Quantitative Glycomics Strategies*

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The correlations between protein glycosylation and many biological processes and diseases are increasing the demand for quantitative glycomics strategies enabling sensitive monitoring of changes in the abundance and structure of glycans. This is currently attained through multiple strategies employing several analytical techniques such as capillary electrophoresis, liquid chromatography, and mass spectrometry. The detection and quantification of glycans often involve labeling with ionic and/or hydrophobic reagents. This step is needed in order to enhance detection in spectroscopic and mass spectrometric measurements. Recently, labeling with stable isotopic reagents has also been presented as a very viable strategy enabling relative quantitation. The different strategies available for reliable and sensitive quantitative glycomics are herein described and discussed. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.R112.026310, 874–884, 2013.***

The glycosylation of proteins is one of the most common protein post-translational modifications. The glycan moieties of membrane or secreted glycoproteins are responsible for modulating and controlling many of the biological roles of these glycoproteins, including cell signaling, adhesion, and communication (1–4). These roles depend on interactions between a glycan and its target protein(s)/glycoprotein(s). Moreover, protein folding, stability, and localization are dependent on protein glycosylation (5). A correlation between changes in the glycan moieties of glycoproteins and many mammalian diseases, including hereditary disorders, immune deficiencies, cardiovascular disease, and cancer, has been suggested (6–9). The diverse biological roles of glycans and their implications in diseases have created a demand for reliable quantitative glycomics strategies permitting sensitive monitoring of glycans in biological systems. These strategies are needed in order to better elucidate the roles and attributes of glycan in biological systems.

Several quantitation strategies for glycomics that permit reliable and sensitive monitoring of glycan changes correlated to different biological conditions and diseases have been developed. These strategies employ several separation and mass spectrometric techniques, including capillary electrophoresis, liquid chromatography (different modes), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS),[1] and electrospray ionization mass spectrometry (ESI-MS). Because of the high diversity of glycan structures, interfacing separation techniques to mass spectrometry is currently deemed necessary for reliable quantitative glycomics. This mini-review is concerned with describing and discussing the different strategies that are currently employed in monitoring glycan changes. The intention is to provide a concise description and discussion of the state-of-the-art strategies currently utilized in quantitative glycomics.

Fluorescence Spectroscopic Strategies—Because protein and lipid glycosylation is a"template-free" enzymatic process, the structural diversity of glycans attached to proteins and lipids is exceptionally high. This high diversity prompts the need for a separation technique capable of resolving positional, structural, and linkage glycan isomers. Separation of these closely related structures is achieved through electrophoretic and chromatographic methods.

Capillary Electrophoresis Laser-Induced Fluorescence Detection Strategy—The derivatization of glycans is always pursued in order to facilitate the separation and enhance the detection of glycans. Capillary electrophoresis (CE) separation and laser-induced fluorescence detection (LIF) of glycans are now routinely achieved using 1-aminopyrene-3,6,8-trisulfonic acid (APTS) (Fig. 1) (10–12). An APTS labeling kit is commercially available from Beckman Coulter, Inc. (Brea, CA). This reagent, which is a fluorophore possessing three negatively charged functional groups, permits both electrophoretic separation and sensitive fluorescence detection of glycans. The derivatization of glycans with APTS is attained through reductive amination chemistry facilitated by acidic

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[1] The abbreviations used are: 2-AA, 2-aminobenzoic acid; 2-AB, 2-aminobenzamide; APTS, 1-aminopyrene-3,6,8-trisulfonic acid; CE, capillary electrophoresis; ESI, electrospray ionization; GT, Girard's reagent T; HILIC, hydrophilic interaction chromatography; LIF, laser-induced fluorescence detection; MALDI, matrix-assisted laser desorption ionization; MCE, microchip capillary electrophoresis; MS, mass spectrometry; PA, 2-aminopyridine; PGC, porous graphitized carbon.

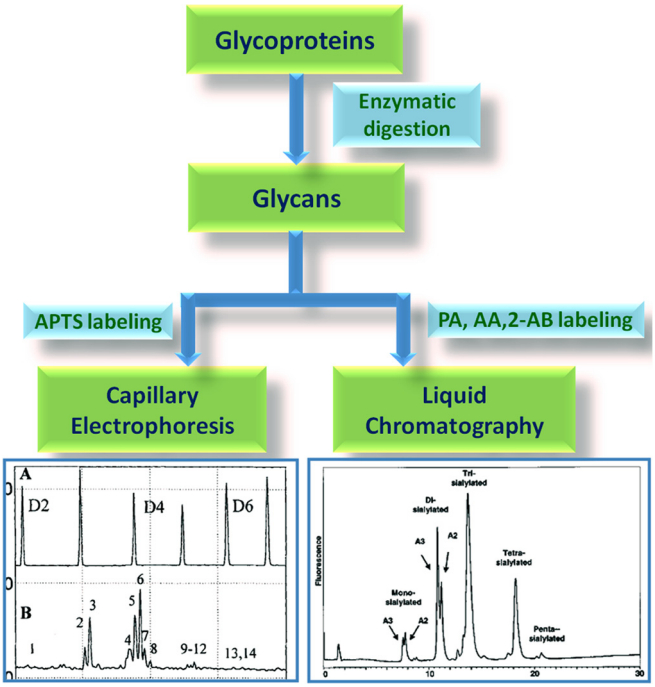

FIG. 1. **CE-LIF and LC-fluorescence detection quantitative glycomic strategies.**

conditions and reducing reagents such as sodium cyanoborohydride (11) and, more recently, 2-picoline-borane (13), which is less toxic. CE-LIF of APTS-labeled glycans has been utilized to quantify N-glycans derived from various glycoproteins, including ribonuclease B (10, 11, 14–16), fetuin (10, 11), recombinant human erythropoietin (10), kallikrein (10), monoclonal antibody (17), and a chimeric recombinant monoclonal antibody (18). Recently, CE-LIF was applied to APTS-labeled serum N-glycans derived from of 376 consecutive chronic hepatitis C virus patients in order to assess liver diseases (19–22). This CE-LIF strategy for quantitative glycomics was labeled as the GlycoFibro test. The strategy appears to be effective in assessing liver fibrosis in chronic hepatitis patients; therefore, it might be considered as an alternative to liver biopsy, which suffers from several disadvantages, including sampling error and up to 20% interlaboratory variance (22).

Microchip capillary electrophoresis (MCE) LIF utilized in quantitative glycomic analysis involves devices that incorporate microchannels, allowing sample injection, preconcentration, and separation. MCE-LIF of APTS-labeled N-glycan derived from human serum collected from patients with liver disease was recently demonstrated (21). This miniaturized strategy (11.5 cm effective length) allows the efficient separation of the major N-glycans in human serum in 12 min with adequate spatial resolution. This separation time is somewhat long, considering that a major advantage of MCE is the speed of separation. The separation of APTS-labeled glycans derived from human glycans was recently attained in 40 s in a microfluidic channel 14 mm in length (23). However, the spatial resolution of such separation was low. MCE with two channel designs, (i) a spiral channel design (24) and (ii) a serpentine channel design with asymmetrically tapered turns (25), was employed recently for the separation of APTS-labeled N-glycans derived from blood serum. Comparable separation was attained in less than 3 min in the case of the former and in less than 2 min in the case of the latter. The serpentine-channel design was effective in differentiating APTS-labeled N-glycans derived from blood serum collected from patients with different diseases of the esophagus (26).

CE- or MCE-LIF of APTS-labeled glycans permits unmatched isomeric separation. The separation of glycan structural and positional isomers has been demonstrated (15, 17, 18). However, the identification of glycans via this strategy heavily depends on the availability of standards, which are not in abundance. An electrophoretic peak is conclusively identified if a standard is available. Nevertheless, limited identification has been demonstrated through the use of exoglycosidases. These limitations are easily alleviated by interfacing CE with MS, enabling effective identification through both MS and tandem MS.

Hydrophilic Interaction Chromatography with Fluorophores—Hydrophilic interaction chromatography (HILIC) of glycans derivatized with fluorophores, such as 2-aminobenzamide (2-AB), 2-amino pyridine (PA), and 2-aminobenzoic acid (2-AA), has been shown to be an effective means of sensitively quantifying glycans (Fig. 1). This derivatization involves the above-described reductive amination. Ludger Ltd. (Oxford, UK) offers derivatization kits for these reagents. 2-AB is a non-ionic hydrophobic derivatizing reagent that has been widely employed in the quantitation of glycans via HILIC with fluorescence detection (FL). A database comprising normalized elution times of 2-AB-labeled glycans on HILIC, represented as glucose units, has been routinely employed for the structural assignment of more than 350 N-glycan structures (27, 28). This strategy has been effectively utilized for the quantitative glycomic analysis of different cancers, including breast (29, 30), lung (31), and ovarian (32).

Similarly, PA is also widely used in conjunction with HILIC-FL to quantify glycan structures, employing a database that also has normalized retention times of PA glycans represented as glucose units (33, 34). The lack of commercially pure PA necessitates the recrystallization of this reagent as an added step, which limits its practical use. In contrast to the other reagents, 2-AA is an ionic derivatizing reagent enabling both electrophoretic and chromatographic separations of labeled glycans (35, 36).

The performance of these reagents was recently compared, and major differences in detection sensitivity were observed (37). The detection of AA-labeled glycans was 3-fold higher than that of 2-AB-labeled counterparts. The sensitivity was highly dependent on the glycan structures. This could be partially attributed to the preparation procedure and the purity

of the reagents used. These findings were also observed in a HUPO Human Disease Glycomic/Proteome Initiative multi-institutional study that involved 20 laboratories, 6 of which used labeling strategies (38). Among these laboratories, there were significant variations in the quantitation of the labeled glycans (38).

Although the identification and quantification of glycans via HILIC-FL have been demonstrated as mentioned above, the identification of glycans is dependent on the availability of standards or the use of exoglycosidases. Also, correlating the retention times of glycans to those of glucose ladders is effective in identifying structures previously identified using standards or exoglycosidases. This strategy does not permit the identification of unknown or unique structures; these are identified only by means of MS and tandem MS.

Mass Spectrometric Strategies—To date, MALDI- and ESI-MS have been effective in the quantification of glycans derived from glycoconjugates. Among the challenges faced when using either ESI- or MALDI-MS are the different ionization efficiencies of neutral and acidic glycans and the weak ESI ionization of native glycans in general. Additionally, ESI-MS of glycans results in the formation of multiple charge state ions and adducts. The derivatization of glycans prior to MS analysis has been used as a means to eliminate the difference in the ionization efficiencies of neutral and acidic glycans. Moreover, the ESI ionization of glycans is enhanced through derivatization with hydrophobic reagents, which is usually achieved via reductive amination chemistry, hydrazide chemistry, and permethylation. Such derivatization not only imposes hydrophobicity, which facilitates efficient ionization in both MADLI and ESI-MS, but also prompts efficient chromatographic separation of the derivatized glycans.

Quantitative Analysis of Glycans Derivatized with Reductive Amination Chemistry—The hydrophilicity of the glycans' sugar functional groups makes desorption from the electrospray droplet difficult in ESI, because hydrophobicity is essential for high ionization efficiency in ESI quantitative applications. As mentioned above, the most common chemistry employed to derivatize glycans is reductive amination. The same reagents described above for HILIC-FL have also been employed for MS-based quantitative glycomics (39). Positive- and negative-mode MALDI-MS have been employed to analyze 2-AA-labeled N-glycans derived from purified glycoproteins and human blood serum and plasma (35, 36, 40–42). Another reagent that has been shown to enhance ESI ionization efficiency by 10- to 50-fold is procainamide (43).

Quantitative Analysis of Glycans Derivatized with Reagents that Introduce a Cationic Charge—Early investigations aimed at increasing ionization efficiency by derivatizing carbohydrates through the addition of a cationic charge began with the use of *p*-aminophenyl ammonium chloride for fast atom bombardment MS (44). Recently, 5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid 4-aminomethyl-benzylamide synthesized via the activation of biotin with a 1,1′-carbonyl diimidazole, which undergoes nucleophilic attack by the aminomethyl groups of xylylenediamine, was introduced as a new reagent imparting a cationic charge to glycans (45). This reagent is coupled to glycans via reductive amination, with the generation of a cation via quaternization of the linking secondary amine. MALDI-TOF and ESI-MS of labeled glycans demonstrated a 100-fold increase in sensitivity (45).

Girard's reagent T (GT) has been used for the quantification of N-glycans derived from Chinese hamster ovary cell lines (46). When used with MALDI-TOF, GT can quantify only neutral glycans. However, methyl esterification in conjunction with GT derivatization permits the rapid qualitative and quantitative analysis of sialylated and neutral N-glycans and allows one to distinguish the differences in glycosylation between two Chinese hamster ovary cell lines (46). GT was also utilized in conjunction with the amidation of sialic acid in order to compare the quantification of glycans labeled with GT and those labeled with 2-AA (42). The relative peak percentage difference between the two derivatized N-glycans was less than 5% when using MALDI-MS, yet the analysis of GT-labeled glycans was 1.7-fold less sensitive than that of 2-AA counterparts (42).

Quantitative Analysis of Glycan Hydrazones—Although reductive amination is effective, additional purification steps are necessary in order to remove salts. This often leads to an increase in preparation time, sample loss, and analytical variability. The reaction of glycans with a reagent that provides a hydrazide end group has been explored as an alternative that does not require extensive sample clean-up after derivatization. Hydrazide labeling is much like reductive amination, but no Schiff base is involved. Once the hydrazone is formed, the sample can be subjected to MS analysis, because no salts are used or produced in the reaction. However, a drawback of this chemistry is the fact that these hydrazone labels are not readily available and have to be synthesized.

Five hydrazide reagents were recently synthesized: 2-phenylacethydrazide, 3-phenylpropane hydrazide, 4-phenylbutane hydrazide, 5-phenylpetane hydrazide, and 4-phenylbenzohydrazide (Phenyl2-GPN) (47). These hydrazides were systematically evaluated to determine which would effectively increase the relative abundance of *N*-linked glycans detected via ESI-MS. Phenyl2-GPN is a hydrazide that has a second phenyl ring, which increases the hydrophobicity and the ionization efficiency of the sample. The intensities of Phenyl2-GPN-labeled N-glycans derived from 50 μl human plasma increased 10% relative to the same glycans labeled with other reagents possessing a single phenyl ring (47).

Quantitative Analysis of Permethylated Glycans—The permethylation of glycans increases MS detection sensitivity by converting highly polar OH–, NH–, and COOH– groups to nonpolar OCH_3–, NCH_3–, and $COOCH_3$– groups, respectively. This derivatization also allows neutral and acidic glycans to be simultaneously detected in MALDI-MS positive mode with high ionization efficiency. Moreover, permethyla-

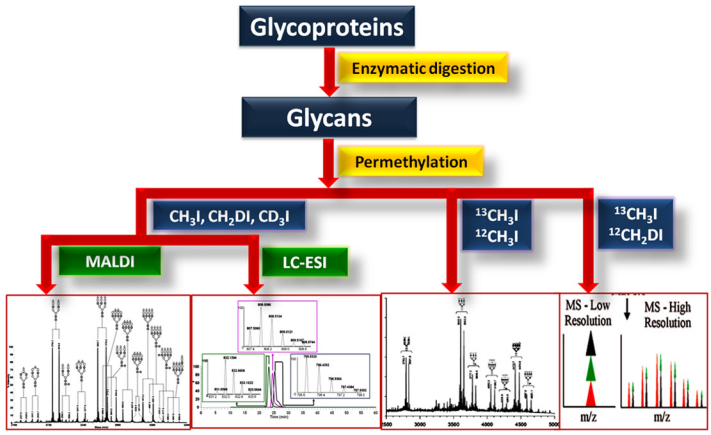

FIG. 2. **Quantitative glycomics of stable isotopically permethylated glycans via ESI-, MALDI-, and LC-ESI-MS.**

tion simplifies the interpretation of tandem mass spectra and allows for easy structural elucidation. The increased hydrophobicity also allows the glycans to be amenable to reverse-phase liquid chromatography separation. Thus, permethylation is one of the most popular strategies for glycan derivation. As discussed above, reductive amination lacks repeatability among different laboratories, but this is not true in the case of permethylation (38).

The conventional permethylation protocol was initially introduced by Ciucanu and Kerek (48) and more recently was modified to eliminate by-product formation (49). The recent development of solid-phase permethylation, in which sodium hydroxide is packed in capillaries (50) or spin columns (51), enhanced the analytical reproducibility of this chemical derivatization. This strategy was employed to quantitatively assess glycomic changes associated with different cancers, including prostate (52), liver (53), breast (54), esophagus (55), and ovarian (56). A high-throughput version of solid-phase permethylation involving the use of 96-well plates packed with sodium hydroxide beads has been introduced recently (57). This approach enables the simultaneous permethylation and purification of 96 samples.

Quantitative Glycomics through Stable Isotopic Labeling—Although label-free quantification methods are widely used, such methods are known to suffer from limited normalization ability. This is due to the different ionization efficiencies of the different analytes, which originate partially from instrument instability. Relative quantification strategies can reduce the run-to-run variation through the normalization of internal standards. The relative quantification of N-glycans involving the incorporation of stable-isotope labeling appears to be the most popular strategy. Isotopic labeling involves the incorporation of different isotopic species onto chemically similar analytes of interest to generate an identifiable mass difference, thereby allowing simultaneous MS analysis of multiple samples. Permethylation (58–63) and reductive amination (13, 64–72) with stable isotopic reagents are currently employed to reliably attain relative and comparative quantitative glycomics.

Stable Isotopic Labeling via Permethylation—Similar to reductive animation, the isotopic labeling of glycans via permethylation has been widely employed in quantitation studies (Fig. 2). Kang *et al.* (58) were the first to introduce the stable isotopic labeling of glycans using iodomethane or iodomethane-*d*3 to permethylate glycans prior to MALDI-MS analysis. This approach was labeled as comparative glycomic mapping. This strategy was recently employed in conjunction with LC/MS for the quantitation of glycans derived from glycoproteins and human blood serum (62). Distinct peaks were observed for each "light" and "heavy" glycan pair. The retention times were different, which might pose a problem, as the ionization might be slightly different; however, this was not observed (62).

Aoki and coworkers employed $^{13}CH_3I$ and $^{12}CH_3I$ for the relative quantification of glycans derived from embryos of *Drosophila melanogaster* aged between 0 and 6 h (early) or between 6 and 24 h (late) after egg laying (63). This relative quantification suggested that there is a significant decrease of some major glycans derived from early embryos (63).

Similar to comparative glycomic mapping, quantization by isobaric labeling is another stable isotopic permethylation strategy that uses $^{13}CH_3I$ and $^{12}CH_2DI$ (60). It has been shown to be effective for the comparative glycomic profiling of N- (60) and O-glycans (61). This strategy indicated for the first time that the differentiation of embryonic stem cells into embryoid bodies is accompanied by a greater than 3-fold decrease in the expression of two difucosylated (Lewis X type) N-glycans. However, this approach is limited by the need for high-resolution MS capable of defining a difference of 0.002922 Da between methylated sites.

Stable Isotopic Labeling by Reductive Amination—As mentioned above, reductive amination is one of the most common strategies for glycan derivatization. Not only do the reductive reagents provide fluorescence or hydrophobic properties that aid in sensitive detection or reverse-phase liquid chromatography separation, but they can be used in conjunction with stable isotopically labeled reductive reagents that can be applied for the relative and comparative quantitation of gly-

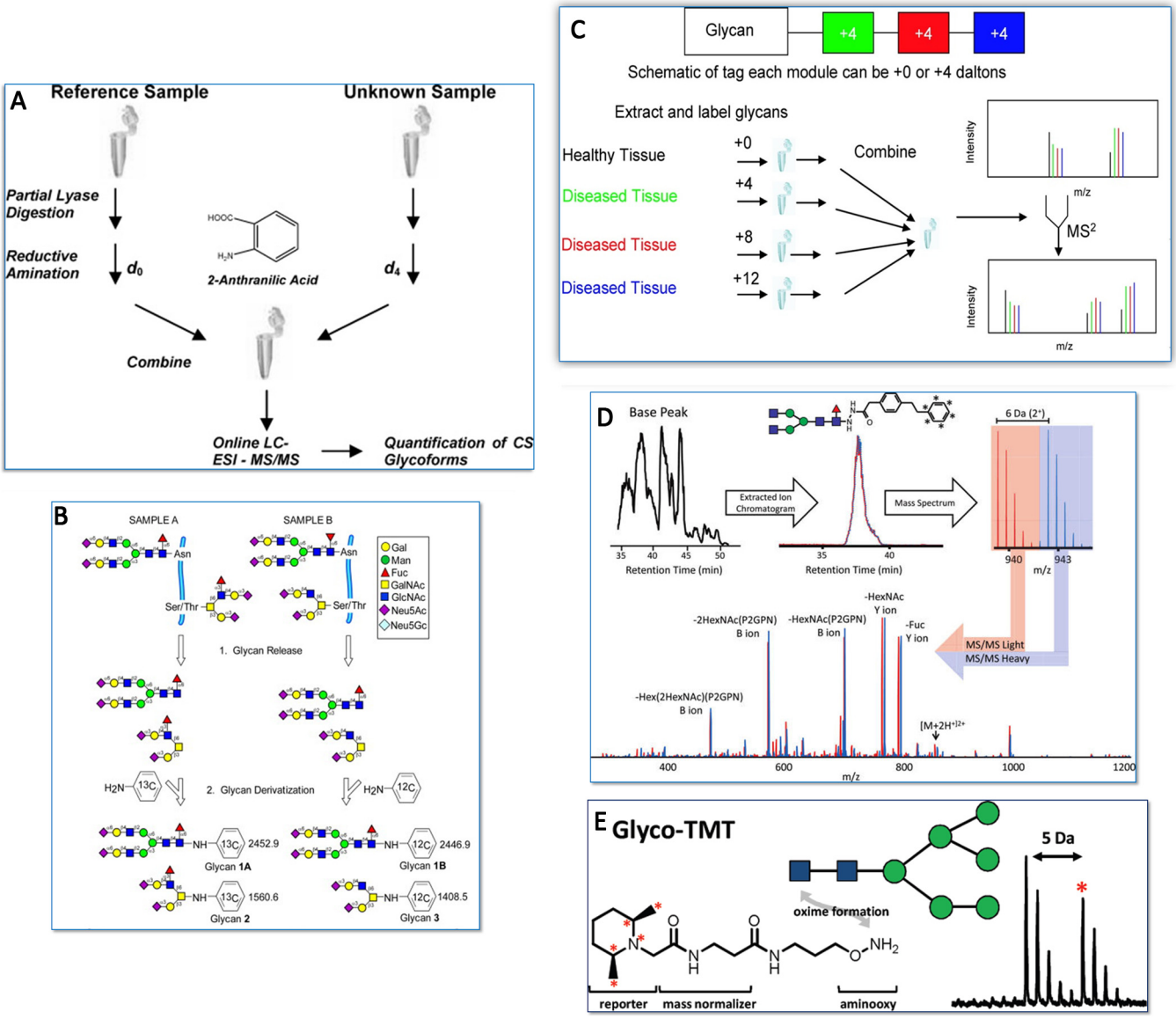

FIG. 3. **Quantitative glycomics incorporating labeling with stable isotopic reagents.** *A*, labeling with (*d*0/*d*4) 2-AA. Reproduced and modified from Ref. 66 with permission. *B*, labeling with [$^{12}C_6$]aniline and [$^{13}C_6$]aniline. Reproduced and modified from Ref. 72 with permission. *C*, labeling via reductive amination using tetraplex tagging reagents. Reproduced and modified from Ref. 64 with permission. *D*, labeling with stable isotope hydrazide reagents. Reproduced and modified from Ref. 47 with permission. *E*, labeling with isobaric tandem mass tag reagents. Reproduced and modified from Ref. 74 with permission.

cans. As mentioned above, several reductive amination reagents have been effectively employed for the quantification of glycans, such as PA, 2-AA, 2-AB, and others.

PA is a reagent commonly employed for N-glycan labeling. Recently, tetradeuterium-labeled pyridylamino and non-deuterated PA have been used for the comparative quantitation of N-glycans derived from recombinant chorionic gonadotropin and human chorionic gonadotropin (73). The results showed that many hybrid-type glycans linked to recombinant chorionic gonadotropin, whereas fucosylated glycans were attached to human chorionic gonadotropin. Although this strategy appears to be effective, the lack of pure PA reagent is hampering its wide use.

Light (*d*0) or deuteriomethyl (*d*4) forms of 2-AA (Fig. 3*A*) have been used to quantitatively assess the levels of chondroitin sulfate derived from cartilage and versican (66) glycosaminoglycans derived from cartilage tissue (67). The mass difference between (*d*0) 2-AA and (*d*4) 2-AA is only 4 Da, which might cause the overlap of isotopic peaks. Moreover, a chromatographic retention time difference for the pairs is associated with this labeling approach, which might influence quantification aspects. 2-$^{12}[C_6]$ aminobenzoic acid (2-$^{12}[C_6]$-

AA) and 2-$^{13}[C_6]$ aminobenzoic acid (2-$^{13}[C_6]$-AA) reagents have been used in conjunction with MALDI-MS and direct infusion ESI-MS to quantitatively assess N-glycans derived from ribonuclease B and porcine thyroglobulin (68). This strategy offers high accuracy and good reproducibility. $^{12}[C_6]$-aniline and $^{13}[C_6]$-aniline have been used for the relative quantification of glycosaminoglycans (69). Quantification was achieved through the simultaneous injection of $^{12}[C_6]$-aniline labeled disaccharides derived from heparin and mixed with $^{13}[C_6]$-aniline disaccharide standards. The same reagents were also applied for the quantitation of N-glycans derived from human and mouse serum glycoproteins (Fig. 3*B*) (72). In contrast to what is observed with deuterium-containing compounds, the retention times of an N-glycan labeled with these two reagents were the same. Thus, the ionization environment of the pairs is the same, permitting comparable ionization efficiency and, subsequently, reliable quantification.

Multiplexing quantitative glycomics employing isotopic tags synthesized in four forms (+0, +4, +8, and +12), allowing the simultaneous analysis of four samples, was recently described (Fig. 3*C*) (64). The advantages of the tetraplex tag were demonstrated for enzymatic depolymerization chondroitin sulfate mixtures of varying percentages. N-glycans produced from 10%, 20%, 30%, and 40% digestions were labeled with *d*0, *d*4, *d*8, and *d*12, respectively. The reproducibility and quantitative nature of the tagging procedure was demonstrated relative to a *d*0-tagged internal standard. The reagents were also effective in the comparative glycomics of sulfate proteoglycans, heparins, and N-glycans derived from four mammalian species (65).

Other Stable Isotopic Labeling Strategies Employed in Quantitative Glycomics—^{18}O-isotopic labeling of glycans released by endo-β-N-acetylglucosaminidase in heavy water was recently employed in a comparative glycomics study of glycans derived from glycoproteins (invertase and ovalbumin) and blood serum of hepatocellular carcinoma patients. N-glycans derived from a normal sample were labeled with ^{16}O, and hepatocellular carcinoma samples were labeled with ^{18}O and mixed in a 1:1 ratio. MS results showed nine glycans increased in hepatocellular carcinoma samples, with the majority existing as complex biantennary types. Isotopic interference was overcome by applying a deconvolution method in that study.

A "light" (^{12}C) and "heavy" ($^{13}C_6$) pair of hydrazide reagents, 4-phenethylbenzohydrazide, were recently synthesized and employed in stable isotopic labeling (Fig. 3*D*) (47). Two samples were labeled as light and heavy, respectively, prior to being mixed at a 1:1 ratio and undergoing LC/MS analysis. The heavy and light N-glycans have identical chromatographic properties, MS results, and MS/MS performance. The reagents are capable of quantifying changes in the glycosylation of simple mixtures and pooled plasma samples in the presence of internal standards (47).

The use of stable-isotope-labeled carbonyl-reactive tandem mass tags was recently described as a strategy enabling comparative quantitative glycomics (Fig. 3*E*) (74). Although the strategy mimics proteomic tandem mass tags, the data do not appear promising. The study in question did not employ labeled tandem mass tags to evaluate the glycans of biological samples. Biological applications for this strategy have not yet been convincingly demonstrated.

Metabolic Labeling Quantitative Glycomics—In the hexosamine biosynthetic pathway, the side chain amide of Gln is the source of nitrogen in the production of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). This was recently implemented in a metabolic labeling strategy known as the isotopic detection of amino sugars with glutamine (75). When amide-^{15}N-Gln media is used to culture cells, ^{15}N is incorporated into glycans (Fig. 4*A*). The isotope patterns of light and heavy $Man_7GlcNAc_2$ are shown in Fig. 4*B*. Mouse embryonic stems cells were incubated in amide-^{15}N-Gln and amide-^{14}N-Gln media for 72 h and mixed together at the beginning of sample preparation. Variations in sample preparation were largely minimized. However, the incorporation of isotopic reagents is limited by the availability of a biological sample. Thus, when an isotopic tag is introduced through glycan derivatization, sample limitations can be overcome.

Incorporating an unnatural synthetic monosaccharide into a glycoprotein is an attractive metabolic labeling method for glycans. However, this is not easily accomplished because of the high complexity of glycan biosynthesis. The metabolic replacement of GlcNAc preserved in the pentasaccharide cores of N-glycans with unnatural counterparts in *Saccharomyces cerevisiae* was recently demonstrated by Breidenbach *et al.* (76). This strategy enables the incorporation of GlcNAc analogs such as N-azidoacetylglucosamine and N-(4-pentynoyl)-glucosamin into the cell surface and secreted glycoproteins of *Saccharomyces cerevisiae*. This strategy potentially can be applied for quantitative comparisons of glycans expressed in organisms or cells subjected to different biological conditions.

MS-based Quantitative Glycomics Strategies with Electrophoretic and Chromatographic Separations—Although CE-LIF and LC-fluorescence detection have been shown to be useful in quantative glycomics, the characterization of unknown glycans that do not have standards (very often) prompts the need for MS. CE and LC resolve isomers that are not mass resolved by MS. Glycans that lack standards are characterized by means of MS. Therefore, CE-MS and LC-MS are powerful analytical tools that permit the unequivocal assignment of glycan structures.

Quantitative Glycomics with CE-MS—The use of CE-MS and tandem MS to quantify glycans has been described and discussed in recent reviews (77, 78). Off-line coupling of CE to MALDI-MS for the structural characterization and quantification of APTS-labeled N-glycans derived from ribonuclease B was first described by Suzuki *et al.* (16). Mannooligosaccha-

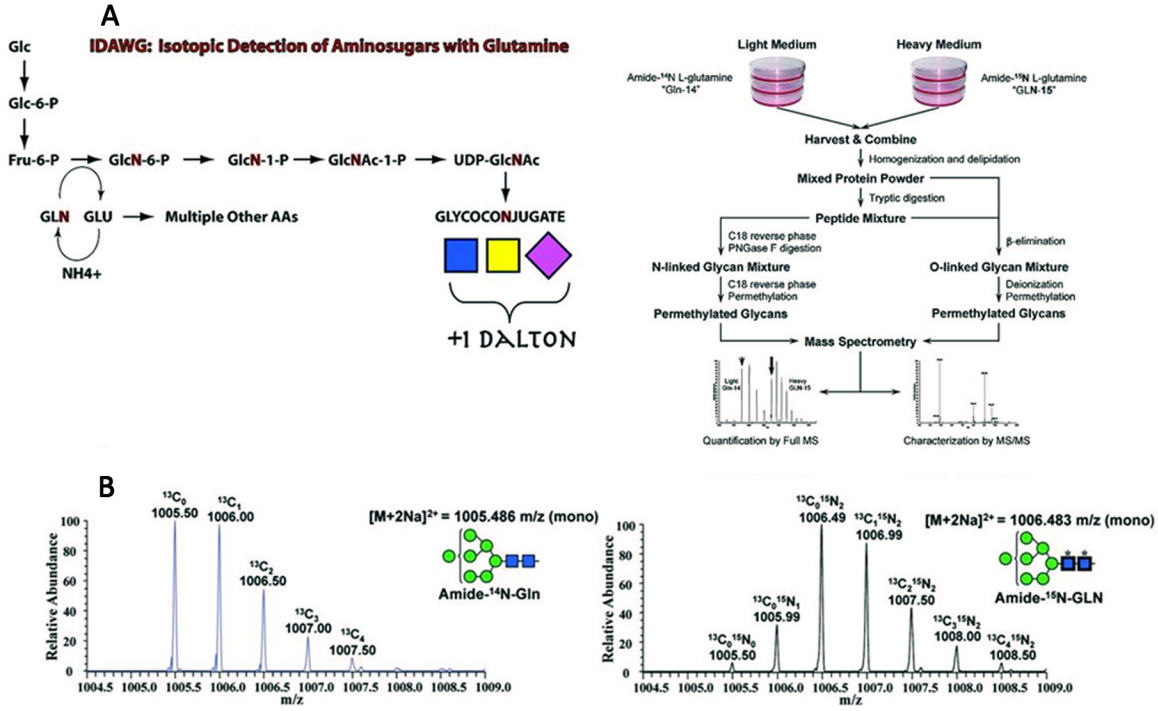

FIG. 4. ***A*, Isotopic detection of aminosugars with glutamine (IDAWG) labeling theory and strategy.** A schematic of the hexosamine biosynthetic pathway that converts the glycolysis intermediate fructose-6-phosphate (Fru-6-P) to UDP-GlcNAc showing the addition of nitrogen into the pathway from the amide side chain of Gln. UDP-GlcNAc goes on to generate UDP-GalNAc and CMP-Neu5Ac so that the aminosugars GlcNAc (blue square), GalNAc (yellow square), or Neu5Ac (purple diamond) in glycoconjugates will have an additional +1 Da added to their mass if amide-15N-Gln is used as the nitrogen donor. Glc, glucose; GlcN, glucosamine. Schematic of the IDAWG *in vivo* cell culture labeling strategy for comparative glycomics. Amide-15N-Gln or Gln is added to the media to label cultured cells. Upon harvesting, cells can be combined, and then N-linked and O-linked glycans can be isolated from digested proteins for permethylation and tandem mass spectrometry analysis. *B*, isotope pattern of light and heavy Man7GlcNAc2. Reproduced and modified from Ref. 75 with permission from the publisher.

ride caps in mycobacterial lipoarabinomannan, which are key molecules in the immunopathogenesis of tuberculosis, were characterized via both CE-ESI-MS (79) and CE in conjunction with MALDI-MS (80). CE and off-line MALDI-MS permitted the analysis of glycans derived from only 5 μg (300 pmol) of *M. tuberculosis* mannooligosaccharide caps in mycobacterial lipoarabinomannan. Interestingly, off-line CE-MALDI-MS was more sensitive than CE-ESI-MS as suggested by the detection of minor compounds observed in CE-LIF analysis (80) that were not identified in the above-mentioned CE-MS study (79). CE interfaced with Q-Trap mass spectrometry permits the analysis of phosphorylated APTS-labeled N-glycans derived from cellobiohydrolase I (81). Gennaro and Salas-Solano were the first to describe the on-line CE-LIF-MS configuration that permits the direct characterization of *N*-linked glycans from therapeutic antibodies (82).

Quantitative Glycomics with HILIC-MS—HILIC separation commonly entails the use of low concentrations of acid or volatile salts and high percentages of organic solvents such as acetonitrile. Therefore, this mode of chromatography is suited for ESI-MS interfacing (for reviews, see Refs. 83 and 84). Nano-flow HILIC-MS has been employed for the quantitative and qualitative analysis of native (85) and labeled glycans (86, 87). Femtomole sensitivity was observed in both labeled and native glycans. Sulfated glycans derived from mucin glycoproteins and glycosaminoglycans were effectively separated and quantified via HILIC-ESI-MS (88, 89). In the latter, the separation was achieved by using a chip packed with hydrophilic interaction media.

Quantitative Glycomics with Porous Graphitized Carbon Columns—Like HILIC, separation on porous graphitized carbon (PGC) columns is compatible with MS and the two can be interfaced, because a gradient of organic solvents containing small amounts of acids, bases, or volatile salts is employed to separate glycans. The separation of glycans on PGC has been attained with excellent efficiency and selectivity (90). However, the mechanism of separation associated with this medium is not fully understood. Alditol-labeled and permethylated glycans have been effectively resolved on PGC chromatographic media (90). Sulfated glycans derived from mucin glycoproteins have been successfully separated and quantified using PGC columns (88). PGC columns facilitate the separation of glycan isomers. The retention time of glycans on PGC columns is predictable, enabling the assignment of structures via the combination of retention times and mass (91, 92).

Software Tools for MS-based Quantitative Glycomics—The success of any quantitative strategy hinges on software tools that enable automated data interpretation. Because MALDI-MS has been the technique of choice for quantitative glycomics for a long time, most existing software tools in glycomics are based on MALDI-MS data, focusing on the annotation of glycans in an experimental MALDI-MS spectrum. Cartoonist (93) is the first automatic N-glycan annotation tool and assigns potential glycans to peaks in an MS spectrum by matching the theoretical glycan mass to the observed mass. Although Cartoonist does not directly report the abundances of the glycan measured in an MS experiment, it is straightforward to combine it with quantitative proteomics tools such as Biomarker Wizard (Ciphergen Biosystems Inc. Fremont, CA) to retrieve the intensities of annotated peaks, used as a measure of glycan quantities compared across multiple samples (*e.g.* disease and control samples for biomarker discovery).

GlycoWorkBench (94), another software package for glycan annotation in mass spectrometric data, supports multiple data formats from different MS instruments. It also provides a glycan drawing tool that allows users to define specific glycan structures to be annotated in the MS data. Similar to Cartoonist, GlycoWorkBench needs to be combined with quantification tools to extract quantitative information about glycans from a glycomic dataset. Notably, both Cartoonist and GlycoWorkBench can be used to identify glycans from their MS/MS spectra, and thus provide additional functionality for glycomic data analysis.

LC/MS recently has been considered as a choice for high-throughput glycomic analysis. Software tools that integrate the functionalities of glycan annotation and quantification have become available. As initial efforts, these tools have been developed for label-free glycan analysis. Once the labeling techniques for quantitative glycomics become mature, we expect that software tools will be developed to assist in the automatic analysis of such data. GlycReSoft (95) is a software tool that takes as input the de-isotoped LC/MS data output from the Decon2LS tool (96), annotates de-isotoped peaks using a default or user-defined glycan library, and reports the intensity of the peaks annotated as glycans. Because the software relies on DECON for de-isotoping, it might be slow when processing large LC/MS datasets. We developed an open-source software tool, MultiGlycan, for quantitative glycomic analysis on both MALDI-MS and LC/MS. The MALDI version of the tool annotates and quantifies glycans in a pre-defined library by matching the theoretical isotopic envelopes to the observed peaks using a linear regression model. As a result, the tool can deconvolute overlapping isotopic envelopes of large glycans, and thereby achieve more accurate quantification results. In the LC/MS version of the software, the de-isotoping algorithm (based on the open-source code used in Decon2LS) is integrated. As a result, it can be used as a stand-alone tool for fast processing of LC/MS data. It also allows users to define various adduct ions (*e.g.* H^+, Na^+, K^+, and NH_4^+) and detect isotopic peaks accordingly. This tool is currently being used to process LC/MS data of permethylated N-glycans derived from blood serum collected from patients diagnosed with liver cancer and esophageal adenocarcinoma.

CONCLUSIONS

Multiple reliable and sensitive quantitative glycomics strategies are currently available, many of which have been employed for the quantitative assessment of glycan changes associated with the development and progression of many diseases. These strategies have enabled a better understanding of the biological attribute of the complex glycosylation machineries of many biological systems. Many strategies employ stable isotopic labeling reagents that enable multiplexing and comparative quantitative glycomics. Although the sensitivity of many of the above-mentioned strategies is adequate, enhancing the sensitivity of quantitative glycomics is a very active area of research with a lot more needing to be done.

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