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An HPLC-MALDI MS method for N-glycan analyses using smaller size samples: Application to monitor glycan modulation by medium conditions

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Abstract Existing HPLC methods can provide detailed structure and isomeric information, but are often slow and require large initial sample sizes. In this study, a previously established two-dimensional HPLC technique was adapted to a two-step identification method for smaller sample sizes. After cleavage from proteins, purification, and fluorescent labeling, glycans were analyzed on a 2-mm reverse phase HPLC column on a conventional HPLC and spotted onto a MALDI-TOF MS plate using an automated plate spotter to determine molecular weights. A direct correlation was found for 25 neutral oligosaccharides between the 2-mm Shim-Pack VP-ODS HPLC column (Shimadzu) and the 6-mm CLC-ODS column (Shimadzu) of the standard two- and three-dimensional methods. The increased throughput adaptations allowed a 100-fold reduction in required amounts of starting protein. The entire process can be carried out in 2–3 days for a large number of samples as compared to 1–2 weeks per sample for previous two-dimensional HPLC methods. The modified method was verified by identifying N-glycan structures, including specifying two different galactosylated positional isomers, of an IgG antibody from human sera samples. Analysis of tissue plasminogen activator (t-PA) from CHO cell cultures under varying culture conditions illustrated how the method can identify changes in oligosaccharide structure in the presence of different media environments. Raising glutamine concentrations or adding ammonia directly to the culture led to decreased galactosylation, while substituting GlutaMAX™-I, a dipeptide of L-alanine and L-glutamine, resulted in structures with more galactosylation. This modified system will enable glycoprofiling of smaller glycoprotein samples in a shorter time period and allow a more rapid evaluation of the effects of culture conditions on expressed protein glycosylation.

Keywords N-glycan analysis · HPLC · Mass spectrometry · Media · Antibody · Cell culture · pH

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Introduction

Secreted proteins often undergo post-translational modifications including the addition of oligosaccharides (glycosylation) to the protein backbone. As the number of commercial glycoproteins used as biotherapeutics increases, efforts to understand and control posttranslational modifications including glycosylation will continue to increase [1]. The value of a recombinant protein is highly dependent on the oligosaccharide pattern that is obtained from the cell producing that glycoprotein. The presence or absence of sugars such as galactose can play a large role in altering the circulatory activity of certain biotherapeutics [2]. The ever increasing requirement for consistency in biotherapeutics will result in the need to verify the details of branching and sugar linkage types of glycans rapidly for these important glycoproteins.

The N-linked glycosylation process begins in the endoplasmic reticulum (ER) with the addition of the $Glucose_3$ $Mannose_9$*N*-acetylglucosamine$_2$ ($Glc_3Man_9GlcNAc_2$) oligosaccharide onto the asparagine (Asn) residue in the consensus sequence of Asn-X-Thr/Ser. As the glycosylated protein (glycoprotein) traverses the ER and moves into the Golgi network, the glucose (Glc) and mannose (Man) residues are trimmed followed by rebuilding with the addition of more *N*-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc), and sialic acid (SA) by means of multiple glycosyltransferases [3]. The variability of how each molecule of a species of glycoprotein is digested and subsequently reconstructed results in a heterogeneous population of glycostructures even for one species of glycoprotein [4, 5]. The nature of the glycan structure can be important to the stability, solubility, intracellular localization, bioactivity, and *in vivo* clearance rate of the glycoproteins [6–9].

A number of methods have been developed for the detailed analysis of the profile of glycan structures on a species of glycoprotein. Two of the major techniques include high performance liquid chromatography (HPLC) and mass spectrometry (MS). The quantity of a sample glycoprotein required for glycan analysis depends on many factors such as complexity of glycan structure, degree of structural details needed, numbers of glycans per protein molecule, molecular weight, and sensitivity of detection. As new technologies such as tandem mass spectrometry and nano-HPLC have been developed, structural analysis of N-glycans has become possible with micrograms or less of a glycoprotein [10]. However, samples have to be ultra pure, operation often requires highly trained personnel, and mass spectrometers are orders of magnitude more expensive than HPLC systems. When using Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), negatively charged glycans cannot be analyzed with the same mode that neutral glycans are best analyzed. In addition, quantification of glycan levels can be difficult. Finally, traditional mass spectrometry such as MALDI-TOF, and even the more expensive and complex forms such as tandem MS/MS, cannot determine isometric branching of glycans easily [11]. Recently only some of the newest high-end mass spectrometers, often unavailable to lower-end users, are able to fractionate the glycans enough to produce large amounts of data required to differentiate isomers and determine branching structures [12].

On the other hand, a complete analysis of glycan structure using well-established columns operated on a conventional HPLC, such as reverse (ODS silica) and normal phase (amide-silica), have generally required a milligram or more of a glycoprotein as a starting material. However, a relatively low cost for the equipment, a capacity to differentiate isomers of glycans conveniently by running samples through reversed and normal phase columns [13], and an ability to analyze relatively impure samples support the continued use of conventional HPLCs for glycan analysis. Further, conventional HPLCs are available in a large number of laboratories and institutions. Finally, typical LC/MS methods have required multiple dimensions, complex forms, and detailed analysis of mass spectrometry to determine complete structures including branching isomers [12, 14, 15]. This includes methods for oligosaccharides, glycopeptides [16], and glycolipds [17, 18].

Given the limitations and advantages of the different methods, the current study was directed at determining if an improved methodology could be obtained by combining one column from an HPLC method and its established literature elution data [13] together with automated sample spotting onto plates for conventional MALDI-MS analysis. This technique would potentially reduce sample size and, to some extent, lengthy processing requirements of the traditional two-dimensional HPLC methods while permitting a more quantitative evaluation of glycans between samples than is typically available for MS analysis. These modifications to the traditional 2- and 3-dimensional HPLC technology would allow analysis of more samples in parallel over only two to three days starting with as little as one hundred micrograms of protein.

Many factors can influence the distribution of structure types in a glycan profile. The collection of structures in a glycosylation profile is not limited to the intracellular enzyme concentrations and the kinetics of association between the enzyme and the substrate [5, 19]. The components and conditions of the medium can have a significant impact on the glycosylation profile as well. The modified method described here can be used to examine in parallel many conditions that affect glycan structure while maintaining isometric branching analysis. Different initial media can affect the glycosylation profile by altering the concentration of sugar-nucleotide precursor concentrations [20] or by altering the activity of the glycosyltransferases by changing the pH of the subcellular compartments [21]. In addition, the changing conditions of the medium itself over the course of the cell culture process can have a significant impact on the glycosylation profile as well [22]. An improved HPLC glycan analysis method would be useful for screening the effects of multiple media conditions simultaneously on glycan processing.

One factor in the medium that can have a significant impact on the glycosylation profile produced by animal cells is the levels of ammonia and ammoniagenic metabolites such as glutamine. The accumulation of ammonia has been shown to have a significant negative impact on the complexity of glycan structure including galactosylation and sialic acid level of N-glycans. Medium ammonia and glucosamine are incorporated into the glycan structures [23,

24] when free ammonia is used to aminate fructose-6-phosphate (from free glucose) in the formation of UDP-hexosamines [25]. The increased metabolites can reduce the branching of glycans in recombinant human erythropoietin [26]. Other experiments showed reduced tetraantennary structures with proportional increase of tri- and di-antennary structures as ammonia levels increased [27, 28]. By altering intracellular pH, ammoniagenic metabolites alter sugar-nucleotide precursor pools [29], negatively affect glycosylational enzymatic activities [30], and change localization of lysozomal degradation enzymes [31].

The impact on glycosylational complexity that comes from the use of ammoniagenic amino acids suggests the need for alternative substrates which could potentially minimize the accumulation of ammonia. Lack of glutamine, a traditional medium additive, causes low viability, while excess glutamine spontaneously degrades to form ammonia and glutamic acid, causing an increase in pH and potential negative affects on the glycosylation profile. A balance between maintaining culture viability and high glycosylation complexity is desired [32]. Simply increasing the pH of the culture alone has been shown to decrease the galactosylation of glycoproteins to indicate that control over the glutamine precursor is important [33].

A potential alternative to maintain the essential elements of glutamine as an energy and amino acid source without the negative affects of ammonia accumulation is the use of a dipeptide in place of glutamine. One such dipeptide is a non-ammoniagenic additive of alanyl-glutamine (Ala-Gln), available as GlutaMAX™-I from Invitrogen that does not degrade spontaneously like glutamine in culture to form ammonia as a by-product. As the cell secretes extracellular peptidases, the enzymes cleave the dipeptide and provide the medium with necessary glutamine for metabolism. In this way, the Ala-Gln dipeptide provides the positive attributes of glutamine availability without increasing the ammonia concentration from spontaneous degradation and its inherent negative impact on glycosylation. Cultures utilizing the Ala-Gln dipeptide exhibit equal growth rates and protein production [34–36]. In addition to developing a new analytical technique for glycan analysis, the combined HPLC-MS methodology has been utilized to examine the effects of excess ammonia and to compare the effects of using the Ala-Gln dipeptide (GlutaMAX™-I) versus a traditional glutamine feeding on the glycosylation profile of a target model protein, a recombinant tissue plasminogen activator (t-PA).

Materials and methods

Cell culture stock maintenance

A Chinese Hamster Ovary cell line expressing tissue plasminogen activator (t-PA) was obtained from ATCC. The cell line, CHO-15 t-PA (CRL-9606), was adapted from medium supplemented with 10% FBS to a CD OptiCHO™ base medium (CD OptiCHO™ Medium, 50 mg/L Gentamicin, and 10 μM Methotrexate), and cells were grown in the same medium supplemented with either 4 mM GlutaMAX™-I (CD OptiCHO™-4X) or 4 mM L-glutamine (CD OptiCHO™-4Q). Cultures were passaged at 2×10^5 viable cells/mL (vc/mL) on a 3-day/4-day schedule. Cultures were grown in a 37°C humidified incubator with 8% CO_2 while being agitated at ~115 rpm in a shake flask.

Media test conditions

The CD OptiCHO™-4X stock culture was used to seed CD OptiCHO™ base medium cultures containing 8 mM GlutaMAX™-I (CD OptiCHO™-8X) and 4 mM GlutaMAX™-I with 30 mM ammonium chloride (CD OptiCHO™-4X30A). The CD OptiCHO™-4Q stock culture was used to seed a CD OptiCHO™ base medium culture containing 8 mM L-glutamine (CD OptiCHO™-8Q). Each condition was seeded at 2×10^5 vc/mL in quintuplicate 200 mL cultures (1 L shake flasks) and grown as mentioned above.

Growth performance assay and harvest

Cultures were grown for 8 days. Viable cell densities were determined using a Coulter Vi-Cell (Coulter Beckmann). On day 8, the quintuplicate cultures of each condition were pooled and centrifuged at 200 g for 15 min. Supernatants were divided into 400 mL fractions, Tween-80 (Calbiochem) was added to 0.01%, and the supernatants were frozen at −80°C.

Tissue Plasminogen Activator (t-PA) purification

t-PA was purified using a slight modification of a previously published procedure [37]. Briefly, samples of supernatant were thawed in a 37°C waterbath, filtered through a 0.45 μm membrane, and loaded onto a packed, pre-equilibrated Lysine Hyper D (Pall) column using an AKTA FPLC (GE Healthcare). Contaminants were removed by a salt wash and the t-PA was eluted with a 500 mM L-arginine buffer, collecting 1 mL fractions. Fractions containing t-PA were pooled and stored at −80°C.

t-PA analysis

Protein concentration in the fractions was determined using a Bio-Rad Protein Assay kit (Bio-Rad). Protein size and purity were determined using SDS-PAGE analysis. Briefly, samples and CandyCane™ glycoprotein molecular weight standards (Invitrogen) were separated on a Novex Precast

4–20% gradient Tris-Glycine SDS gel for 140 min at 125 volts. The gel was stained overnight with Simply Blue™ Safe Stain.

Lysosomal staining

The acidity of lysosomes and other organelles was investigated using LysoSensor™ Yellow/Blue DND-160 probes following the manufacturer's protocol (Invitrogen). Briefly, the LysoSensor probe was diluted to 1 μM in 37°C pre-warmed medium. Cells were centrifuged at maximum speed for 2 min in a microfuge and the cell pellet was resuspended in the pre-warmed diluted LysoSensor probe and incubated at 37°C for 2 min. Stained cells were placed on microscope slides, covered with coverslips, and visualized using a fluorescent microscope with a FITC filter. Images were captured using Spot v4.0.4 software (Diagnostics Instruments, Inc.).

Supplies for modified glycan analysis

PNGaseF and accompanying buffers (New England Biolabs), ethanol (Sigma-Aldrich), butanol (Sigma), 25 mg graphitized carbon cartridge (Alltech), acetonitrile (Fisher), trifluoroacetic acid (Sigma), 2-aminopyridine [(Aldrich), recrystalized in house], acetic acid (JT Baker), borane-dimethylamine complex (Fluka), microcrystalline cellulose, Sigmacell type 50 (50 μm) powder (Sigma), hydrochloric acid (Sigma), ammonium formate (Sigma), ammonium hydroxide (JT Baker), 2,5 dihydroxybenzoic acid (Sigma), ammonium phosphate monobasic (Sigma), sodium chloride (JT Baker), Poly-Prep chromatography columns (Bio-Rad), human apo-transferrin (Sigma), human immunoglobulin G (IgG) from serum (Sigma), ribonuclease B from bovine pancreas (Sigma), human α_1-acid glycoprotein (Sigma), and fetuin from fetal calf serum (Sigma).

Modified glycan analysis methodology

A process flow chart for the modified method is shown in Fig. 1. Typically, a glycoprotein of interest is used on the order of 100 ug of protein in order to yield tens of nanomoles of glycan for subsequent HPLC and MS analysis. The protein sample need not be absolutely pure as long as the glycoprotein of interest is the only glycoprotein in the mixture. Glycoprotein purity can be verified by a glyco detection assay kit such as Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (Invitrogen). All glycans from the protein backbone are released in the first step of sample preparation as follows. The glycoprotein is dissolved in water (1 mg/mL), mixed with the denaturing buffer (New England Biolabs), and denatured according to the manufacturer's protocol. To the mixture, 1 NEB unit of PNGaseF was added

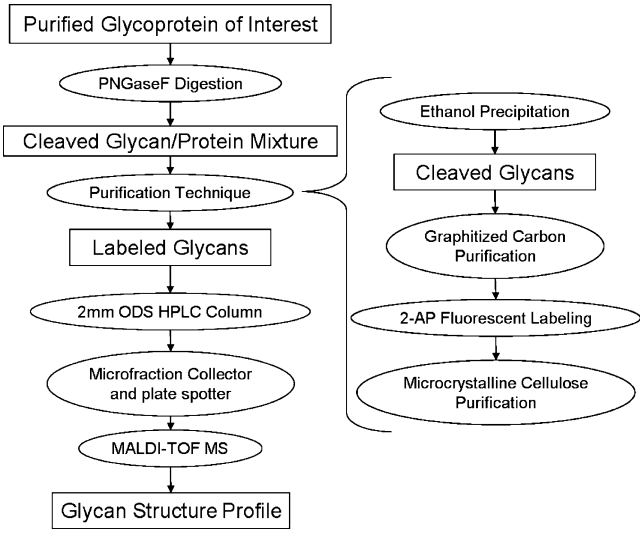


Fig. 1 Flow Chart representation of modified glycan analysis method including a chart representation of glycan purification and labeling scheme

per nanomole of glycan and incubated for 7–8 h with hourly vortexing to release N-glycans. After terminating the reaction by boiling for 10 min at 100°C, the proteins are precipitated from the mixture using cold ethanol at 4°C overnight. The supernatant containing the released glycans is collected after centrifugation, dried on Speed Vac, and resuspended in 50 μl water. Glycans are purified from remaining proteins, salts, and amino acids using a 25 mg graphitized carbon cartridge according to the previously reported method [38]. The glycans are derivatized with 2-aminopyridine by the Kondo method [39] and the derivatized glycans are purified on a column of microcrystalline cellulose. Briefly, the derivatized glycans are loaded onto a column of Sigmacell type 50 (50 μm) (400 μL) pre-equilibrated with a mixture of BuOH, EtOH, and water (4:1:1, v/v). The column is washed with 24 bed volumes of the same solvent and the bound PA-glycans are eluted with three bed volumes of water and dried on a Speed Vac. Sialic acids were removed by treating the sample in 10–20 mM HCl (pH~2.0) at 80°C for 1 h, followed by drying and reconstitution in 50 μl water. The glycan sample is run alongside an IgG glycan mixture and isomaltose oligomer standard on a 2 mm Shimadzu VP-ODS HPLC column with running buffers A: 10 mM ammonium formate pH 4.0 and B: 10 mM ammonium formate, pH 4.0 containing 0.5% 1-BuOH, from 5% to 70% B over 80 min with 20 min equilibration between. The eluant from the column is then spotted on-line onto MADLI-TOF MS plates every 1 min with a matrix solution of 5 mg/mL DHB [40], sodium chloride (10 mM), ammonium phosphate monobasic (10 mM) [41] in 50/50 water/ethanol [42] using a microfraction collector/plate spotter such as the Dionex Probot™. The plate is then subjected to MALDI-TOF MS analysis. HPLC elution times from the reverse phase column are converted

into Glucose Units (GU) using the isomaltose oligomer standard. Glucose units and mass ratios from the MALDI-TOF MS are used as two dimensional data to plot out detailed structural information and compared with literature data published at www.glycoanalysis.info or [13].

Results

Design of modified HPLC-MALDI MS glycan analysis method

A modified method (Fig. 1) has been developed based on an established HPLC multidimensional mapping protocol involving sequential HPLC runs [13]. It uses a combination of reverse phase HPLC and MALDI-TOF MS with automated, on-line sample spotting on a target plate. The method allows a significant scale down in terms of quantity of a glycoprotein required for structural analysis by using a narrower HPLC column and modified sample preparation. The method would be most suitable for those who have easy access to a conventional HPLC and a MALDI-TOF MS instrument. In this scheme, PA-oligosaccharides are first separated on an ODS-silica reverse phase HPLC column. The dilution of the sample in the traditional method's 6 mm column can be reduced by substituting a 2 mm column which allows for detection of the smaller glycan sample on the same HPLC system. With the previous HPLC method, every peak emerging from the ODS column would be collected, concentrated, and run on another type of HPLC column, expanding the number of samples up to 10 fold. Alternatively, each peak can be directly subjected to MS analysis.

To minimize the time required for sample collection and dry-up of fractions on a reverse phase column, an automated plate spotter was used in order to collect peaks directly onto MALDI plates. The spotter used in this study can be programmed independent of the HPLC system to collect peaks every minute, eliminating the cost of specialized HPLC-Plate spotter interaction software. The samples are then analyzed on a MALDI-TOF MS to provide the second dimension of data. In order to specify and standardize the specific peaks, elution times are determined in terms of a glucose oligomer standard. A two dimensional data set comprised of the elution data from the reverse phase column (GU values) and molecular weight determined by MS are then compared with a literature library to identify the glycan structure [43–46].

Comparison of small diameter and large diameter columns

Multiple 2-mm HPLC ODS columns were compared using an IgG glycan mixture created from purified IgG protein from Sigma to determine the optimal column which could scale down but still separate positional isomers as effectively as the 6-mm Shimadzu CLC-ODS column used in the established method. Several columns from different manufacturers were chosen based on their capacity factors to evaluate columns with similar or different packing material compared to the traditional 6-mm Shimadzu column. In choosing a column for the new method, focus was put on the column's ability to separate two positional isomers of glycans present in IgG glycans, which have a terminal galactose on a different branch with the same linkage (β1-4). The separation of these two isomers represents an ideal test for evaluating how well isomers can be separated on an HPLC column. The elution profiles of IgG glycans on the different reverse phase columns are shown in Fig. 2. Human IgG glycans consist of the three major groups of glycans shown in Table 1. Each of the groups shown in Table 1 contains a core structure with two GlcNAc and three Man residues, and two extending GlcNAc residues attached to the α1-6 or the α1–3 Mannose. Later eluting glycans in each group also contain a terminal galactose on the α1–6Man branch, α1–3Man branch, or both, in this order. Group I, eluting between 18.5 and 25 min, contains the core structure and additional galactoses. Group II, eluting between 30 and 35 min, contains only an addition of a core fucose and is the set of major structures for human IgG. The core structure of Group III oligosaccharides, which elute between 47 and 56.5 min on the 6-mm Shimadzu column, are fucosylated and contain a bisecting GlcNAc in addition to galactose residues at different positions. Within Group II are the positional isomers of galactose on the α1–6Man branch (II.2) and α1–3Man branch (II.3) (circled in Fig. 2) used to specify the optimal 2-mm column based on the ability to differentiate these two structures. While the Waters Symmetry C18 column was not able to retain glycans sufficiently under current buffer conditions, the Phenomenex Luna column showed acceptable elution with non-ideal separation using the same buffer conditions as the traditional 6 mm method. The 2-mm Shimadzu VP-ODS column showed the best separation of the positional isomers (Fig. 2). It should be possible to optimize buffer conditions in order to improve the retention and separation of glycans on the Symmetry and Luna columns but the approach was not necessary for this technique with an effective column in hand. All structures were confirmed by literature comparison and a second phase HPLC (data not shown).

Correlation of 2-mm ODS column elutions to 6-mm ODS column elutions

With a smaller diameter column that separated IgG glycan mixtures as efficiently as the literature 6-mm column, a

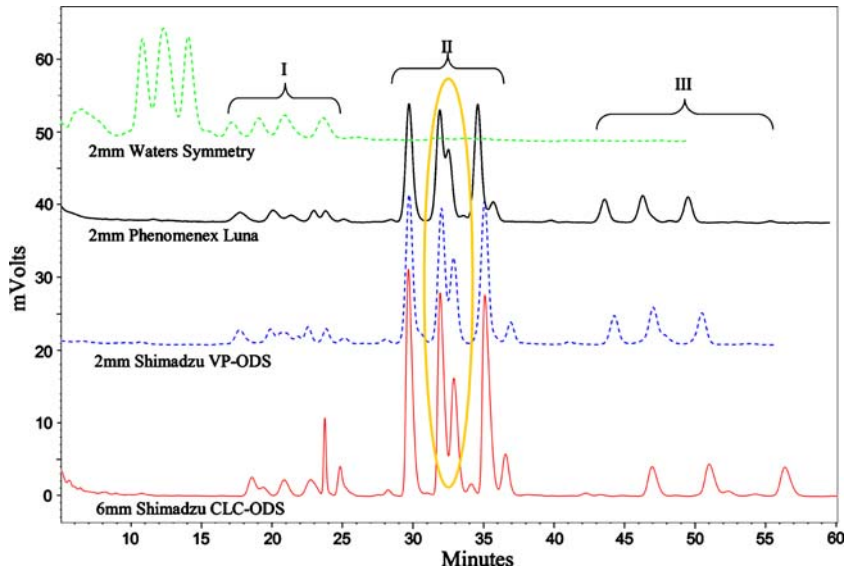


Fig. 2 HPLC ODS Column Effectiveness Comparison. Three 2 mm HPLC ODS columns were used to compare elution profiles of an IgG oligosaccharide to a standard 6 mm HPLC column used in literature

library of glycans was created and analyzed on both 2-mm and 6-mm Shimadzu ODS columns (Table 2). This library contained a wide range of common types of non-sialylated glycans to determine if the elution properties from a 2-mm Shimadzu column could be transferred directly and used in conjunction with literature library information obtained using elution positions from a 6-mm Shimadzu column [13]. Neutral glycans were obtained from five major glycoproteins acquired from Sigma. Twenty structures were determined explicitly (Table 2) and five more were determined to be highly branched tri- or tetra-antennary glycans whose structure were not determined. Oligomannose-type oligosaccharides (M5-M9) controls were obtained from Ribonuclease B of bovine pancreas; di-antennary structures with variations of terminal galactose, core fucosylation, and bisecting GlcNAc were obtained from human immunoglobulin g (IgG) of serum [47] and human apotransferrin; tri-antennary samples were collected from fetuin of fetal calf serum [48]; and tetra-antennary glycans were made from α_1-acid glycoprotein (AGP) of human plasma [49]. The retention time for the samples through both columns on the HPLC was converted into Glucose Units (GU) using isomaltose oligomers run alongside the samples. The average difference between the two columns in glucose units of each library glycan was shown to be 0.01±0.2 (Table 2). Shown in Fig. 3 is the correlation between the 2-mm and 6-mm column elution positions. The 2-mm and 6-mm column elution data correlated directly with a correlation coefficient of 1.000. It is then assumed that elution positions of all non-tested neutral structures are similarly correlated between columns within the standard deviation of GU differences. Thus, identification of structures from the 2-mm Shimadzu column can be directly obtained from reference standards compiled previously using the 6-mm Shimadzu column.

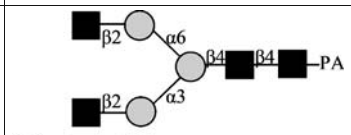
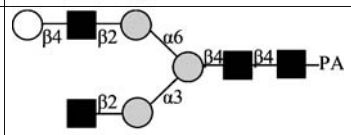
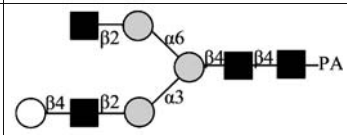
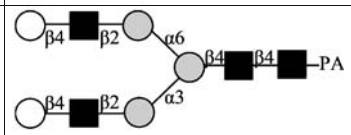
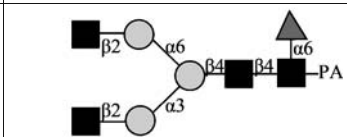
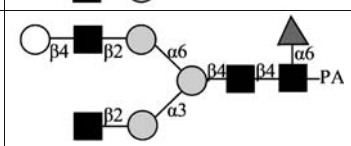
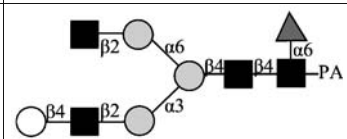
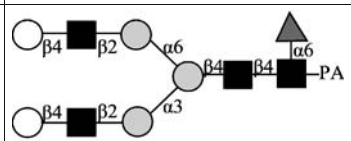
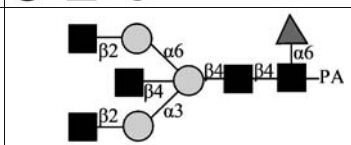
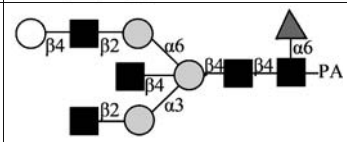
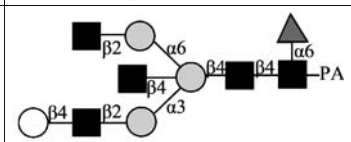
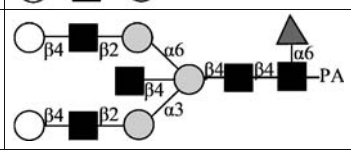
Analysis of IgG glycans using HPLC and MS

The new method was used to analyze the major glycans of a human IgG antibody sample which is well documented [47]. For this purpose, an IgG glycan sample used to determine the appropriate column choice was prepared using the modified method and subjected to the combined one dimensional HPLC and MALDI-TOF MS analysis. Shown in Fig. 4a is the elution profile of the glycans from the IgG sample described previously in Fig. 2 and Table 1. In Fig. 4b, the mass spectra resulting from the four major peaks are shown. Expected mass values based on their known structures including the fluorescent 2-aminopyridine tag are also listed. The method allowed a quick determination of a composition of the four major glycans attached to human IgG using mass spectrometry (Table 3). However, smaller peaks from HPLC did not provide sufficient sample to generate a detectable peak in the fractions provided to the MALDI-MS. Most significantly, this combined HPLC-MALDI MS method was able to differentiate between branching isomers including terminal galactosylation represented by peaks 2 and 3 in Fig. 4a.

Modified method analysis of major glycan profiles for proteins affected by Media Additives

In order to test the method on another protein sample, recombinant human tissue plasminogen activator (rt-PA) was expressed in CHO cells. Glycan structures for t-PA are also well documented so structure results could be compared with those found in the literature [50–52]. One

Table 1 Structures and elution times of hIgG major glycans. (■, GlcNAc; ◯, mannose; ◯, galactose; ▲, fucose)

Group	Structure Number	Structure	Elution Time (min)
I	1		18.5
	2		21
	3		23
	4		25
II	1		30
	2		32
	3		33
	4		35
III	1		47
	2		51
	3		52
	4		56.5

of the ways in which a culture medium can change is the accumulation of ammonia. In order to examine if any effect of ammonia on the glycan profile could be elucidated with the new method, CHO cells producing t-PA were exposed to different media additives that may potentially alter ammonia levels. Culture media were treated with either 4 mM or 8 mM glutamine (abbreviated 4Q and 8Q, respectively), which represent normal additives used in many culture techniques. Alternatively, two other culture samples were treated with the glutamine substitute GlutaMAX^{TM}-I, a dipeptide additive of alanyl-glutamine (Ala-Gln), in concentrations of 4 mM and 8 mM (abbreviated 4X and 8X, respectively). A direct culture addition of 30 mM ammonium chloride to a 4 mM GlutaMAX^{TM}-I culture (A) was included to represent cells exposed to high levels of the by-product of glutamine metabolism.

Before glycans were analyzed, the CHO cells were grown for 8 days. At the end of the culture period, t-PA was purified using established techniques [37]. The purified protein samples were examined by SDS-PAGE, staining with Coomassie and Glycoprotein stain as shown in Fig. 5. Both the expressed t-PA and a sample of a clinical t-PA (not shown) showed the same molecular weight. The only glycosylated protein appearing in the purified sample is the band corresponding to t-PA as indicated by a stain with ProQ Emerald 300 glycoprotein stain.

These protein samples were then subjected to the new glycan analysis method using the 2-mm ODS Shimadzu HPLC column. Glycans were de-sialylated in one reaction step creating a sample of neutral core structures in order to do a quick comparison of glycan profiles rather than full structural analysis. Shown in Fig. 6 are the HPLC plots for the different culture media additives normalized by the total peak area along with the major peaks labeled A–K. The two major peaks provided sufficient sample size for MALDI analysis as shown in Fig. 6d. The size and structure of the glycans identified by MALDI are consistent with the predicted PA-labeled species from t-PA and can be found in Fig. 6d and Table 4. The structures of the glycans from many of the remaining peaks can be estimated by combining their elution positions, expected glycan structures from t-PA, and relations to other positively identified peaks. The peaks were also collected and run on the traditional second dimension of HPLC for verification (Table 5).

The levels of the major glycans in the different media formulations were compared using this modified method. The results from this comparison for some of the major galactosylated glycans are summarized in Table 4. The total percentage of fully galactosylated structures represented between 43 and 46% of total glycans for the traditional glutamine-fed cultures. These include di-antennary and tri-antennary structures with a core fucose on some structures.

Table 2 Structures and elution positions of 6 mm versus 2 mm Shimadzu columns; abbreviations from Tomiya N, *et. al.*, *Anal Biochem*, (1988) [13]

Source	Structure	6mm Elution (GU)	2mm Elution (GU)	Difference
RNAseB	M5.1	6.9	6.7	0.20
	M6.1	6.0	6.1	−0.10
	M7.2	5.3	5.6	−0.30
	M7.1	5.8	5.9	−0.10
	M7.7	6.5	6.4	0.10
	M8.1	5.2	5.5	−0.30
	M9.1	5.4	5.7	−0.30
hIgG	210.1	12.4	12.4	0.00
	210.2	13.1	13.1	0.00
	210.3	13.4	13.4	0.00
	210.4	14.1	14.1	0.00
	211.1	17.4	17.4	0.00
	211.2	18.7	18.7	0.00
	211.3	19.0	19.3	−0.30
	211.4	20.4	20.5	−0.10
hApo Transferrin	200.11	7.2	7.5	−0.30
	200.4	10.6	10.8	−0.20
Fetuin	400.15	9.5	9.4	0.10
	300.22	13.3	13.0	0.30
	n/d	13.9	13.5	0.40
	n/d	10.0	9.9	0.10
	n/d	13.8	13.5	0.30
AGP	400.22	11.1	11.0	0.10
	n/d	13.2	13.0	0.20
	n/d	8.0	8.0	0.00
Average				−0.01
S.D.				0.20

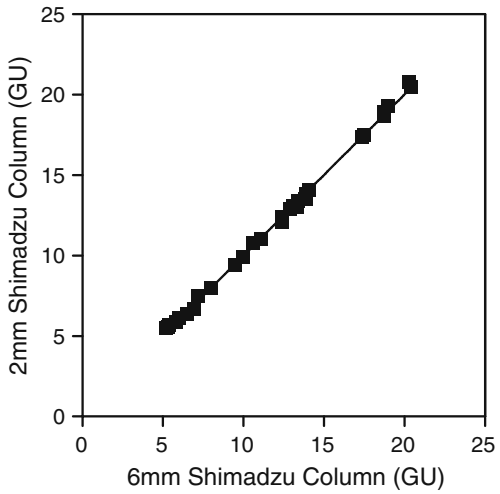


Fig. 3 Correlation of 25 neutral glycan structures analyzed on 6-mm and 2-mm HPLC ODS columns show literature library of elution data can be used directly with 2-mm column. The correlation coefficient of the 25 structures was 1.000

The percentage of each of the three galactosylated species characterized in Table 4 was reduced in the culture fed with ammonia as compared to all others cultures. For instance, the total percentage of fully galactosylated structures for ammonia addition resulted in a 15% reduction from the 4 mM glutamine sample with an 11% reduction of peak G level alone. A similar reduced galactosylation level was seen in the fucosylated tri-antennary galactosylated structures of peak K. Further, another trend is also evident when comparing the media containing glutamine and GlutaMAX^{TM}-I. At both 4 mM (4Q versus 4X) and 8 mM (8Q versus 8X), the levels of all but one fully galactosylated structure were elevated when glutamine was replaced with GlutaMAX^{TM}-I. Overall, the fully galactosylated structures were increased by 10% or more when using GlutaMAX^{TM}-I as opposed to glutamine.

Examining lysosomal pH using an acidic sensitive indicator

In order to understand how the different media components may affect cell physiology, cells exposed to the different additives were treated with a fluorescent

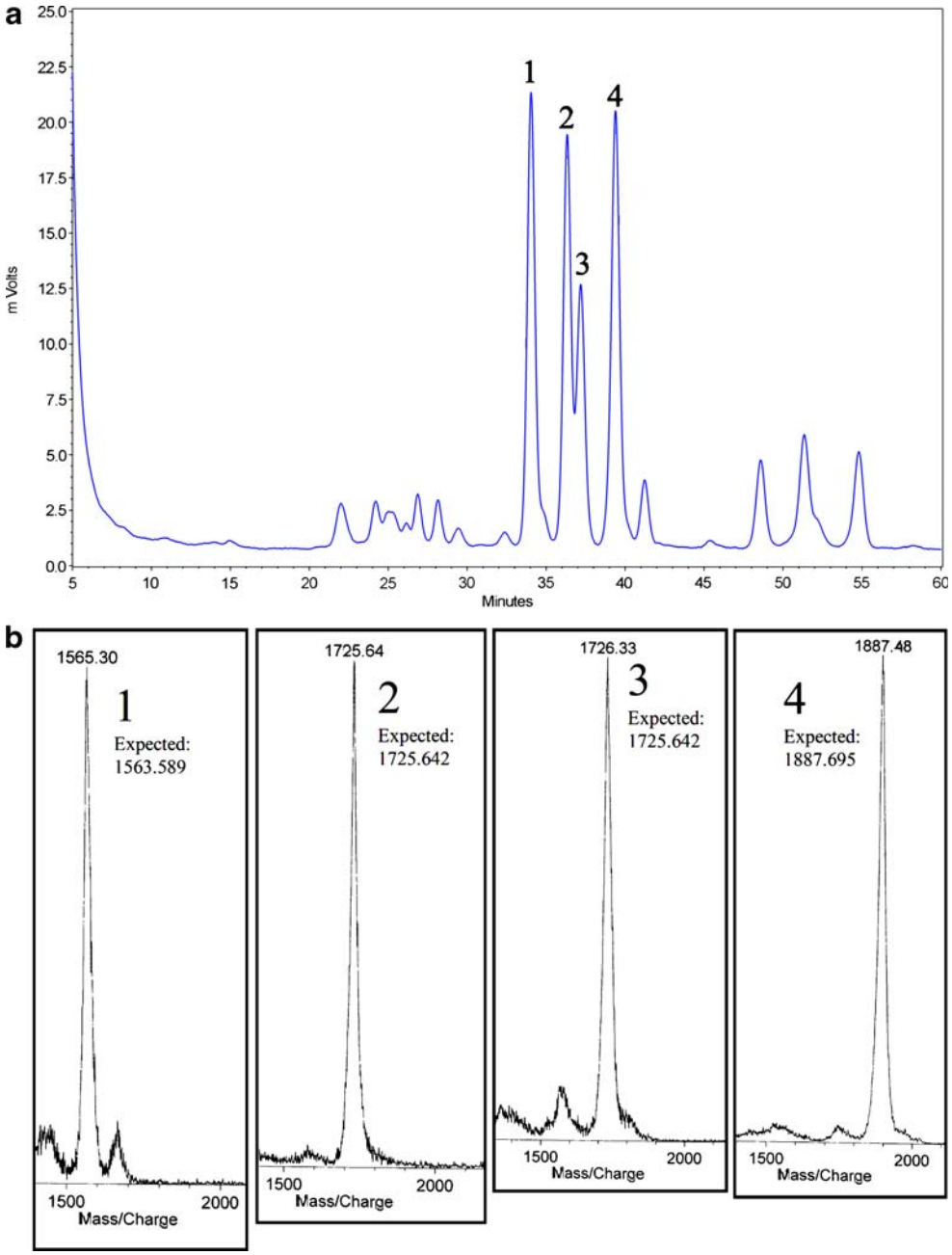


Fig. 4 Analysis of major hIgG glycan 2 mm column profile (**a**) and corresponding mass spectrometry data (**b**) using modified method for four major N-glycans

Table 3 Structure and percent of major glycans of antibody sample analyzed by HPLC/MS method

Peak	Major structure (Table1)	%
1	II.1	21
2	II.2	19
3	II.3	12
4	II.4	21
	Fully galactosylated core structures total sum	29

indicator, LysoSensor™, and examined for relative fluorescence between samples. The LysoSensor™ provides a relative measure of the cellular pH under different culture conditions in which the higher the fluorescence, the more acidic the labeled lysosomes. The LysoSensor™ images taken of samples from 8 day cultures treated with 4 mM GlutaMAX™-I, 4 mM glutamine, and 30 mM ammonia are presented in Fig. 7a. Shown in Fig. 7b are the relative mean cellular fluorescence intensities of the images. Cells with added ammonia had the lowest fluorescence as to be expected as this represents the highest intracellular pH. In contrast, cells treated with GlutaMAX™-I had a higher relative fluorescence and

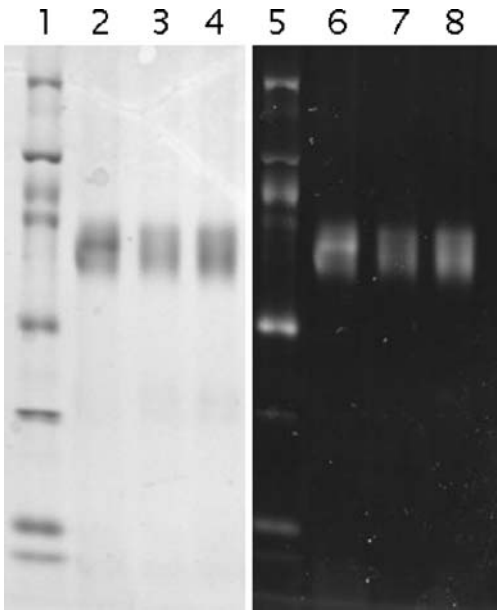


Fig. 5 Coomassie (lanes 1–4) and Pro-Q® Emerald 300 Glycoprotein stain (lanes 5–8) of rt-Pa produced by CHO cells cultured under various conditions. Lanes 1 and 5 CandyCane™ glycoprotein molecular weight standards, Lanes 2 and 6 ammonium treated, Lanes 3 and 7 4 mM GlutaMAX™-I treated, and Lanes 4 and 8 4 mM glutamine treated CHO cultures producing t-PA

thus a lower intracellular pH relative to both glutamine and ammonia supplementation.

Discussion

A new glycan analysis method has been developed which takes advantage of the widely used reverse phase ODS HPLC method for identifying, quantifying, differentiating, and determining structural isomers [13] but utilizes less sample through a narrower column and modified preparation protocol. Furthermore, the approach can be coupled to mass spectrometry as opposed to a second column for structural confirmation. With the reduction in number of required columns, sample analysis is reduced from 20 plus column runs per sample to only one per sample. Sample preparation time was reduced from weeks required by many standard-size chromatography steps down to full preparation in 2 days running samples in parallel. By reducing the column size and number, sample dilution was limited. This is accomplished with traditional HPLC equipment and it is not necessary to acquire additional equipment for this method provided a MALDI-TOF MS instrument is available. The smaller 2-mm Shimadzu column provided highly

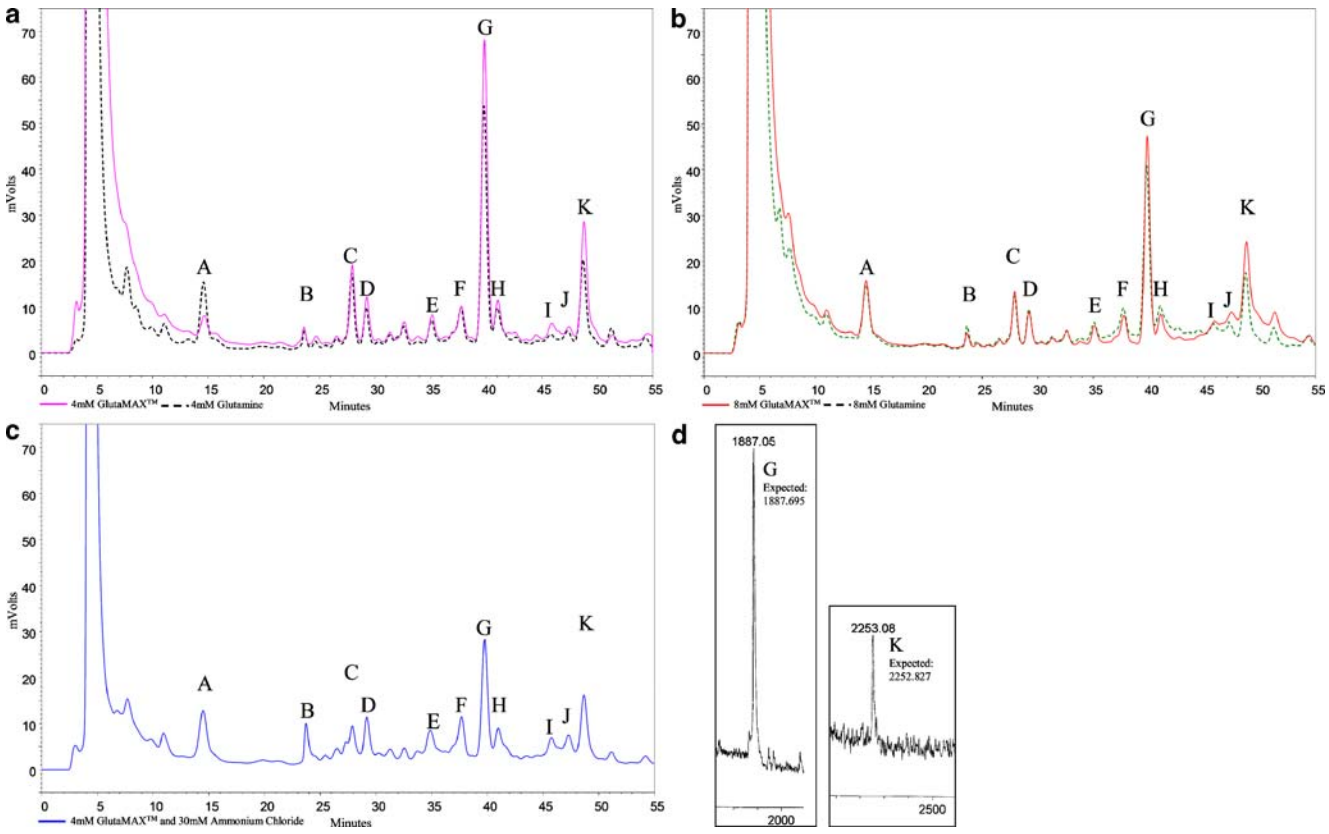
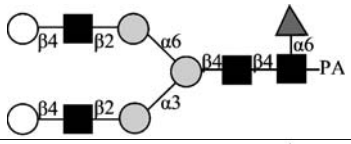
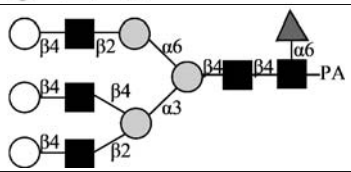


Fig. 6 Normalized comparison of 5 treatment samples. (**a**) 4 mM GlutaMAX™-I versus 4 mM glutamine treatment, (**b**) 8 mM GlutaMAX™-I versus 8 mM glutamine treatment, (**c**) 4 mM GlutaMAX™-I plus 30 mM Ammonium chloride. All samples were normalized together by total area from 7–55 min. Peaks G and K verified by MALDI-TOF MS (**d**)

Table 4 Galactosylated major structures identified by HPLC/MS Method. (■, GlcNAc; ◯, mannose; ◯, galactose; ▲, fucose)

Peak	Major Structure	Percentage of Structures				
		4X	4Q	8X	8Q	A
G		34	27	29	25	16
K		17	11	16	11	10
	Total sum of percentage of structures for fully Galactosylated Di- and Tri-antennary glycans (Includes Peak C)	59	46	53	43	31

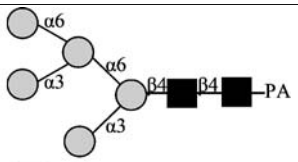
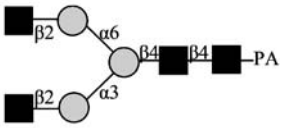
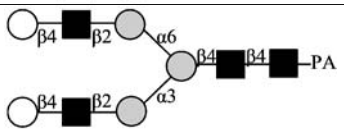
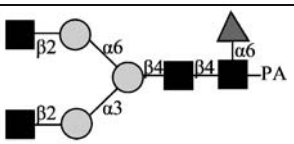
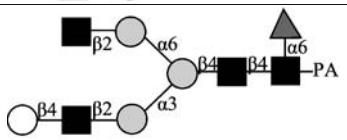
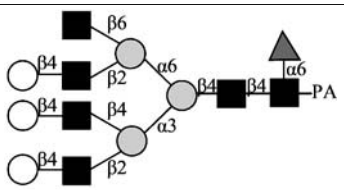
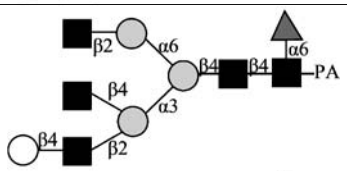
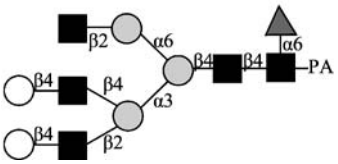
Relative compositions of glycan profile samples of major structures along with specific structural isometric information. The peak area percents allow a measure of relative amounts of glycan structures between profiles rather than using peak height alone

reproducible results compared with the 6-mm column data which is advantageous as it allows utilization of numerous glycan structures available in many literature sources [13, 46]. However, there is room for further improvement of the method by switching traditional HPLC equipment to nano- or capillary HPLC fed to the plate spotter. The literature library could still be used for structure identification after a correlation coefficient is obtained for the nano or capillary column. This could lead to a further reduction of necessary starting sample or, by loading the same amount of sample to the HPLC, an increase in sample available for mass spectrometry detection on the MALDI spots. One of the limitations in the revised method was the ability to obtain sufficient spotted sample for MALDI due to the dilution of the sample flowing through the HPLC system and a flow splitter before the plate spotter. Many of the major peaks in Table 5 would probably be identifiable using the same MALDI MS by doubling the initial sample size of the glycoprotein. This would still be much less than the traditional sample requirement. Alternatively, a more sensitive MALDI-TOF MS can be used in conjunction with sample sizes used here. The method could also be modified to analyze sialylated glycans by verifying the correlation of charged structures from 6-mm to the same 2-mm column and analyzing negatively charged glycans using a different MALDI-TOF MS flight method. In this study, however, sialic acids were removed in order to increase the accuracy and reproducibility of analyzing neutral glycans on both ODS and MALDI-TOF MS. Sialylated glycans could not be easily analyzed without introducing another HPLC column or different mode or matrix for MS. The method represents the most efficient means for comparing core structures upon which sialic acid would be added.

A demonstration of the modified method's efficient resolution is the capacity to differentiate positional isomers of galactosylation on different branches in a human IgG antibody sample. These structures could not easily be separated by many other HPLC systems and also not by using standard one-dimensional mass spectrometry techniques alone. Further, the separation will allow a better understanding of which glycan isomers are preferentially generated from particular cell lines and media conditions. The addition of mass spectrometry to one dimension of HPLC allows for confirmation of peaks from well known HPLC profiles such as IgG, allows for identification of peaks from samples with multiple unknown structures such as t-PA, and is required for elucidating any structures which overlap at the same position on the ODS column.

The practical value of this method was illustrated by examining changes in the glycan profile that occur due to different media additives. In this study, the addition of ammonia to the culture medium of five different samples run from multiple shake flasks in parallel was shown to alter the complexity of glycans formed. Using the modified method, this analysis was performed in 3 days, 10 to 20 times faster than the time to run the same samples on multi-dimensional HPLC, saving weeks of analysis time. Specifically, the method showed that addition of ammonia led to a reduction in galactosylation of di- and tri-antennary structures attached to t-PA. More complex structures are generally preferred to improve desired protein properties such as increased serum half-life. Increased glycan structural complexity can also change biological activity. For example, reduction in galactosylation of an IgG is associated with rheumatoid arthritis [2]. The effect of ammonia on CHO cell physiology was also detected by LysoSensor™, which indicated an increase in the intracellular pH and

Table 5 Other minor structures verified by 2-D HPLC Method. (■, GlcNAc; ◯, mannose; ◯, galactose; ▲, fucose; N/D, not determined)

Peak	Major Structure	Percentage of Structures				
		4X	4Q	8X	8Q	A
A		3	8	9	10	8
B		1	2	1	2	4
C		8	8	8	7	5
D	N/D					
E		3	4	2	4	6
F		5	5	4	5	6
H		7	5	5	7	6
I		4	2	2	3	4
J		2	2	4	3	4

Relative compositions of glycan profile samples of major structures along with specific structural isometric information. The peak area percents allow a measure of relative amounts of glycan structures between profiles rather than using peak height alone

suggested possible causes for the results found by the analysis method. Interestingly, the inclusion of glutamine as opposed to GlutaMAX^{TM}-I also indicated a general increase in the intracellular pH as shown by decreased average cellular fluorescent intensities of LysoSensor^{TM} in the glutamine-supplemented cultures.

Glutamine is known to degrade more rapidly in the medium and lead to a rise in culture pH while GlutaMAX^{TM}-I degrades more slowly. Furthermore, as shown by the modified analysis method, the GlutaMAX^{TM}-I cultures also exhibited a relatively higher level of galactosylation as compared to the glutamine cultures, which in turn contained more fully galactosylated structures than those of the ammonia supplemented cultures. Thus, the inclusion of GlutaMAX^{TM}-I may represent the preferred medium alternative to glutamine in order to generate higher levels of complex galactosylated

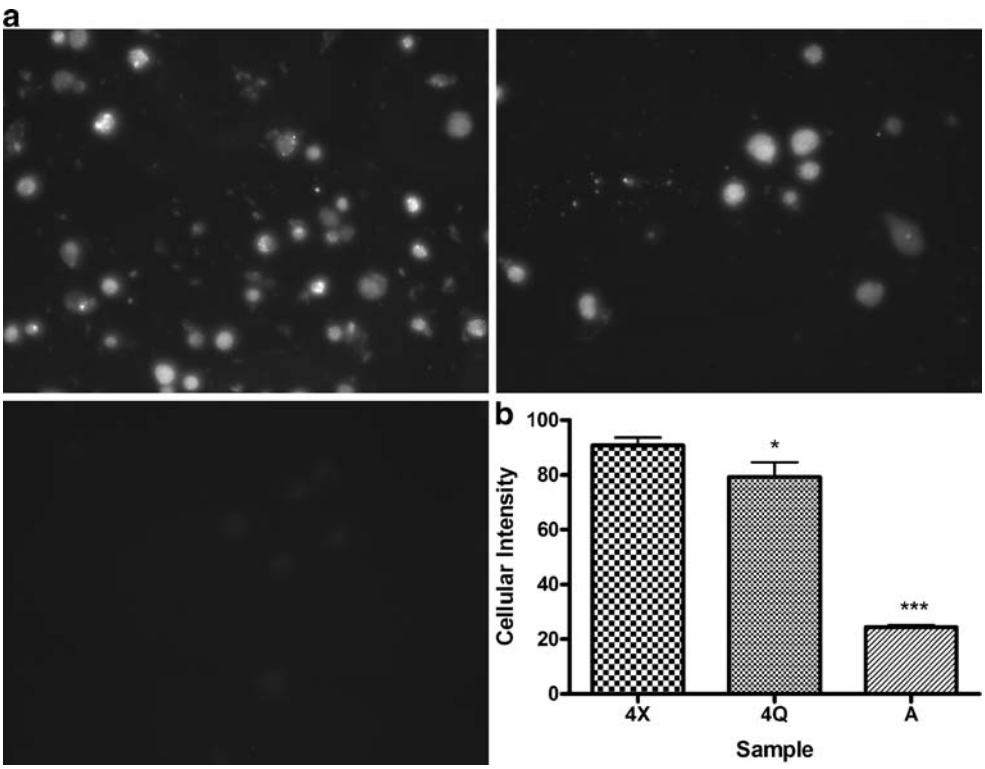


Fig. 7 **a** Lysosomal staining showing the effect of the media additives on acidity of intracellular organelles, typically lysosomes. **b** Mean cellular intensity of fluorescence is plotted with SEM between cells. $p<0.05$ (vs 4Q) and $p<0.0001$ (vs A)

structures. This improvement in complexity may be due in part to the lower physiological pH obtained in the cultures supplemented with the dipeptide. GlutaMAX^{TM}-I prevents spontaneous degradation of glutamine in the medium producing ammonia by remaining in dipeptide form until the cell releases peptidases when it requires a glutamine source [36].

Increasing the glutamine concentration above a normal culture concentration or direct addition of ammonia has been shown previously to reduce the structural complexity of the glycans [21, 26–28, 53] and was demonstrated with this analysis technique. There are multiple possible reasons for the change in glycan structures in cultures with higher ammonia levels and intracellular pH. These include alteration of *trans* Golgi pH affecting glycosyltransferase activities, mistargeting of lysosomal enzymes, and redistribution of glycosyltransferases as they are recycled through the Golgi. One possible reason is due to the effect of pH changes on the galactosyltransferase (GalT) activity in the *trans*-Golgi network (TGN). The pH optimum of GalT occurs at 6.5 and drops at higher pH levels [21]. The TGN, the most acidic portion of the Golgi apparatus, has a normal acidic pH of about 6.0–6.5 [54] but the pH can rise above 7.0 in the presence of ammonia. Gawlitzek *et al.* [21] proposed this change in glycosyltransferase activity under increasing TGN pH as the major contributing mechanism for reduced galactosylation of IgG in CHO with increased ammonia.

A second possible explanation involves redistribution of lysosomal enzymes as a result of the pH change. The cell produces lysosomal hydrolases, including glycosidases, which are targeted to the lysosome by a pH sensitive receptor in the endosome. However, if the pH is altered such that the Golgi pH is increased, the receptor may not be properly transported to the lysosome [31]. These glycosidases can accumulate in secretory compartments or traffic to the medium rather than their lysosomal target [55]. In these locations, the glycosidases can act on secreted protein and reduce the level of complex galactosylation.

A third possible contributing factor involves improper localization of GalT and other enzymes. Like hydrolases destined for lysosomes, the localization of glycosyltransferases in the TGN is affected by pH as well. Increasing the pH of the Golgi apparatus can alter the physical infrastructure. Vesicles can form on the Golgi and decrease the likelihood of a secreted protein seeing the glycosylational enzymes [56]. The GalT can accumulate in the swollen vesicles which prevents it from acting on secreted proteins [57]. The improper pH can also inhibit proper recycling of Golgi enzymes from endosomes back to the Golgi [58].

The modified method described in this study will serve to reduce sample size and lower the time needed to compare different N-glycans subjected to various culture conditions. Such an approach will be extremely valuable for comparing a choice of media formulations and understanding the role different additives and the culture environment can play. As the number of commercial and research glycoproteins produced increases, these higher

throughput methods will allow users to optimize the cell culture environment in order to obtain a more desirable N-glycan profile. The application of stable dipeptides offers one such strategy to reduce ammonia and retain a lower intracellular pH while generating more complex N-glycans with higher galactosylation levels.

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References

1. Butler, M.: Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl. Microbiol. Biotechnol. **68**, 283–291 (2005). doi:10.1007/s00253-005-1980-8
2. Nimmerjahn, F., Anthony, R.M., Ravetch, J.V.: Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. Proc. Natl. Acad. Sci. USA **104**, 8433–8437 (2007). doi:10.1073/pnas.0702936104
3. Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E.G., Warren, G., Nilsson, T.: Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. J. Cell Sci. **108**(Pt 4), 1617–1627 (1995)
4. Umaña, P., Bailey, J.E.: A mathematical model of N-linked glycoform biosynthesis. Biotechnol. Bioeng. **55**, 890–908 (1997). doi:10.1002/(SICI)1097-0290(19970920)55:6<890::AID-BIT7>3.0.CO;2-B
5. Krambeck, F.J., Betenbaugh, M.J.: A mathematical model of N-linked glycosylation. Biotechnol. Bioeng. **92**, 711–728 (2005). doi:10.1002/bit.20645
6. Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B., Rasmussen, J.R.: The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. Biotechnology (N. Y.) **9**, 1347–1355 (1991). doi:10.1038/nbt1291-1347
7. Wright, A., Morrison, S.L.: Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol. **15**, 26–32 (1997). doi:10.1016/S0167-7799(96)10062-7
8. Jenkins, N., Parekh, R.B., James, D.C.: Getting the glycosylation right: implications for the biotechnology industry. Nat. Biotechnol. **14**, 975–981 (1996). doi:10.1038/nbt0896-975
9. Betenbaugh, M.J., Tomiya, N., Narang, S., Hsu, J.T., Lee, Y.C.: Biosynthesis of human-type N-glycans in heterologous systems. Curr. Opin. Struct. Biol. **14**, 601–606 (2004). doi:10.1016/j.sbi.2004.09.001
10. Stubiger, G., Marchetti, M., Nagano, M., Reichel, C., Gmeiner, G., Allmaier, G.: Characterisation of intact recombinant human erythropoietins applied in doping by means of planar gel electrophoretic techniques and matrix-assisted laser desorption/ionisation linear time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. **19**, 728–742 (2005). doi:10.1002/rcm.1830
11. Choi, O., Tomiya, N., Kim, J.H., Slavicek, J.M., Betenbaugh, M.J., Lee, Y.C.: N-glycan structures of human transferrin produced by Lymantria dispar (gypsy moth) cells using the LdMNPV expression system. Glycobiology **13**, 539–548 (2003). doi:10.1093/glycob/cwg071
12. Maslen, S., Sadowski, P., Adam, A., Lilley, K., Stephens, E.: Differentiation of isomeric N-glycan structures by normal-phase liquid chromatography-MALDI-TOF/TOF tandem mass spectrometry. Anal. Chem. **78**, 8491–8498 (2006). doi:10.1021/ac0614137
13. Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., Takahashi, N.: Analyses of N-linked oligosaccharides using a two-dimensional mapping technique. Anal. Biochem. **171**, 73–90 (1988). doi:10.1016/0003-2697(88)90126-1
14. Suzuki, Y., Miyazaki, M., Ito, E., Suzuki, M., Yamashita, T., Taira, H., Suzuki, A.: Structural characterization of N-glycans of cauxin by MALDI-TOF mass spectrometry and nano LC-ESI-mass spectrometry. Biosci. Biotechnol. Biochem. **71**, 811–816 (2007). doi:10.1271/bbb.60599
15. Maslen, S.L., Goubet, F., Adam, A., Dupree, P., Stephens, E.: Structure elucidation of arabinoxylan isomers by normal phase HPLC-MALDI-TOF/TOF-MS/MS. Carbohydr. Res. **342**, 724–735 (2007). doi:10.1016/j.carres.2006.12.007
16. Stadlmann, J., Pabst, M., Kolarich, D., Kunert, R., Altmann, F.: Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. Proteomics **8**, 2858–2871 (2008). doi:10.1002/pmic.200700968
17. Kirsch, S., Zarei, M., Cindric, M., Muthing, J., Bindila, L., Peter-Katalinic, J.: On-line nano-HPLC/ESI QTOF MS and tandem MS for separation, detection, and structural elucidation of human erythrocytes neutral glycosphingolipid mixture. Anal. Chem. **80**, 4711–4722 (2008). doi:10.1021/ac702175f
18. Zarei, M., Kirsch, S., Muthing, J., Bindila, L., Peter-Katalinic, J.: Automated normal phase nano high performance liquid chromatography/matrix assisted laser desorption/ionization mass spectrometry for analysis of neutral and acidic glycosphingolipids. Anal. Bioanal. Chem. **391**, 289–297 (2008). doi:10.1007/s00216-008-1932-0
19. Gawlitzek, M., Valley, U., Nimtz, M., Wagner, R., Conradt, H.S.: Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. J. Biotechnol. **42**, 117–131 (1995). doi:10.1016/0168-1656(95)00065-X
20. Hills, A.E., Patel, A., Boyd, P., James, D.C.: Metabolic control of recombinant monoclonal antibody N-glycosylation in GS-NS0 cells. Biotechnol. Bioeng. **75**, 239–251 (2001). doi:10.1002/bit.10022
21. Gawlitzek, M., Ryll, T., Lofgren, J., Sliwkowski, M.B.: Ammonium alters N-glycan structures of recombinant TNFR-IgG: degradative versus biosynthetic mechanisms. Biotechnol. Bioeng. **68**, 637–646 (2000). doi:10.1002/(SICI)1097-0290(20000620)68:6<637::AID-BIT6>3.0.CO;2-C
22. Andersen, D.C., Bridges, T., Gawlitzek, M., Hoy, C.: Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol. Bioeng. **70**, 25–31 (2000). doi:10.1002/1097-0290(20001005)70:1<25::AID-BIT4>3.0.CO;2-Q
23. Valley, U., Nimtz, M., Conradt, H.S., Wagner, R.: Incorporation of ammonium into intracellular UDP-activated N-acetylhexosamines and into carbohydrate structures in glycoproteins. Biotechnol. Bioeng. **64**, 401–417 (1999). doi:10.1002/(SICI)1097-0290(19990820)64:4<401::AID-BIT3>3.0.CO;2-M
24. Gawlitzek, M., Papac, D.I., Sliwkowski, M.B., Ryll, T.: Incorporation of 15 N from ammonium into the N-linked oligosaccharides of an immunoadhesin glycoprotein expressed in Chinese hamster ovary cells. Glycobiology **9**, 125–131 (1999). doi:10.1093/glycob/9.2.125
25. Ryll, T., Valley, U., Wagner, R.: Biochemistry of growth inhibition by ammonium ions in mammalian cells. Biotechnol. Bioeng. **44**, 184–193 (1994). doi:10.1002/bit.260440207
26. Yang, M., Butler, M.: Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. Biotechnol. Prog. **18**, 129–138 (2002). doi:10.1021/bp0101334

27. Yang, M., Butler, M.: Effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotechnol. Prog. **16**, 751–759 (2000). doi:10.1021/bp000090b
28. Yang, M., Butler, M.: Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol. Bioeng. **68**, 370–380 (2000). doi:10.1002/(SICI)1097-0290(20000520)68:4<370::AID-BIT2>3.0.CO;2-K
29. Grammatikos, S.I., Valley, U., Nimtz, M., Conradt, H.S., Wagner, R.: Intracellular UDP-N-acetylhexosamine pool affects N-glycan complexity: a mechanism of ammonium action on protein glycosylation. Biotechnol. Prog. **14**, 410–419 (1998). doi:10.1021/bp980005o
30. Elices, M.J., Goldstein, I.J.: Ehrlich ascites tumor cell UDP-Gal: N-acetyl-D-glucosamine beta(1,4)-galactosyltransferase. Purification, characterization, and topography of the acceptor-binding site. J. Biol. Chem. **263**, 3354–3362 (1988)
31. Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., Sly, W.S.: Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol. **85**, 839–852 (1980). doi:10.1083/jcb.85.3.839
32. Chee Furng Wong, D., Tin Kam Wong, K., Tang Goh, L., Kiat Heng, C., Gek Sim Yap, M.: Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol. Bioeng. **89**, 164–177 (2005). doi:10.1002/bit.20317
33. Muthing, J., Kemminer, S.E., Conradt, H.S., Sagi, D., Nimtz, M., Karst, U., Peter-Katalinic, J.: Effects of buffering conditions and culture pH on production rates and glycosylation of clinical phase I anti-melanoma mouse IgG3 monoclonal antibody R24. Biotechnol. Bioeng. **83**, 321–334 (2003). doi:10.1002/bit.10673
34. Butler, M., Christie, A.: Adaptation of mammalian cells to non-ammoniagenic media. Cytotechnology **15**, 87–94 (1994). doi:10.1007/BF00762382
35. Christie, A., Butler, M.: The adaptation of BHK cells to a non-ammoniagenic glutamate-based culture medium. Biotechnol. Bioeng. **64**, 298–309 (1999). doi:10.1002/(SICI)1097-0290(19990805)64:3<298::AID-BIT6>3.0.CO;2-U
36. Christie, A., Butler, M.: Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase. J. Biotechnol. **37**, 277–290 (1994). doi:10.1016/0168-1656(94)90134-1
37. Xu, Y., Cacia, J.: A reversed-phase HPLC assay for plasminogen activators. J. Liquid Chromatogr. Relat. Technol. **23**, 1841 (2000). doi:10.1081/JLC-100100455
38. Packer, N.H., Lawson, M.A., Jardine, D.R., Redmond, J.W.: A general approach to desalting oligosaccharides released from glycoproteins. Glycoconj. J. **15**, 737–747 (1998). doi:10.1023/A:1006983125913
39. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., Ikenaka, T.: Improved method for fluorescence labeling of sugar chains with sialic acid residues. Agric. Biol. Chem. **54**, 2169–2170 (1990)
40. Fournier, I., Marinach, C., Tabet, J.C., Bolbach, G.: Irradiation effects in MALDI, ablation, ion production, and surface modifications. Part II. 2,5-dihydroxybenzoic acid monocrystals. J. Am. Soc. Mass Spectrom. **14**, 893–899 (2003). doi:10.1016/S1044-0305(03)00347-7
41. Zhu, X., Papayannopoulos, I.A.: Matrix with noise reduction additive and disposable target containing the same. Applied biosystems, Framingham (2005)
42. Mohr, M.D., Bornsen, K.O., Widmer, H.M.: Matrix-assisted laser desorption/ionization mass spectrometry: improved matrix for oligosaccharides. Rapid Commun. Mass Spectrom. **9**, 809–814 (1995). doi:10.1002/rcm.1290090919
43. Lee, Y.C., Lee, B.I., Tomiya, N., Takahashi, N.: Parameterization of contribution of sugar units to elution volumes in reverse-phase HPLC of 2-pyridylaminated oligosaccharides. Anal. Biochem. **188**, 259–266 (1990). doi:10.1016/0003-2697(90)90603-7
44. Tomiya, N., Lee, Y.C., Yoshida, T., Wada, Y., Awaya, J., Kurono, M., Takahashi, N.: Calculated two-dimensional sugar map of pyridylaminated oligosaccharides: elucidation of the jack bean alpha-mannosidase digestion pathway of Man9GlcNAc2. Anal. Biochem. **193**, 90–100 (1991). doi:10.1016/0003-2697(91)90047-W
45. Takahashi, N., Nakagawa, H., Fujikawa, K., Kawamura, Y., Tomiya, N.: Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. Anal. Biochem. **226**, 139–146 (1995). doi:10.1006/abio.1995.1201
46. Tomiya, N., Takahashi, N.: Contribution of component monosaccharides to the coordinates of neutral and sialyl pyridylaminated N-glycans on a two-dimensional sugar map. Anal. Biochem. **264**, 204–210 (1998). doi:10.1006/abio.1998.2849
47. Suzuki, N., Lee, Y.C.: Site-specific N-glycosylation of chicken serum IgG. Glycobiology **14**, 275–292 (2004). doi:10.1093/glycob/cwh031
48. Townsend, R.R., Hardy, M.R., Wong, T.C., Lee, Y.C.: Binding of N-linked bovine fetuin glycopeptides to isolated rabbit hepatocytes: Gal/GalNAc hepatic lectin discrimination between Gal beta (1,4)GlcNAc and Gal beta(1,3)GlcNAc in a triantennary structure. Biochemistry **25**, 5716–5725 (1986). doi:10.1021/bi00367a055
49. Nakano, M., Kakehi, K., Tsai, M.H., Lee, Y.C.: Detailed structural features of glycan chains derived from alpha1-acid glycoproteins of several different animals: the presence of hypersialylated, O-acetylated sialic acids but not disialyl residues. Glycobiology **14**, 431–441 (2004). doi:10.1093/glycob/cwh034
50. Pfeiffer, G., Schmidt, M., Strube, K.H., Geyer, R.: Carbohydrate structure of recombinant human uterine tissue plasminogen activator expressed in mouse epithelial cells. Eur. J. Biochem. **186**, 273–286 (1989). doi:10.1111/j.1432-1033.1989.tb15206.x
51. Nimtz, M., Noll, G., Paques, E.P., Conradt, H.S.: Carbohydrate structures of a human tissue plasminogen activator variant expressed in recombinant Chinese hamster ovary cells. FEBS Lett. **271**, 14–18 (1990). doi:10.1016/0014-5793(90)80361-L
52. Spellman, M.W., Basa, L.J., Leonard, C.K., Chakel, J.A., O'Connor, J.V., Wilson, S., van Halbeek, H.: Carbohydrate structures of human tissue plasminogen activator expressed in Chinese hamster ovary cells. J. Biol. Chem. **264**, 14100–14111 (1989)
53. Zanghi, J.A., Mendoza, T.P., Knop, R.H., Miller, W.M.: Ammonia inhibits neural cell adhesion molecule polysialylation in Chinese hamster ovary and small cell lung cancer cells. J. Cell. Physiol. **177**, 248–263 (1998). doi:10.1002/(SICI)1097-4652(199811)177:2<248::AID-JCP7>3.0.CO;2-N
54. Demaurex, N., Furuya, W., D'Souza, S., Bonifacino, J.S., Grinstein, S.: Mechanism of acidification of the trans-Golgi network (TGN). In situ measurements of pH using retrieval of TGN38 and furin from the cell surface. J. Biol. Chem. **273**, 2044–2051 (1998). doi:10.1074/jbc.273.4.2044
55. Robbins, A.R., Peng, S.S., Marshall, J.L.: Mutant Chinese hamster ovary cells pleiotropically defective in receptor-mediated endocytosis. J. Cell Biol. **96**, 1064–1071 (1983). doi:10.1083/jcb.96.4.1064
56. Geuze, H.J., Slot, J.W., Strous, G.J., Hasilik, A., von Figura, K.: Possible pathways for lysosomal enzyme delivery. J. Cell Biol. **101**, 2253–2262 (1985). doi:10.1083/jcb.101.6.2253
57. Schaub, B.E., Berger, B., Berger, E.G., Rohrer, J.: Transition of galactosyltransferase 1 from trans-Golgi cisterna to the trans-Golgi network is signal mediated. Mol. Biol. Cell **17**, 5153–5162 (2006). doi:10.1091/mbc.E06-08-0665
58. Starr, T., Forsten-Williams, K., Storrie, B.: Both post-Golgi and intra-Golgi cycling affect the distribution of the Golgi phosphoprotein GPP130. Traffic **8**, 1265–1279 (2007). doi:10.1111/j.1600-0854.2007.00607.x