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A comparison of three techniques for quantitative carbohydrate analysis used in characterization of therapeutic antibodies

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Abstract—A comparison of three techniques for quantitative analysis of galactosylation present on immunoglobulins is described. ESIMS, MALDI-TOF MS, and anion-exchange chromatography with fluorescence detection were evaluated in terms of repeatability, limit of quantitation, selectivity, and linearity. A recombinant monoclonal IgG was enzymatically modified in vitro to produce essentially completely galactosylated and degalactosylated forms of the immunoglobulin. Samples of known galactosylation levels were prepared by mixing the modified forms with the native form of the immunoglobulin. Good repeatability and linearity were demonstrated for all three assays (RSDs <1.0%, correlation coefficients >0.99). Differences in selectivity, sensitivity, and other performance aspects of the three techniques are also discussed in this paper.

Keywords: 2-AA/ESIMS; Galactosylation; Immunoglobulin; MALDI-TOF MS

1. Introduction

Immunoglobulins class G (IgG) are proteins of approximately 150,000 Da molecular mass with a single asparagine-linked (N-linked) glycosylation site present on each of the heavy chains of the IgG. The glycosylation site is contained in the C_H2 domain and is typically occupied with complex-type asialobiantennary carbohydrate structures, although there has been shown some variation according to species.[1] The carbohydrate structures of immunoglobulins are known to influence effector function[2–4] and thermodynamic stability.[5] The principal structures found on an IgG produced recombinantly in Chinese hamster ovary (CHO) cells are shown in Figure 1. Carbohydrate structures found on immunoglobulins have been analyzed using a variety of techniques such as capillary electrophoresis,[6–8] chromatography,[9,10] ESIMS,[11,12] and MALDI-TOF MS.[13–15] The objective of this publication is to compare three techniques used for carbohydrate analysis in our laboratory: ESIMS of intact immunoglobulins, anion-exchange chromatography with fluorescence detection, and MALDI-TOF MS of released carbohydrates. The three techniques are evaluated for precision, linearity, and accuracy. The first approach, ESIMS analysis of intact immunoglobulins is rapid, simple, automated, and requires minimal sample preparation. Reliable and reproducible data can be obtained quickly, which is invaluable for in-process testing where short turnaround times are essential. In another approach, MALDI-TOF of enzymatically released carbohydrates, 2-aminobenzoic acid (2-AA) is utilized as a matrix that has shown to be quite advantageous for neutral carbohydrate detection and relative quantitation. Finally, fluorescence labeling of released carbohydrates using 2-aminobenzoic acid coupled with anion-exchange chromatography (2-AA AEC) is demonstrated to provide good selectivity and relative quantitation for neutral

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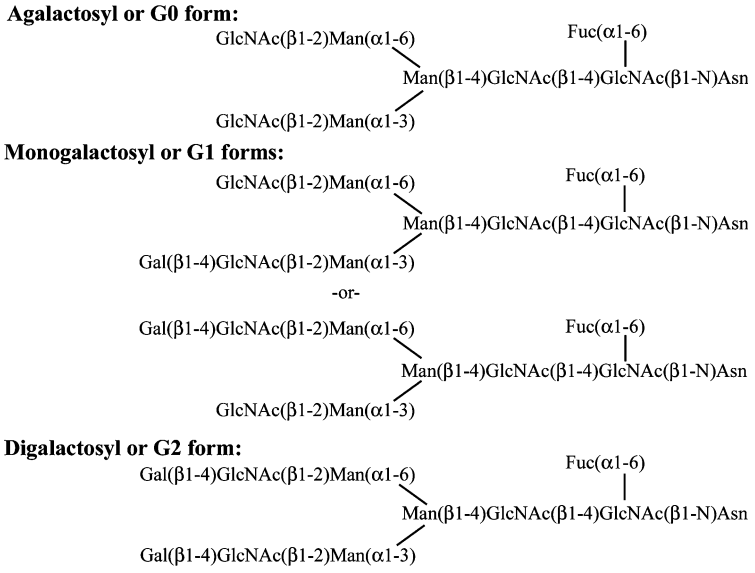

Figure 1. Structures of the typical asialobiantennary carbohydrates found on IgGs as expressed in CHO cells. The two structural isomers for the monogalactosyl (G1) carbohydrates are shown, although these structures are not resolved by the techniques studied.

and charged carbohydrate structures. In order to compare the data obtained by the three techniques, percent galactosylation is calculated based on peak areas as shown below:

For released carbohydrates

$$\text{Percent galactosylation} = \frac{2 \times \text{G2 peak} + \text{G1 peak}}{2 \times (\text{G2 peak} + \text{G1 peak} + \text{G0 peak})} \times 100 \quad (1)$$

For mass spectrometric analysis of intact immunoglobulins

$$\text{Percent galactosylation} = \frac{4 \times \text{Gal}_4 \text{ peak} + 3 \times \text{Gal}_3 \text{ peak} + 2 \times \text{Gal}_2 \text{ peak} + 1 \times \text{Gal}_1 \text{ peak}}{4 \times (\text{Gal}_4 \text{ peak} + \text{Gal}_3 \text{ peak} + \text{Gal}_2 \text{ peak} + \text{Gal}_1 \text{ peak} + \text{Gal}_0 \text{ peak})} \times 100 \quad (2)$$

Carbohydrate peaks are named as presented in Figure 1, and immunoglobulin glycoform peaks detected by ESIMS are named as presented in Figure 2. For clarity, the carbohydrates are referred to as G2, G1, and G0, while the immunoglobulin glycoforms are named Gal_4 through Gal_0.

A representative immunoglobulin molecule chosen for this study contains typical carbohydrate structures and glycoform distribution compared to a number of other IgGs studied in our laboratory. The resolution capabilities of the techniques studied were also considered. For example, the antibody chosen has low levels of afucosyl carbohydrate structures, which are apparent in MALDI-TOF analysis of the released carbohydrates, but are not sufficiently resolved in either ESIMS of the intact immunoglobulin or 2-AA AEC of the released glycans to allow quantitation of these structures. In order to present a consistent comparison of the three techniques, only responses attributed to the three principal carbohydrate structures were used for galactosylation calculations.

2. Results and discussion

2.1. Sample preparation and MS analysis

To compare the three analytical approaches, a set of samples with different percent galactosylations was prepared. A sample with 34% galactosylation (as determined by 2-AA AEC) was enzymatically modified to produce essentially completely galactosylated or degalactosylated immunoglobulins. Considering the possible effect of glycan hindrance within the immunoglobulin Fc loop, both reactions were performed with significant enzyme excess and allowed to proceed overnight. Completeness of both reactions was assessed by ESIMS of intact and reduced antibody and 2-AA AEC of released glycans. Although low levels of incompletely modified material were detected in 2-AA AEC, no masses corresponding to galactosylated and undergalactosylated species in the respective samples were observed by ESIMS. The modified samples were considered essentially completely agalactosylated or galactosylated and were designated as 0% and 100% galactosylated. Samples with different levels of immunoglobulin galactosylation were created by mixing the native (34% galactosylated) and

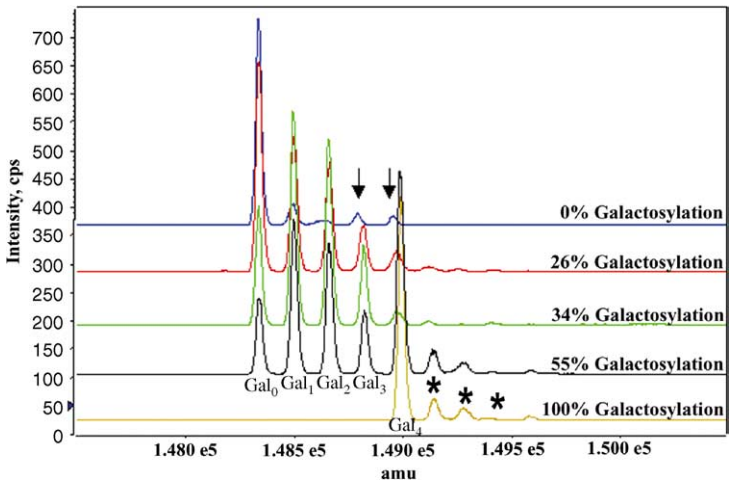

Figure 2. Representative deconvoluted mass spectra of intact immunoglobulin samples used in method linearity study. Samples with different degrees of galactosylation were desalted via a protein desalting cartridge and their masses determined by the ESI-Q-TOF mass spectrometer Qstar. Analyses were performed in 2000–4000 *m*/*z* range in the positive-ion mode. Deconvoluted spectra were obtained using BioAnalyst 1.1 software. The arrows in the top spectrum indicate peaks corresponding to monosialylated species. The asterisks in the bottom spectrum indicate glycated immunoglobulins and/or immunoglobulins with sialylated carbohydrates.

Table 1. Table of nomenclature used for immunoglobulin glycoforms and their description

Immunoglobulin peak name	Carbohydrates present on immunoglobulin[a]
Gal_0	G0/G0
Gal_1	G1/G0
Gal_2	G2/G0 or G1/G1
Gal_3	G2/G1
Gal_4	G2/G2

[a] Glycoform designations separated by '/' represent glycoforms attached to either heavy chain of the immunoglobulin molecule.

enzymatically modified (0% and 100% galactosylated) samples and used for the comparative analyses. Carbohydrate structures are named as presented in Figure 1. Table 1 defines the immunoglobulin glycoforms labeled Gal_0 through Gal_4 in Figure 2.

2.2. ESIMS analysis of intact immunoglobulins

Mixtures of IgG with different galactosylation levels have been analyzed by ESI-Q-TOF mass spectrometry. Representative deconvoluted ESI mass spectra are shown in Figure 2. The top trace represents a spectrum of an essentially completely degalactosylated sample. The peaks labeled with arrows correspond to immunoglobulins containing monosialylated complex-type or hybrid carbohydrates, which are also observed by both MALDI-TOF MS and 2-AA AEC (Figs. 3 and 4, respectively). The bottom trace in Figure 2 represents completely galactosylated species. The peaks labeled with asterisks are glycated (the nonenzymatic reaction of reducing sugars with lysine, arginine or the amino terminus of a protein or peptide) and sialylated species. Low levels of glycosylation were confirmed by ESI-Q-TOF MS analysis of deglycosylated as well as reduced and deglycosylated IgG. Low levels of sialylation were confirmed by sialidase treatment and ESIMS of desialylated species (data not shown). Both glycosylation and sialylation contribute to slightly elevated percent galactosylation calculated from ESIMS data and could be corrected for if percent glycosylation and sialylation were taken into account. In the example described, the contribution of glycosylation and sialylation was considered negligible for simplicity. This approach would be appropriate if glycosylation and/or sialylation contribution is small and constant. If a more accurate assessment is needed, the enzymatic deglycosylation and desialylation must be performed separately, the enzymatically treated samples analyzed by ESIMS, and the percent glycosylation and sialylation determined and accounted for in calculations of galactosylation.

2.3. MALDI-TOF MS analysis of enzymatically released carbohydrates

Enzymatically released carbohydrates from the series of IgG samples with different levels of galactosylation were analyzed using MALDI-TOF mass spectrometry. Representative MALDI-TOF mass spectra are presented in Figure 3. Monosaccharide composition and average masses for the various carbohydrates present on an IgG are listed in Table 2. Low-level signals observed in the MALDI-TOF mass spectra may be attributed to afucosyl complex-type carbohydrates and afucosyl complex-type carbohydrates with an additional *N*-acetylglucosamine residue (either branched or bisecting). These structures are neglected in the galactosylation calculation for consistency of comparison with ESIMS and 2-AA AEC.

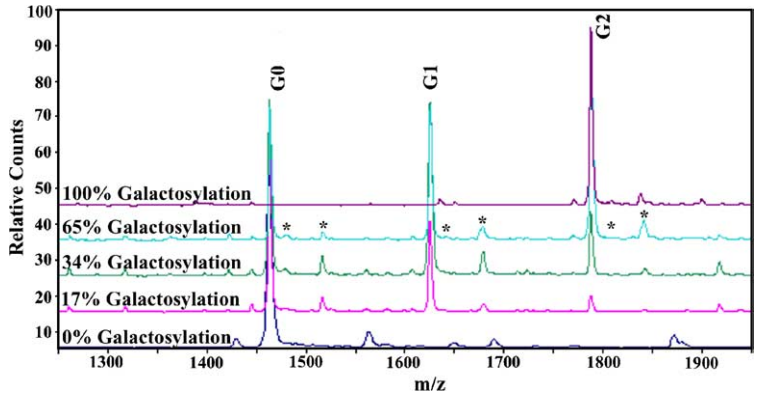

Figure 3. MALDI-TOF mass spectra of PNGase F released carbohydrate samples. The carbohydrate samples were analyzed using 2-AA as a matrix on an ABI Voyager DE-STR operated in linear mode with negative-ion detection as described in the Experimental. Primary peaks of interest have been labeled; lower level peaks (marked with asterisks) can be attributed to afucosyl glycoforms with either branched or bisecting *N*-acetylglucosamine.

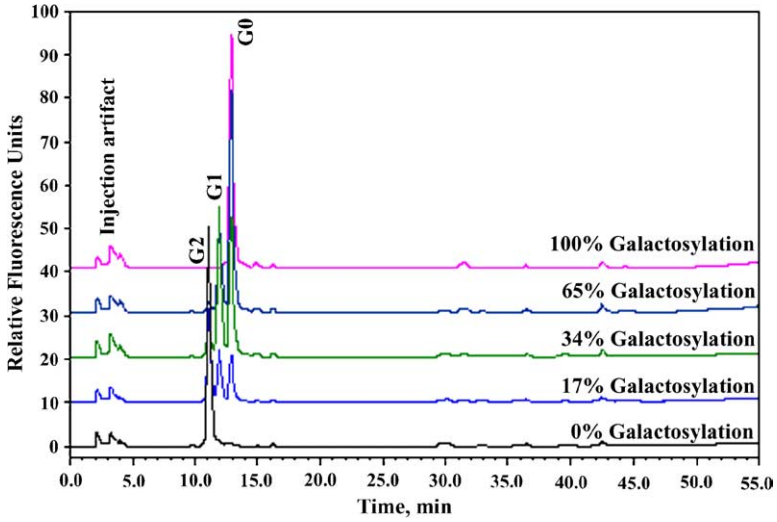

Figure 4. Anion-exchange separation of 2-AA labeled, PNGase F released carbohydrate samples. The labeled carbohydrates were separated on an Asahi Pak NH2P carbohydrate column using a linear gradient of ammonium acetate at 0.5 mL/min, as described in the Experimental. Column effluent was monitored by fluorometry with an excitation wavelength of 330 nm and an emission wavelength of 420 nm. The peaks at 3–4 min denoted as 'injection artifact' are due to residual labeling reagents and are also present in labeled blanks. The group of small peaks eluting at approximately 30–43 min represents sialylated species.

Table 2. Table of nomenclature used for predominant neutral biantennary carbohydrates present on immunoglobulins

Carbohydrate	Composition	Average molecular mass/Da
G2	$Man_3GlcNAc_4Gal_2Fuc$	1787.7
G1	$Man_3GlcNAc_4GalFuc$	1625.5
G0	$Man_3GlcNAc_4Fuc$	1463.4

2.4. 2-AA Chromatographic data

Enzymatically released carbohydrates from the series of IgG samples with different levels of galactosylation were labeled with 2-aminobenzoic acid and analyzed by anion-exchange chromatography with fluorescence detection. Representative chromatograms are shown in Figure 4. Low levels of neutral carbohydrates other than the principal structures found on IgGs have been identified as high-mannose carbohydrates by mannosidase treatment (data not shown). Also evident in the chromatography are monosialyl (retention time approximately 30 min) and disialyl carbohydrate structures (retention time approximately 42 min). These structures are also observed by ESIMS, as described above.

2.5. Comparison of the three methods

Comparison of assay system properties (repeatability, linearity, etc.) for the three methods are presented in Tables 3 and 4 and in Figure 5. The precision data (Table 3) was determined for the glycoform distributions

Table 3. Precision statistics for the three methods: standard deviations for determination of the percent galactosylation of the unmodified immunoglobulin

Technique	Galactosylation repeatability ($n = 6$)/%	Galactosylation interassay precision ($n = 3$)/%
Intact mass analysis	0.9	0.3
MALDI-TOF MS	1.1	0.8
2-AA AEC	0.1	0.3

Table 4. Theoretical and experimental values of galactosylation with linear fit statistics for the three methods studied

Theoretical galactosylation	ESIMS	MALDI-TOF MS	2-AA AEC
0%	3.5%	1.2%	1.3%
17%	ND	20.8%	19.3%
26%	27.5%	ND[a]	ND[a]
34%	36.1%	34.1%	34.8%
55%	58.6%	ND[a]	ND[a]
65%	ND[a]	56.7%	61.0%
100%	100.0%	96.2%	96.0%
Least-squares, linear fit statistics			
Slope	0.97	0.91	0.93
y-Intercept	3.5%	2.4%	2.2%
Correlation coefficient (r^2)	0.999	0.992	0.999

[a] ND: not determined.

obtained from the unmodified IgG. All three techniques demonstrate comparable levels of repeatability and interassay precision. Good linearity (Table 4) was also demonstrated for the three techniques. There are differences in the slope and intercept of the least-squares linear fit for the ESIMS of intact IgG method as compared to the techniques analyzing released carbohydrates.

While analysis of enzymatically released carbohydrates by MALDI-TOF MS or 2-AA AEC allowed detection of low levels of incompletely galactosylated material, analysis by ESIMS of intact IgG did not detect any incompletely galactosylated species. This inability to detect low levels of incomplete galactosylation by ESIMS is due to the contribution of sialylated carbohydrates that are not resolved from Gal_3 and Gal_4 glycoforms as well as contribution of low levels of glycosylation. The lack of resolution for isobaric species (i.e., those of equal mass), as well as for species that are close in mass, is a disadvantage of the ESIMS method; percent galactosylation calculated from ESIMS experiments is slightly higher than for the other methods due to the contribution of unresolved by mass species. As has been noted earlier, contribution of glycosylation and sialylation can be accounted for if necessary; however, a number of additional experiments would be required. It must be noted that discrepancy in galactosylation values determined by the three techniques at the extreme of galactosylation may not be critical, as these high levels are virtually never attained (in our experience) during recombinant IgG production.

The three methods for quantitation of immunoglobulin glycosylation described in this paper vary in their fundamental approaches—and hence the degree of sample preparation required, and the type of data produced. The relative strengths and weaknesses of the techniques are compared in Table 5. While ESIMS analysis suffers from the lack of resolution of isobaric species, the resolution of the 2-AA AEC method does not sufficiently resolve high-mannose structures from the agalactosyl forms, which may result in overestimation of agalactosyl forms. MALDI-TOF MS results obtained for the extreme galactosylation sample showed measurable signal for low levels of undergalactosylated material; however, the detected levels were below the lower limit of quantitation for the technique.

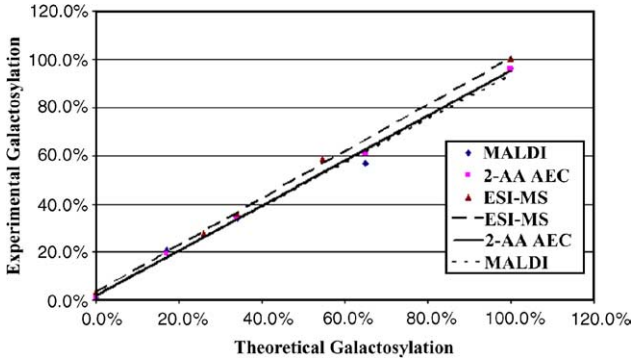

Figure 5. Graphical comparison of linearity for test mixtures. Each data point is the average of triplicate analyses. Least-squares linear regression was performed using Microsoft Excel 2000. Regression statistics are shown in Table 4.

While quantitation of proteins by mass spectrometry has been a controversial issue, it is implemented in this study assuming comparable ionization efficiency of IgG glycoforms. In this application, the comparability

Table 5. Comparison of relative strengths and weaknesses of ESIMS, MALDI-TOF MS, and 2-AA AEC

	ESIMS	MALDI-TOF MS	2-AA AEC
Typical turnaround time (eight samples)	1 day	2 days	5 days
Detection of aglycosyl IgG	Yes	No	No
Interference from protein/peptide heterogeneity	Yes	No	No
Resolution of afucosyl versus degalactosyl structures	No	Yes	No
Sensitivity	+	++	++
Ability to quantitate both charged and neutral structures	Yes	No	Yes

of the ESIMS results with other independent techniques demonstrates the validity of the relative quantitation approach. Mass spectrometry of an intact immunoglobulin requires virtually no sample preparation. Rapid desalting by reversed-phase liquid chromatography is used on-line before the sample is introduced into the mass spectrometer. An integrated software package allows for deconvolution of the raw data and could also

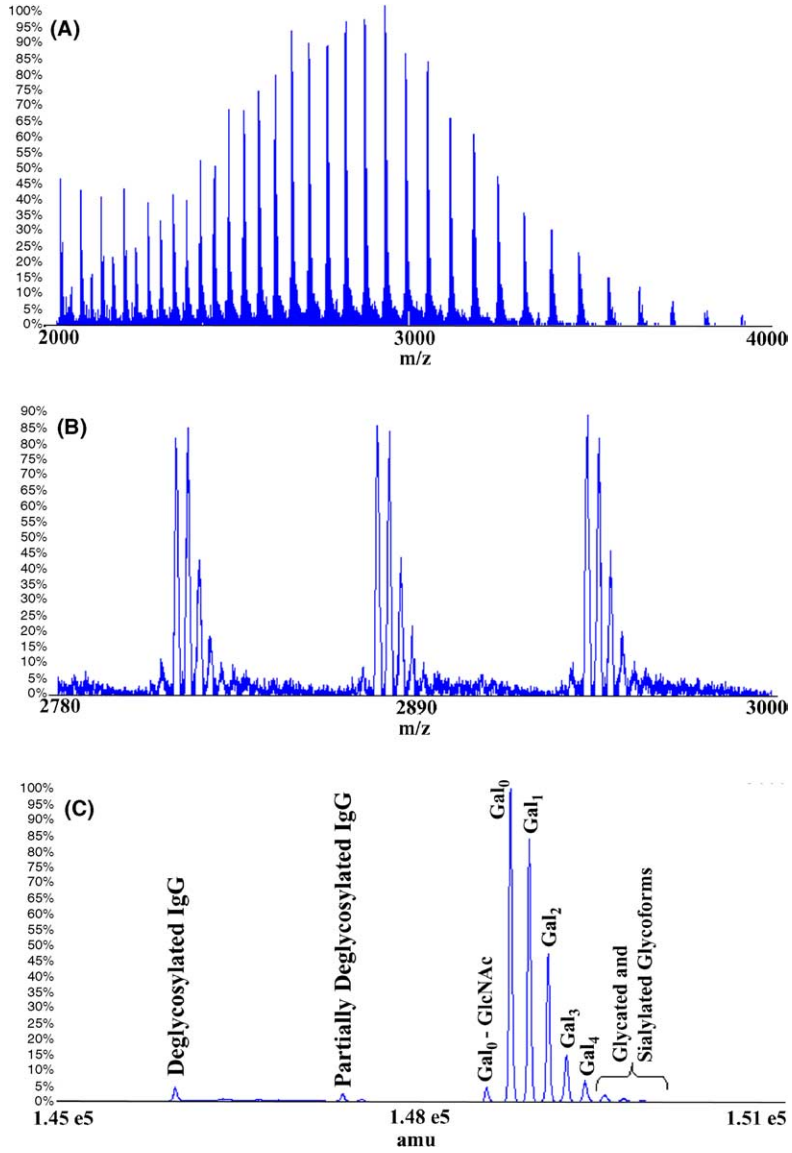

Figure 6. Intact mass spectra of native immunoglobulin. Experimental conditions are detailed in Figure 2. (A) Raw mass spectrum illustrating the distribution of charge states observed from *m*/*z* 2000 to 4000. (B) Area of the raw mass spectra scaled to show three charge states from *m*/*z* 2780 to 3000. (C) Deconvoluted mass spectra of intact immunoglobulin containing low levels of completely deglycosylated, as well as partially deglycosylated species (with one deglycosylated and one glycated heavy chain), and typical IgG glycoforms. The peak labeled 'Gal_0-GlcNAc' is an IgG with two G0 glycans, one of which is missing an *N*-acetylglucosamine (see Fig. 1 for structures).

directly provide quantitative information. The rapid turnaround time of analysis is ideal for in-process monitoring during the manufacture of recombinant antibodies. Furthermore, information on levels of nonglycosylated immunoglobulins (Fig. 6) as well as on protein heterogeneity (e.g., carboxy or amino terminal heterogeneity) can be obtained, which is of course not possible during analyses of released carbohydrates. It has been demonstrated that less than 2% of aglycosyl immunoglobulin may be detected by ESIMS of intact protein (Fig. 6), which is a unique advantage of the intact IgG mass measurement by ESIMS. Immunoglobulin glycoforms containing both charged and neutral carbohydrate structures can also be detected by ESIMS. The data shown in Figure 7 demonstrates ESIMS analysis of an antibody species containing monosialylated (*N*-glycolylneuraminic acid containing) as well as neutral glycoforms. Stability of sialylated IgG glycoforms during electrospray process has been demonstrated by quantitative comparison with 2-AA AEC. Levels of sialylation calculated from the ESIMS data were in agreement with quantitative data obtained by 2-AA AEC[16] (data not shown).

The methods based on analysis of enzymatically released carbohydrates have the advantage of a reduced complexity of the analytes; variability in the protein backbone of the IgG is irrelevant. These techniques exhibit different selectivities with respect to carbohydrate structure detection. Analysis of carbohydrates by MALDI-TOF MS using the 2-AA matrix provides sufficient mass resolution to differentiate loss of a fucose versus loss of a galactose. Carbohydrate structures,

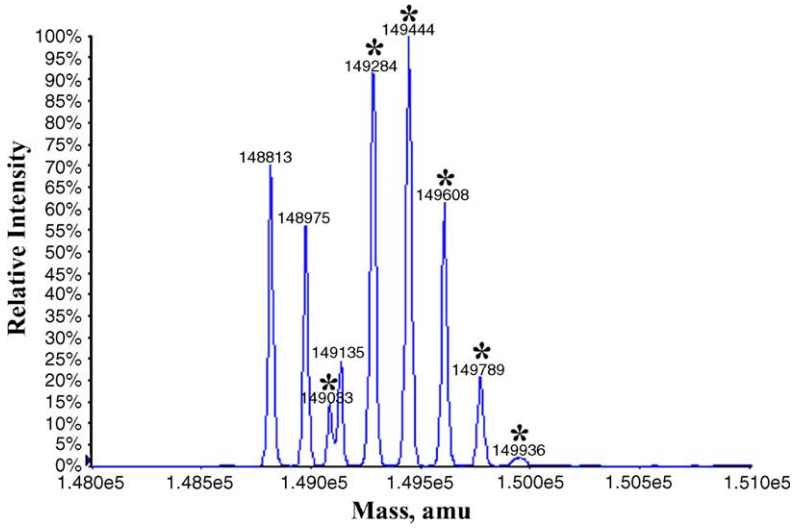

Figure 7. Deconvoluted mass spectrum of an intact IgG sample containing charged and neutral glycoforms. Experimental conditions are detailed in Figure 2. Mass peaks corresponding to the sialylated (*N*-glycolylneuraminic acid) IgG species are marked with asterisks (*). Percent monosialylated glycoforms was calculated using deconvoluted peak areas and determined to be approximately 65%.

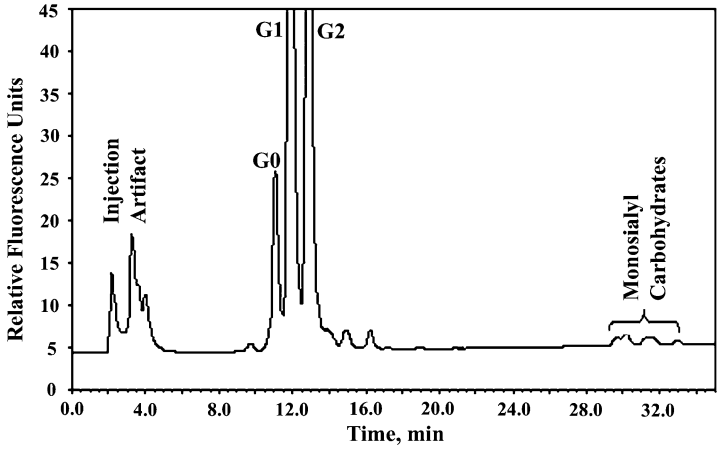

Figure 8. Anion-exchange separation of 2-AA labeled carbohydrates from IgG-containing sialylated carbohydrate structures. Peaks identified as monosialyl carbohydrates were confirmed by treatment with Sialidase A (data not shown).

which co-elute in anion-exchange separations can be resolved by MALDI-TOF MS, such as some high-mannose structures and biantennary complex-type glycans. If effector function is an important parameter for IgG characterization, the level of fucosylation may be a critical determinant. Fucosylation could not be accurately determined by either ESIMS analysis of the intact immunoglobulin or by anion-exchange chromatography of the fluorophore labeled carbohydrates. MALDI-TOF MS analysis of released carbohydrates has also been demonstrated to be a highly sensitive technique[12] that has been used for glycoform analysis of drugs in pharmacokinetic studies.

The fraction of charged carbohydrate structures (sialylated species in this study) could not be accurately quantified using the described MALDI-TOF technique. Unlike neutral glycans, charged species have not been shown to respond linearly in this system. While the MALDI-TOF MS analysis is rapid and amenable to automation, the carbohydrates must be released from the immunoglobulin and isolated from the digest mixture with minimal salts in order to achieve good sample ionization as well as reproducible quantitative results. Although turnaround time for the method is not as rapid as ESIMS analysis of intact immunoglobulins, it is considerably shorter than that for 2-AA AEC.

Fluorophore labeling of a carbohydrate structure yields consistent response, independent of the glycan composition. An advantage of chromatographic techniques based on fluorophore labeling of carbohydrates is the ability to quantitate neutral and charged carbohydrates. A representative chromatogram of an IgG with approximately 2% sialylation is presented in Figure 8. Carbohydrate structures that differ by a single neutral monosaccharide (e.g., loss of a fucose vs loss of a galactose) are not well resolved by 2-AA AEC. Lack of resolution for these closely related structures would be problematic for quantitation of glycosylation for IgGs with variable fucosylation. Also at issue is the time required for assay performance. Typically, 3 days are required for overnight PNGase F digestion, carbohydrate isolation (the column effluent is typically dried under vacuum overnight), fluorophore labeling, and excess dye removal (again, the cartridge effluent is typically dried under vacuum overnight). Since each sample requires approximately 80 min to chromatograph (including column cleaning and re-equilibration time), an eight-sample set (20 injections including blanks and assay controls) can easily take up to 5 days to analyze by 2-AA AEC.

The percent galactosylation values determined in this study are consistent across the three methods for a wide range of galactosylation levels, indicating a high degree of accuracy for all three methods. Assays intended for quantitation of immunoglobulin glycoforms must provide accurate and reproducible data to be useful during characterization or release of recombinant drug candidates. Since the three methods studied all provide excellent linearity, repeatability, and reproducibility, the choice of assay then becomes dependent on the other requirements such as (1) the type of information needed, (2) specific properties of the immunoglobulin (e.g., primary structure, degree of glycosylation site occupancy), and (3) assay turnaround time or other assay operability considerations.

3. Experimental

3.1. Enzymatic carbohydrate modifications of recombinant immunoglobulin G (rIgG)

An aliquot of recombinant IgG was treated with β-(1→4)-galactosidase (*Streptococcus pneumoniae*, CalBiochem, 345806). Degalactosylation was performed in 50 mM ammonium acetate, pH 5.0 at 37 °C for 24 h using an enzyme-to-substrate ratio of 30 mU enzyme per mg IgG (1 mU = release of 1 μmol galactose min^{-1}), corresponding to a 1400-fold excess of galactosidase.

Completely galactosylated IgG was produced using β-(1→4)-galactosyl transferase (bovine, CalBiochem, 345650) in the presence of UDP-galactose (CalBiochem, 670111). Hypergalactosylation was performed in 50 mM 3-*N*-(morpholino)propanesulfonic acid (MOPS), 20 mM $MnCl_2$, pH 7.2 buffer at 37 °C for 24 h. An enzyme-to-substrate ratio of 7 mU galactosyl transferase (1 mU = addition of mol galactose min^{-1}), and 0.5 mg of UDP-galactose per mg of IgG was used, corresponding to a 6400-fold excess of enzyme and a 120-fold excess of UDP-galactose per IgG molecule.

The enzymatically modified samples were affinity purified on a recombinant Protein A column (Applied Biosystems, 1-5559-05). The enzymatically treated samples were applied to the column without any buffer or pH adjustment. The column was washed with 75 mM phosphate, 100 mM NaCl to remove glycosidase/glycotransferase and other enzymatic treatment components. The bound immunoglobulin was eluted from the Protein A columns with 75 mM NaOAc, pH 3.4. Column eluates were neutralized by addition of 1 M NaOAc. The samples with 0%, 34%, and 100% galactosylation were combined to produce a set of samples corresponding to approximately 0%, 17%, 26%, 34%, 55%, 65%, and 100% galactosylation.

3.2. PNGase F digestion

Following protein A purification, samples of known percent galactosylation were prepared by mixing known amounts of the native IgG and enzymatically modified IgG. The samples were analyzed directly by intact mass spectrometry. Samples to be analyzed by

MALDI-TOF MS or by fluorescent labeling coupled with anion-exchange chromatographic separation (2-AA AEC) were treated with peptide N-glycosidase F (PNGase F, Prozyme, GKE5006) in a 50 mM ammonium bicarbonate at 37 °C for 24 h. An enzyme-to-substrate ratio of 150 mU enzyme per mg IgG was used. Complete removal of carbohydrate structures from immunoglobulins under these conditions has been confirmed by SDS-PAGE analysis (data not shown).

3.3. Carbohydrate sample preparation for 2-AA AEC

Upon digestion, samples to be analyzed by 2-AA AEC were isolated via reversed-phase solid-phase extraction (RP-SPE). Reversed-phase 96 well blocks (Waters, WAT058951) were washed with 50% MeOH and conditioned with 10% MeOH prior to sample application. Samples were drawn through the HLB blocks by vacuum, and the sample flow through containing the carbohydrate structures were collected. The HLB wells were washed with 0.5 mL of 10% MeOH, and the wash pooled with the sample flow through. The effluents from RP-SPE were evaporated to dryness in a vacuum centrifuge.

3.4. Carbohydrate sample preparation for MALDI-TOF MS

Carbohydrates to be analyzed by MALDI-TOF MS were isolated by EtOH precipitation of the PNGase F digests. Samples were EtOH precipitated by the addition of 5 vol of cold EtOH, followed by incubation at −20 °C for 10 min, and centrifugation at 10,000*g* for 20 min. The supernatants containing the released carbohydrates were aspirated off the proteins pellets and the recovered supernatants were evaporated to dryness in a vacuum centrifuge.

3.5. ESIMS of intact immunoglobulins

Immunoglobulin samples were diluted to approximately 10 pmol/μL in 100 mM Tris, pH 8.0. Approximately 2–5 μg of sample was used per injection. Prior to MS detection, the samples were desalted on-line by reversed-phase HPLC using a Macrotrap protein cartridge (Michrom BioResources, 004-25108-53). The sample was loaded onto the column and desalted with 95% mobile phase A (0.08% HCO_2H, 0.02% TFA in water) and 5% mobile phase B (0.08% HCO_2H, 0.02% TFA in CH_3CN). The flow through was diverted to waste. Protein sample was eluted with a step gradient of 95% B and 5% A and detected by the ESI-Q-TOF mass spectrometer Qstar (MDS Sciex/Applied Biosystems). The TOF region was calibrated using multiply charged ions of bradykinin and angiotensin. The instrument was scanned from 2000 to 4000 m/z in the positive-ion mode for the intact samples. The protein spectra were deconvoluted using BioAnalyst 1.1 software. The mass accuracy of the measurements was $\leqslant$0.005%. The analyses of mixtures with different percent galactosylations were performed in triplicate, and percent galactosylation was determined according to Eq. 2 using deconvoluted peak areas.

3.6. MALDI-TOF MS of released carbohydrates

A saturated matrix solution was prepared by mixing 10 mg of 2-aminobenzoic acid (Fluka, 10678) with 1 mL of 20% CH_3CN in water. The matrix solution was used without further modification. Samples of dried, released carbohydrates from 200 μg of IgG were reconstituted in 50 μL of HPLC-grade water and briefly vortexed. Samples were stored at 2–8 °C prior to analysis. Samples were mixed with matrix solution at a 1:2 ratio (i.e., 5 μL sample with 10 μL matrix solution). The mixture was vortexed briefly prior to spotting on a stainless steel target plate, each sample was spotted three times (0.5 μL per spot). Each spot represented the carbohydrates obtained from approximately 1.3 μg IgG. An external calibration mixture of G0 and G2 carbohydrates (CalBiochem, 436401 and 436405, respectively) was spotted adjacent to each set of analyte spots. MALDI-TOF MS data were collected on an ABI DE-STR BioSpectrometry Workstation in linear mode with delayed extraction (150 ns) and negative-ion polarity. The accelerating voltage was maintained at 20 kV with grid and guide wire voltages set to 95% and 0.2%, respectively. Typically, 100–150 laser shots were acquired and averaged to produce a spectrum. Raw mass and area count data were exported from Data Explorer V4.0 software to Microsoft Excel 2000. The analyses of mixtures with different percent galactosylations were performed in triplicate, and percent galactosylation was determined according to Eq. 1 using deconvoluted peak areas.

3.7. Fluorescent labeling and chromatography of released carbohydrates

Released carbohydrates to be analyzed by anion-exchange chromatography were labeled with 2-aminobenzoic acid (2-AA) as per the method described by Bigge and Patel,[17] except the reaction temperature was altered from 65 to 45 °C to preserve carbohydrate sialylation. Excess labeling reagents were removed by paper chromatography on LudgerClean S® cartridges (Ludger, LC-S-01) according to manufacturer instructions. Cartridge effluents were dried under vacuum overnight. Samples of labeled, dried carbohydrates were reconstituted with 50 μL of HPLC-grade water and briefly vortexed prior to chromatographic analysis. Labeled carbohydrate mixtures were separated by anion-

exchange chromatography on an AsahiPak NH2P 4D carbohydrate column developed with a gradient of ammonium acetate at pH 4.8 (mobile phase B: 250 mM NH4OAc, 160 mM HOAc, mobile phase A: 0.2% of mobile phase B in water). The column was eluted with a two segment linear gradient from 0% to 15% B in 15 min, followed by 15% to 100% B in 5 min. The column was washed at 100% B for 25 min, and then re-equilibrated at 0% B for 19 min prior to the next injection. Column effluent was monitored by fluorometry using an excitation wavelength of 330 nm, and an emission wavelength of 420 nm (emission bandwidth = 40 nm). The analyses of mixtures with different percent galactosylation were performed in triplicate, and percent galactosylation was determined according to Eq. 1 using deconvoluted peak areas.

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