mixing by vortex, glycans in DMSO-AA (100 μ L) was aliquoted to two vials. One vial was added 100 μ L/100 mM of iARTs¹¹⁴ and the second vial added 100 μ L/100 mM of iARTs¹¹⁵. Equal volume of DMSO-AA in the presence of 100 mM sodium cyanoborohydride was added to each sample. Both vials were placed in temperature-controlled microwave at 50% of power for 20 min (Temperature was set to 60°C; EMS-820; Electron Microscopy Sciences; Hatfield, PA).

The labeled samples were mixed, which contain 114 and 115 iARTs-labeled glycans, unreacted iARTs, sodium cyanoborohydride, DMSO and AA. To remove non-glycan molecules, samples were purified over Carbograph column. Cleaned samples were eluted by adding 800 μ L of 0.1% formic acid in 80% ACN. The elution was dried in Speed-Vac at room temperature and re-suspended in 50 μ L water.

MS Analysis

One μ L of labeled glycans and one μ L of DHB-DMA were deposited on μ Focus MALDI plate and analyzed by Shimadzu Resonance MALDI-MS at power of 100. Data was acquired after 200 profiles at 100 different locations. For each peak, MS/MS was conducted

for glycan structure identification (collision energy setting: 180–250). Further necessary MSⁿ fragmentation is performed on those structures which cannot be determined at MS/MS level. For ESI-MS/MS, 10 µl of labeled gp120 glycans was diluted in 90 µl of 0.1% TFA, which was directly injected into Orbitrap (Velos Pro Mass Spectrometer; Thermo Fisher Scientific Inc.; Waltham, MA) through a 100-µl needle. Flow rate was set to 2 µl/min and each test was run for 10 min. The instrument was operated in data-dependent mode with m/z ranging from 300–2000 Da, in which a full MS scan (mass resolution = 60,000) was followed by ten MS/MS scans. The normalized collision energy of higher energy collisional dissociation was 40%, and the dynamic exclusion duration was 5 min. Ions without assigned charge states were rejected for MS/MS analysis. The heated capillary was maintained at 200°C, while the ESI voltage was maintained at +2.2 kV. Each sample was analyzed in triplicate on the Orbitrap. To determine the retention time of un-labeled and iARTs-labeled glycans, the un-labeled and iARTs-labeled glycans were mixed and analyzed by ESI-MS using TSQ Quantum (Thermo Fisher) with online reverse phase separation. (C18, 150 × 1 mm, particle size 1 µm). Chromatographic separation was accomplished using a ramp from 100% mobile A (0.2% formic acid) to 90 % mobile B (acetonitrile + 0.2% formic acid) over four minutes.

Glycan Data Analysis

Glycan data from MS and MSⁿ were analyzed by a suite of software tools. First, we calculated a list of glycans containing multiple HexNAc, Hexose, Sialic acid, and Fucose. The accurate mass of each possible glycan was compared with MS data. Second, the potential structures from our list were determined by comparing the glycan mass with those in the *N*-glycan database we previously identified, in which the matched glycan structure could be achieved. Third, we used those structures as input for GlycoWorkbench, which is able to perform virtual MS/MS fragmentation. The virtual MS fragmentation was verified by MALDI-MS/MS data ³⁶.

RESULTS

The iARTs labeling strategy

The molecular structure of a pair of 2-plex iARTs is illustrated in Figure 1, which consists of three parts, including a reporter, a balancer and a primary amine group that can be used for reductive amination. The reporter group is C₇H₁₆¹⁴N and C₇H₁₆¹⁵N, thus the molecular weight is 114 and 115 in MS/MS spectra respectively. The mass difference is compensated by the balance group: one carbon uses ¹³C in iARTs¹¹⁴ whose reporter ion is 114 Da; while the same carbon is a ¹²C in iARTs¹¹⁵ (Figure 1A and Supporting Information Figure 1).

Reductive amination has been widely used to label reduce ends of glycans with different fluorophore tags ¹⁶. Typically, reductive amination is performed at 65°C for 3–4 h, or 70°C for 2 h, or 80°C for 30 min ¹⁶. Studies showed that labeling glycans in high temperature (80°C) and low pH (2 M acetic acid) for 3 h resulted in acid-catalyzed loss of sialic acids ³⁸. To prevent sialic acid hydrolysis during reductive amination, we used temperature-controlled microwave to irradiate and accelerate glycan labeling for 20 min. The temperature was kept at 60°C and the power of the microwave was 50%.

Isobaric labeled glycans using iARTs are shown in Figure 1A. The reporters can be fragmented from the balancer by using a hybrid HCD acquisition in Orbitrap, generating singly positive-charged ions at 114 and 115. Glycan quantification by iARTs is illustrated in Figure 1B. Glycans from different sources for quantitative comparison are first labeled by iARTs¹¹⁴ and iARTs¹¹⁵ respectively, pooled together and purified to remove non-glycan

species. The iARTs labeled glycans were then analyzed by MS/MS with the intensity of reporter ions representing the relative abundance of original samples.

We first studied the labeling efficiency using different ratios of iARTs over a mixture of four standard oligosaccharides (DP4, DP5, DP6 and DP7) at a concentration of 1 mM for each oligosaccharide (Supporting Information Figure 2). Equal amount of oligosaccharides (10 μ l at 1 mM, 10 nmol) was applied and iARTs was added at a ratio of 0.1, 1, 10, 50, 75 and 100 (iARTs/glycan; molar ratio). The reaction was carried out in a microwave reactor and the resulting sample was purified by Carbohydrate column. MALDI-MS was performed to determine the completeness of labeling. As shown in Supporting Information Figure 2 and Supporting Information Figure 3, negligible amount of iARTs-labeled oligosaccharides was observed at the ratio below 10 (Supporting Information Figure 3B), while labeled glycans were significantly increased with the addition of more iARTs. DPs were completely labeled with iARTs when concentration of the labeling reagent was 100-fold over that of glycans (Supporting Information Figure 3A). Therefore, we used this condition for iARTs-glycan labeling thereafter.

The effectiveness of glycan quantification by iARTs labeling may be determined by its MS and MS/MS pattern. Ideally, one would expect to observe reporter ion and glycan fragments in MS/MS spectra. Fragmentation between the reporter and the balancer leads to the detection of reporter ion 114 or 115; fragmentation of glycosidic bonds generates a series of species consisting of glycan structural information. The single charged fragment ions ($[M+H]^+$) of iARTs-labeled DP5 include reporter ion 114 or 115, monosaccharide-iARTs (438.29 Da), disaccharide-iARTs (600.31 Da), trisaccharide-iARTs (762.23 Da), and tetrasaccharide-iARTs (924.12 Da) (Figure 2A). We also noted that no other fragment ions generated from oligosaccharides overlap with reporter ions 114 and 115. Therefore, the fragments from MS/MS provide not only structural information for glycan identification, but also intensity of reporter ions for glycan quantification.

For MS analysis, it is crucial to identify how many charges a labeled glycan has and what adduct ions it carries. iARTs-labeled oligosaccharides usually carry one or two positive charges, including $[M+H]^+$, $[M+2H]^{2+}$, and $[M+Na]^+$, depending on ionization methods. As a result, we observed different mass spectra for the same sample using MALDI-MS or ESI-LTQ-Orbitrap (Supporting Information Figure 3A and Figure 2B). All labeled DPs were singly charged with a sodium ion by MALDI (Supporting Information Figure 3); DPs were doubly charged with two protons by ESI (Figure 2B), even though we did observe small amount of singly charged oligosaccharides (< 5%). The observed m/z values of four labeled glycans of DP4, DP5, DP6, and DP7 were 945.74, 1107.88, 1270.02, 1432.16 Da in MALDI spectra; while they were 462.37, 543.44, 624.51 and 705.58 Da respectively in ESI-LTQ-Orbitrap spectra. The respective ions were chosen as precursors for MS/MS quantification in Orbitrap. HCD MS/MS fragmentation of iARTs-DP5 is given in Figure 2(C). MS/MS of iARTs-DP5 includes single-charge Y^+ ion, such as 924.12, 762.23, 600.31 and 438.29 Da, and double-charge Y^{2+} ion, such as 462.06, 381.55, 300.16, 219.64 Da. Noticeably, the reporter ions are dominant in the spectrum, in contrast to weak peaks generated from TMT-labeled glycans³². This better fragmentation of iARTs is a significant advantage over other isobaric tags such as TMT and iTRAQ in term of quantifying glycans.

Quantitative analysis of isobarically labeled oligosaccharides

The accuracy and dynamic range of isobaric iARTs labeling on oligosaccharides was assessed with standard glycans having free reducing ends. Four standard oligosaccharides were labeled by iARTs¹¹⁴ or iARTs¹¹⁵. Detailed protocol for labeling of DPs with iARTs is given in Supporting Information Table 1. The reactions were conducted in microwave as described in experimental section. After Carbohydrate purification and vacuum dried,

differentially labeled oligosaccharides were dissolved in water and pooled together. Several ratios of iARTs¹¹⁴ vs. iARTs¹¹⁵ were mixed, including 1:4, 1:3, 1:2, 4:1, and 10:1. The concentration of each sample was 100 μ M, assuming no sample loss and complete labeling efficiency. We used one μ L of each sample (or 0.1 nmol) for MALDI (MS and MS/MS), while each sample was diluted to 10-fold for ESI MS/MS quantification.

Both MALDI-MS and ESI-MS indicated complete labeling of DPs with iARTs. As an example shown in Figure 3A, the pooled iARTs-DP5 with an expected ratio of 1:3 (114/115) showed the intensity ratio at 1:3.27 in ESI MS/MS. We tested all DP samples with assigned ratios listed in Supporting Information Table 1, in which each condition was analyzed in triplicate to obtain mean and standard deviation. The observed ratios were plotted versus expected ratios (Figure 3B). Overall, report ions from MS/MS spectra on iARTs-DPs show good quantification agreement with theoretical ratios and dynamic range. At an expected ratio of 10:1, the observed ratio is approximately 8.5. Clearly, iARTs is a useful tag for glycan quantification considering its labeling efficiency, report ion fragmentation, quantification accuracy and dynamic range.

Sensitivity improvement on iARTs-labeled glycans

It is challenging to analyze glycans by MS due to poor ionization efficiency. Glycans are more hydrophilic than peptides so they do not fly as well as peptides. However, the ionization of glycans can be improved by increasing its hydrophobicity via modification^{31,39}. iARTs are hydrophobic tags so they can potentially increase the ionization of labeled glycans. To test this possibility, we made a serial dilution of iARTs-labeled DPs and native DPs at the concentrations of 1500, 375, 150, 75, 25, 12.5 μ M. Each concentration of non-labeled DPs and iARTs-labeled DPs were characterized by MALDI-MS analyses for comparison of ionization efficiency. An internal standard, Angiotensin (1 μ L at 1 μ M; Sigma), was added to sample to normalize the signal. We then plotted relative intensities (to Angiotensin) of iARTs-labeled and un-labeled DPs at each concentration as shown in Figure 4A and B. iARTs-labeled DPs exhibited better ionization efficiency, whose intensity is approximately five-fold (DP5) and two-fold (DP6) over respective non-labeled DPs. The difference was more prominent at lower concentrations. The MALDI-MS spectra on 25 IM of DP6 and iARTs-DP6 (Figure 4 (B)) clearly demonstrate this pattern, in which DP6 was hardly detectable at this concentration while iARTs-labeled DP6 was evident with high signal-to-noise ratio (> 10). This feature is extremely useful for glycan analysis by mass spectrometry.

To determine whether the iARTs-labeling changed glycan hydrophobicity, we tested the retention time of un-labeled and iARTs-labeled glycans and observed on C18 column that the iART-labeled glycans had slightly but consistent delay in retention time, indicating an increased hydrophobicity after iART-labeling (Supporting Information Table 2).

HIV gp120 glycan quantification

The glycoprotein 120 subunit (gp120) is an important part of the envelope spikes that decorate the surface of HIV and a major target for neutralizing antibodies⁴⁰. It has been recognized that the outer domain of gp120 is largely covered by glycans which present as weak immunogenic antigens⁴¹. In addition, glycans on gp120 render the underlying protein surface invisible to the immune system. It was reported that gp120 contains high-mannoses and many other complex/hybrid glycans⁴². The structures of these glycans are quite sensitive to environments and could be completely different on wild type (WT) and mutants⁴². Even the same glycans can display different relative abundance under various conditions⁴². Therefore, it is important to identify and profile glycans of gp120 as this information can provide novel targets for HIV vaccines.

N-glycans were isolated from gp120 via glycoprotein immobilization for glycan extraction (GIG) and dissolved in 100 μL of water. We took equal amount of glycans (20 μL) in two microcentrifuge tubes, spiked 10 μL of 10 μM DP7 into each sample, dried them in vacuum, and dissolved sample in 20 μL of DMSO-AA solution. We then labeled equal amount of gp120 glycans from HIV with iARTs. One tube of glycans was labeled with iARTs¹¹⁴ and the other labeled with iARTs¹¹⁵. After labeling as described previously, both samples were pooled and purified by Carbograph. Samples were re-suspended in 40 μL of water in the presence of 0.1% TFA after dried by vacuum. Over 30 iARTs-labeled glycans were detected and spiked DP7 indicated complete labeling of glycans. As shown in Table 1, we mixed a ratio of 1:1 of gp120 glycans labeled by iARTs¹¹⁴ and iARTs¹¹⁵. The doubly-charged ions listed in Table 1 were detected in Orbitrap HCD. No sodium adducts of glycans were observed in Orbitrap. Quantification on gp120 glycans whose molecular weight is less than 2000 Da showed that this technique was reproducible with a CV less than 25%. For large glycans (with molecular weight more than 2000 Da), the results showed significant deviation from the expected ratio of 1. This may be caused by the different energy requirement for the fragmentation of iARTs and glycans with different size.

DISCUSSION

Protein glycosylation is associated with many diseases by changing protein biological activities. Quantitative and qualitative characterization of glycoprotein associated glycans is thus biologically significant. Many efforts have been devoted to the derivatization of reducing ends⁴³ or sialic acids⁴⁴, or the permethylation of hydroxyl groups^{45,46}. Quantification by modifying glycans at their reducing ends with fluorophore tags has advantages including high sensitivity and high throughput^{18,47}, but the detection resolution is limited by front-end separation techniques. Stable isotope-coded tags have been developed and shown excellent resolution when combined with LC-MS analysis^{29,31,48}. Yet most isotope tags are mass-shift based, which may complicate spectra and lead to difficulty in data analysis. Isobaric tags, on the other hand, do not introduce more peaks on MS spectra and make it possible to analyze multiple samples concurrently.

There are several methods to introduce isobaric labeling into glycans, one of which is to derivatize the single reducing end of glycan. Various chemical reactions can be applied, including reductive amination, hydrazone formation through hydrazide³¹, or oxime formation via aminoxy reagents^{32,49}. It has been demonstrated that aminoxy-TMT is superior to hydrazide-TMT in terms of labeling efficiency²⁸. We synthesized iARTs with amine as active group to react with aldehyde at the reducing end of glycans through reductive amination and demonstrated complete labeling. Due to the isobaric nature of the iARTs, differentially labeled glycans do not differ in mass (Table 1) and quantitative information is provided by the isobaric-encoded reporter ions generated from MS/MS or MS³ spectra. Quantitative information is thus derived from signal of the reporter ions on the same precursor. A remarkable feature of iARTs, compared to similar TMT and iTRAQ, is that reporter ions are much easier to fragment. Therefore, stronger reporter ions with better signal-to-noise can be obtained for accurate quantification from iARTs-labeled glycans.

iARTs glycan labeling described in this study provides several advantages for glycan analysis. First, it significantly increases glycan detection sensitivity by one order of magnitude. iARTs-oligosaccharides show much stronger signal on MALDI-MS for oligosaccharide structure identification. Second, labeled glycans are more hydrophobic than native glycans (Supporting Information Table 2), so that they can be more efficiently separated by hydrophilic interaction liquid chromatography or reverse-phase graphitized carbon liquid chromatography³⁰. Third, reporter ions are well fragmented in Orbitrap-HCD and their intensity can be compared to reliably quantify glycans. The accuracy and dynamic

range of iARTs-oligosaccharides has also shown its potential for glycan quantification (Figure 3). Although we only synthesized and characterized two iARTs reagents, iARTs can be readily expanded to six-plex tags using the 6-plex DiART reagents for labeling six glycan samples for simultaneous quantitative glycomic analysis.

We applied the iARTs to the analysis of HIV gp120 glycans for demonstration of quantification by iARTs¹¹⁴ and iARTs¹¹⁵. Over 30 glycans are identified and quantified, in which 80% iARTs-glycans are accurately quantified with CV less than 25%. The deviation of quantifying higher molecular weight glycans might be caused by different energy requirements for the fragmentation of iARTs and glycans with different size. Although iARTs is easier to fragment than other isobaric tags (e.g. TMT and iTRAQ), it still needs higher collision energy than glycosidic bonds. In small glycans, there are few glycosidic bonds to break apart so strong reporter ions can be obtained to provide reliable quantification results. With the size of glycans increasing, the intensity of reporter ions becomes lower, which compromises the accuracy of quantification. To generate report ions when low abundant report ions presented in MS/MS spectrum, further fragmentation (MS³) of a fragment glycan from MS/MS containing the iARTs could be used for quantitation.

CONCLUSION

We synthesized iARTs for quantitative analysis of glycans from biological samples. The amine group of iARTs directly reacts with the single reducing end of each glycan via reductive amination. Derivatization at reducing end with isobaric iARTs simplifies the quantification in contrast to other isotopic labeling tags. Isobaric labeling makes no difference on other parts of glycans so that it is straightforward to interpret the spectra and characterize the labeling efficiency. The relative glycan abundance is quantified by comparing the reporter ions for each labeled glycans on tandem MS spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A: iARTs-Amine

B: glycan labeling and quantitation

Figure 1.

Structure of the iARTs-labeled glycans and the strategy for glycan quantification. (A) iARTs consists of three elements: a reporter group, a balance group, and a primary amine, which is used to covalently react with the reducing end of glycans through reductive amination. (B) Glycans from different samples are labeled with different iARTs reagents containing different reporter ions. The relative quantification is determined by the ratio of the reporter ions from tandem mass spectrometry.

Figure 2. MS/MS spectrum of iARTs-labeled oligosaccharides. (A) Structural illustration of reporters (114 or 115) and oligosaccharide pattern (Y ions) observed in MS/MS spectra. (B) Doubly positive-charged iARTs-labeled oligosaccharides (DP4, DP5, DP6, DP7) detected by HCD-Orbitrap. (C) A representative MS/MS spectrum of an iARTs-labeled polysaccharide acquired in Orbitrap (iARTs-DP5). Fragments include Y-ions with one- or two-charge.

Figure 3. Dynamic range and accuracy of iARTs quantitation of oligosaccharides. (A) MS/MS reporter ions of glycans with expected ratio of 1:3 (114/115). (B) The linear correlation between expected and observed ratios of iART-labeled glycans. Differentially labeled glycans by iART¹¹⁴ and iART¹¹⁵ (Mixture of DP4, DP5, DP6, and DP7) were mixed in known ratios (1:4, 1:3, 1:2, 4:1, and 10:1) in which each experiment was analyzed in triplicate.

Figure 4.

Improvement of oligosaccharide ionization efficiency by iARTs labeling. (A) Signal enhancement of iARTs labeled DP5 over native DP5. (B) MALDI-MS spectra of labeled and native oligosaccharides (DP4, DP5, DP6, DP7, 25 μM). Intensities of labeled DP5 increase up to 10 folds.

Table 1

Quantification of iARTs labeled glycans using Orbitrap. Glycans were extracted from gp120 glycoprotein via solid-phase glycan extraction (GIG).

Labeled glycans (2H ⁺ , Da)	Glycan composition	Observed Ratio (114/115)	Standard Deviation(±)	Expected Ratio	%, error
623.29 [*]		1.119	0.084	1	11.9
643.38 [*]		0.976	0.041	1	2.4
655.38 [*]		1.155	0.035	1	15.5
674.94 [*]		1.173	0.391	1	17.3
705.81	H7	0.636	0.015	1	36.4
717.96 [*]		1.051	0.115	1	5.1
737.83	H5N2	0.856	0.078	1	14.4
743.39 [*]		0.897	0.067	1	10.3
757.41	H4N3	0.836	0.158	1	16.4
773.32 [*]		0.921	0.373	1	7.9
807.50 [*]		0.849	0.360	1	15.1
818.85	H6N2	0.766	0.127	1	23.4
824.52 [*]		1.111	0.877	1	11.1
831.37	H4N3F1	1.023	0.371	1	2.3
838.54	H5N3	1.085	0.532	1	8.5
841.02 [*]		0.847	0.547	1	15.3
859.88	H4N4	0.772	0.194	1	22.8
862.38 [*]		0.707	0.251	1	29.3
868.55 [*]		0.565	0.120	1	43.5
888.5 [*]		0.620	0.390	1	38.0
899.88	H7N2	0.793	0.657	1	20.7
903.89 [*]		0.610	0.333	1	39.0
926.56 [*]		0.961	0.547	1	3.9
932.91	H4N4F1	0.321	0.498	1	67.9
940.91	H5N4	0.633	0.209	1	36.7
949.43 [*]		0.581	0.078	1	41.9

Labeled glycans (2H ⁺ , Da)	Glycan composition	Observed Ratio (114/115)	Standard Deviation(±)	Expected Ratio	%, error
953.42	H3N5F1	0.499	0.343	1	50.1
1013.94	H5N4F1	0.404	0.245	1	59.6
1025.43 [*]		0.689	0.096	1	31.1
1034.45	H4N5F1	0.287	0.155	1	71.3
1042.95	H5N5	0.530	0.375	1	47.0
1086.45	H4N4F2	0.356	0.143	1	64.4

^{*} Structures of these glycans have not been assigned, yet their MS/MS spectra indicate that they are glycans.

N = n-Acetyl hexosamine; H = Hexose; F = Fucose