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Journal of Chromatography B, 859 (2007) 178–191
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A novel HPLC–UV–MS method for quantitative analysis of protein glycosylation

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Received 11 July 2007; accepted 19 September 2007
Available online 29 September 2007

Abstract

Monoclonal antibody samples derived from transgenic plants (plantibodies) may often contain significant amounts of aglycosylated variants. Because glycosylated and non-/de-glycosylated proteins exhibit different functional and pharmacokinetic properties, accurate measurement of non- and de-glycosylated glycoprotein abundances is important. Glycosylation of plant-derived glycoproteins presents specific challenges. Here we describe a novel method to accurately measure relative and absolute amounts of non-glycosylated, de-glycosylated, and total glycosylated protein using an HPLC–UV–MS methodology. Additionally, these results were compared with glycopeptide profiling by MALDI MS. Our studies demonstrated that the quantitative aspect of HPLC–UV method was superior to MALDI MS profiling, which significantly overestimated the relative amounts of aglycosylated species in the isolated glycopeptide fractions.

Keywords: Protein glycosylation; Non-glycosylated; De-glycosylated; Glycoforms; Antibodies; HPLC; UPLC–UV–MS

1. Introduction

The biotechnology industry is developing and producing products such as recombinant proteins and vaccines. One of the key aspects of characterization of recombinant proteins, vaccines, and antibodies from non-bacterial expression systems is their glycosylation. The oligosaccharides contained in glycoproteins are often responsible for diverse biological functions, such as cellular cytotoxicity (e.g. complement-dependent cellular cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC)) and specific receptor binding. Also, glycosylation often confers to a protein stabilization of its folded structure in solution, increased resistance to proteolytic destruction, and enhanced *in vivo* half-life. Glycosylated and non-/de-glycosylated protein variants often have different functional and pharmacokinetic properties [1]. The glycoform distribution of a protein product is in many cases dependent on the methods and conditions of its production. It is therefore important to be able to correctly measure the different quantities of non- and de-glycosylated species within samples of recombinant therapeutic glycoproteins, and MAbs in particular.

We have previously observed that some monoclonal antibody (MAb) samples (especially from plant of plant cell culture expression systems) contained a large fraction of aglycosylated (no glycans attached) variant, as determined by MALDI MS profiling of isolated tryptic glycopeptide fractions (Karnoup, unpublished results). Reports of detecting significant fractions of non- and de-glycosylated protein, as well as protein modified with only a single GlcNAc residue, in glycoprotein samples were also published in literature [2–5]. It has been proposed that the presence of non-glycosylated protein can be due to inadequate glycosylation efficiency [2,4], whereas the presence of single GlcNAc residues attached to Asn in the *N*-linked glycosylation sites evidences degradation due to endoglycosidase-H-type

Abbreviations: N, peptide EEQY**N**STYR (Glu-Glu-Gln-Tyr-**Asn**-Ser-Thr-Tyr-Arg); D, peptide EEQY**D**STYR (Glu-Glu-Gln-Tyr-**Asp**-Ser-Thr-Tyr-Arg); ACN, acetonitrile; Hex (H), hexose (Man, Gal); HexNAc (N), *N*-acetyl-hexosamine (GlcNAc, GalNAc); GlcNAc, *N*-acetyl-glucosamine; GalNAc, *N*-acetyl-galactosamine; Xyl (X), xylose; Man, mannose; Fuc (F), fucose; Gal, galactose; TDCC, The Dow Chemical Company; MALDI MS, matrix-assisted laser desorption/ionization mass-spectrometry; Asn, asparagine; TFA, trifluoroacetic acid; HC, heavy chain; LC, light chain; IAA, iodoacetamide; DTT, dithiothreitol; PNGase A, peptide *N*-glycanase A (from almonds); HPLC, high-performance liquid chromatography; MAb, monoclonal antibody; UPLC, ultra performance liquid chromatography; CE, capillary electrophoresis; SEC, size-exclusion chromatography.

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doi:10.1016/j.jchromb.2007.09.030

activity (EC 3.2.1.96) [3,4]. The presence of de-glycosylated protein may reflect degradation due to *N*-glycosidase activity (EC 3.5.1.52) [4].

Human IgG1 antibodies contain a single site for *N*-linked glycosylation in their conserved Fc-region. When an *N*-linked glycan is enzymatically removed from the protein, the corresponding Asn residue is converted to Asp (+1 Da mass-shift). Although such +1 Da mass-shift may also result from a deamidation reaction, such reaction was not observed under the typical sample-handling conditions [6]. In addition, it was reported recently [6] that the Fc fragment containing the site of *N*-linked glycosylation in IgG1 antibodies is not prone to spontaneous deamidation during typical sample preparation conditions (reduction, alkylation, and digestion with trypsin). Consistent with the above observations, the degradation of this kind was not observed in our control experiments using commercially available recombinant human IgG1 preparations (Karnoup, unpublished). Therefore, the presence of non-glycosylated Fc-region fragment EEQYNSTYR should be indicative of incomplete glycosylation of the antibody, and the presence of the corresponding de-glycosylated fragment EEQYDSTYR should be indicative of *in vivo* enzymatic de-glycosylation. Additionally, the presence of abundant EEQYNSTYR fragment modified with only a single GlcNAc in many samples that also exhibit abundant de-glycosylated species adds to the enzymatic degradation hypothesis: because the *N*-glycosylation machinery of the cell transfers full-sized oligosaccharide precursors (not single GlcNAc) to the specific Asn residues [8], so the presence of a single GlcNAc on such Asn is indicative of oligosaccharide degradation.

For mammalian-expressed glycoproteins, the fraction of aglycosylated protein is possible to assess by analyzing the intact protein before and after enzymatic de-glycosylation, using electrophoretic or chromatographic methods for protein detection and quantitation [7]. However, this approach is not suitable for glycoproteins expressed in plants or plant cell cultures because of a combination of three technical issues: (a) different linkage of core-linked fucose (α-1,3 in plants *vs.* α-1,6 in mammals) [8] does not allow the use of a mammalian enzyme PNGase F to cleave *N*-glycans off Asn residues of intact glycoproteins; (b) available plant-specific enzyme PNGase A can only use peptides (and not intact protein) as substrate in the de-glycosylation reaction [9]; and (c) chemical release of glycans from glycoproteins results in destruction of either the protein or the glycan components [10]. Therefore, there is a need to develop a method for the analysis of glycopeptides generated by proteolytic digestion of recombinant glycoproteins to determine relative and absolute concentrations of non-glycosylated, de-glycosylated, and total glycosylated protein.

In this study we investigated the dependence of relative intensities of EEQYNSTYR and EEQYDSTYR peptides in MALDI mass-spectra on their relative quantities in binary mixtures, and the ability to use HPLC–UV–MS for quantitatively determining amounts of non-glycosylated, de-glycosylated, and glycosylated protein fragments. We developed a method for determining relative and absolute amounts of non-glycosylated, de-glycosylated, and total glycosylated protein using HPLC–UV–MS to analyze glycoprotein tryptic digests. For this application, we used a Waters UPLC system and a reversed-phase (C18) column packed with small-diameter particles (1.7 μm) to increase chromatographic resolution. For simplicity, this methodology is further referred to as "UPLC–UV–MS" in the text, and HPLC on a Waters UPLC system is dubbed "UPLC". The developed methods may be applied to determining the quantities of non-glycosylated, de-glycosylated, and glycosylated protein in antibodies and other recombinant protein samples.

2. Experimental

2.1. Materials

Dithiothreitol (DTT), iodoacetamide (IAA), guanidine hydrochloride (Gu:HCl), and ammonium bicarbonate were purchased from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) was from Fisher Scientific (Pittsburgh, PA). Modified sequencing grade trypsin was from Roche Diagnostics (Indianapolis, IN).

2.1.1. Chemicals and synthetic peptides

Ten mg of each peptide EEQYNSTYR ("N") and EEQYDSTYR ("D") were synthesized by Genemed Synthesis Inc. (South San Francisco, CA) using a solid-phase peptide synthesis approach, and purified by reversed-phase HPLC to >98% purity. Stock solutions of peptides were prepared by dissolving ∼10 mg of each peptide solid sample in 1.6 mL of Milli-Q deionized water (18 Ω cm, 6 ppb TOC). Dilutions of stock solutions with Milli-Q water (∼10×) were prepared, and the final concentration was measured by UV absorption at 280 nm (UV280) using the calculated extinction coefficients (2.1527 (mg/mL) cm^{-1} for EEQYNSTYR, and 2.1509 (mg/mL) cm^{-1} for EEQYDSTYR) [11]. The final concentration of EEQYNSTYR peptide was 0.3166 mg/mL (0.266 mmol/L), and the final concentration of EEQYDSTYR peptide was 0.3152 mg/mL (0.265 mmol/L). A Shimadzu UV-3101-PC UV–vis–NIR scanning spectrophotometer was used to record UV absorption spectra. Starna quartz optical cells with 1 cm path length were used in all spectral measurements.

2.1.2. Glycoproteins

Herceptin IgG1 expressed in CHO cell culture was obtained from Genentech (lot no. M14176). Human polyclonal IgG (pooled from human serum) ("Sigma IgG") was purchased from Sigma (cat. no. I-4506; lot no. 044K7616). Recombinant plant-expressed antibodies and glycoproteins were produced at Dow AgroSciences. Maize-expressed recombinant chicken avidin was from Sigma (cat. no. A-8706, lot no. 76H9504).

2.1.3. Glycopeptides from IgG1 antibodies

Procedures for reduction, alkylation, proteolysis, and isolation of tryptic glycopeptides from IgG antibodies by HPLC were as follows. Two hundred micrograms of protein was dissolved in 180 μL of 6 M guanidine hydrochloride/0.4 M ammonium bicarbonate, reduced by addition of 20 μL of aqueous 0.1 M DTT and incubation at 65 °C for 0.5 h. The sample was then

alkylated by addition of 40 μL of aqueous 0.2 M iodoacetamide and incubating at room temperature for 1 h in the dark. The reaction was quenched by addition of 80 μL of DTT solution. The reduced and alkylated protein was desalted using NAP-5 cartridges (GE Healthcare, Piscataway, NJ), according to the manufacturer's procedure, and digested with trypsin at 1:40 enzyme-to-protein ratio (16 h at 37 °C). The tryptic digest was concentrated to ∼250 μL in a vacuum concentrator and 200 μL (∼160 μg) was injected onto HPLC column. A Jupiter Proteo C18 90A column (4.6 mm × 15 cm, Phenomenex) and a Hitachi LC system were used for the separation of the tryptic digests. Chromatography was run at a constant flow rate of 1 mL/min and at room temperature. The separation of peptides was accomplished using the following gradient: 100% solvent A (3% acetonitrile/0.06% TFA) isocratic for 2 min, 0–12% solvent B (80% acetonitrile/0.05% TFA) in 10 min, and 12–100% solvent B in 30 min. The column was then washed with 100% solvent B for 3 min and re-equilibrated in 100% solvent A (100–0% B in 5 min). Elution of peptides was monitored by UV absorption at 205 nm. One-milliliter fractions were collected in siliconized microcentrifuge tubes and dried in a centrifugal evaporator. Before analysis by MALDI MS, fractions were redissolved in 4 μL of 50% acetonitrile/0.1% TFA, and 1 μL of the material in each fraction was examined by MALDI MS. For each antibody, HPLC fractions containing glycopeptides were combined and analyzed by MALDI MS to obtain full glycosylation profiles. Only IgG1 fragment (EEQYNSTYR) variants of human polyclonal IgG ("Sigma IgG") were isolated (IgG2 fragment (EEQFNSTFR) variants, eluting later in HPLC chromatogram, were discarded). For each antibody, glycopeptide fractions from 11 HPLC runs were combined.

For quantitative UPLC–UV–MS and MALDI MS experiments, Herceptin IgG1 and Sigma IgG1 glycopeptide isolates were spiked with various amounts of synthetic EEQY**N**STYR ("N") and EEQY**D**STYR ("D") peptides.

2.2. MALDI MS of synthetic peptides and isolated glycopeptide fractions

Mixtures of the two synthetic peptides were prepared immediately prior to MALDI MS measurements. The following volume ratios of the peptides EEQYNSTYR to EEQYDSTYR were prepared for MALDI MS measurements—1:0, 80:1, 60:1, 40:1, 30:1, 10:1, 8:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:10, 1:30, 1:40, 1:60, 1:80, 0:1. All mixtures were prepared and measured in duplicates, except for 1:1 ratio, which was prepared and measured in a triplicate.

Fractions of glycopeptides isolated from protein digests were dissolved in 50% acetonitrile containing 0.1% TFA prior to MALDI MS analysis.

One microliter of each sample was deposited onto a MALDI plate, overlaid with 1 μL of CHCA (α-cyano-4-hydroxycinnamic acid; Fluka) matrix solution and air-dried.

Voyager DE-STR (Applied BioSystems) MALDI-Tof mass spectrometer operated in reflectron mode was used. The acceleration voltage was set to 20 kV. The grid voltage was set to 62% of the acceleration voltage. The delay time was set to 215 ns. The laser setting varied between 2300 and 2500. Five hundred acquisitions were averaged in each spectrum. The mass scale was calibrated using a Sequazyme peptide mass standard kit (Applied BioSystems). MALDI MS data were analyzed using Data Explorer v4.0 software (Applied BioSystems). Molecular masses and amino acid sequences of peptides and glycopeptides were attributed to the amino acid sequences of the corresponding proteins using MassLynx v3.5 software (Waters/Micromass).

2.3. UPLC–UV–MS

2.3.1. UPLC system parameters

A Waters Acquity UPLC equipped with a TUV detector and interfaced with Waters/Micromass Q-Tof Micro ESI mass-spectrometer was used. A BEH C18 2.1 × 150 mm, 1.7 μm column (Waters) was used. Column temperature was kept at 50 °C. Autosampler temperature was set to 7 °C. Mobile phase A was 3% acetonitrile, 0.1% FA (formic acid). Mobile phase B was 80% acetonitrile, 0.1% FA. Flow rate was 0.150 mL/min. Injection volume was 20 μL (full loop method, 2× loop overfill). UV signal was detected at 214 nm (sampling rate: 2 pts/s).

The following gradient conditions were used for IgG1 tryptic peptides and synthetic peptides: 1% mobile phase B isocratic for 2 min, 1–7% mobile phase B in 10 min, and 7–100% mobile phase B in 2 min. The column was then washed with 100% mobile phase B for 5 min and re-equilibrated back to 1% mobile phase B (100–1% B in 1 min, then 1% B for 10 min).

2.3.2. Mass-spectrometer parameters

Q-Tof Micro ESI mass-spectrometer was operated in the positive ionization mode. The following settings were used. Capillary: 2600.0 V, sample cone: 15 V, extraction cone: 1 V, desolvation temperature: 250 °C, source temperature: 100 °C. The following lock-spray configuration was used. Reference scan frequency: 5 s; reference cone voltage: 20 V.

3. Results and discussion

3.1. MALDI MS glycopeptide profiling

MALDI MS is often used for semi-quantitative profiling of *N*-linked glycoforms (as free glycans, and/or as glycopeptides). Typically such an approach produces results for glycans or glycosylated peptides that correlate reasonably well with separation-based methods ([9,12]; also: Karnoup, unpublished data). However, for glycoproteins from some expression systems (e.g. some plant-expressed glycoproteins), apparent high abundance of non- and de-glycosylated species in MALDI MS profiles of isolated glycopeptides was observed (Fig. 1A and B). This posed a question whether such high abundance in MALDI MS profiles truly reflected a high content of non- and de-glycosylated species in the samples, or it was merely an artifact of MALDI MS. In experiments with control fully glycosylated glycoproteins it appeared that the MALDI MS signals for non- and de-glycosylated species were either absent or detected at only trace levels (Fig. 1C). But it was still not clear whether the dependence of concentration of non-/de-glycosylated species on

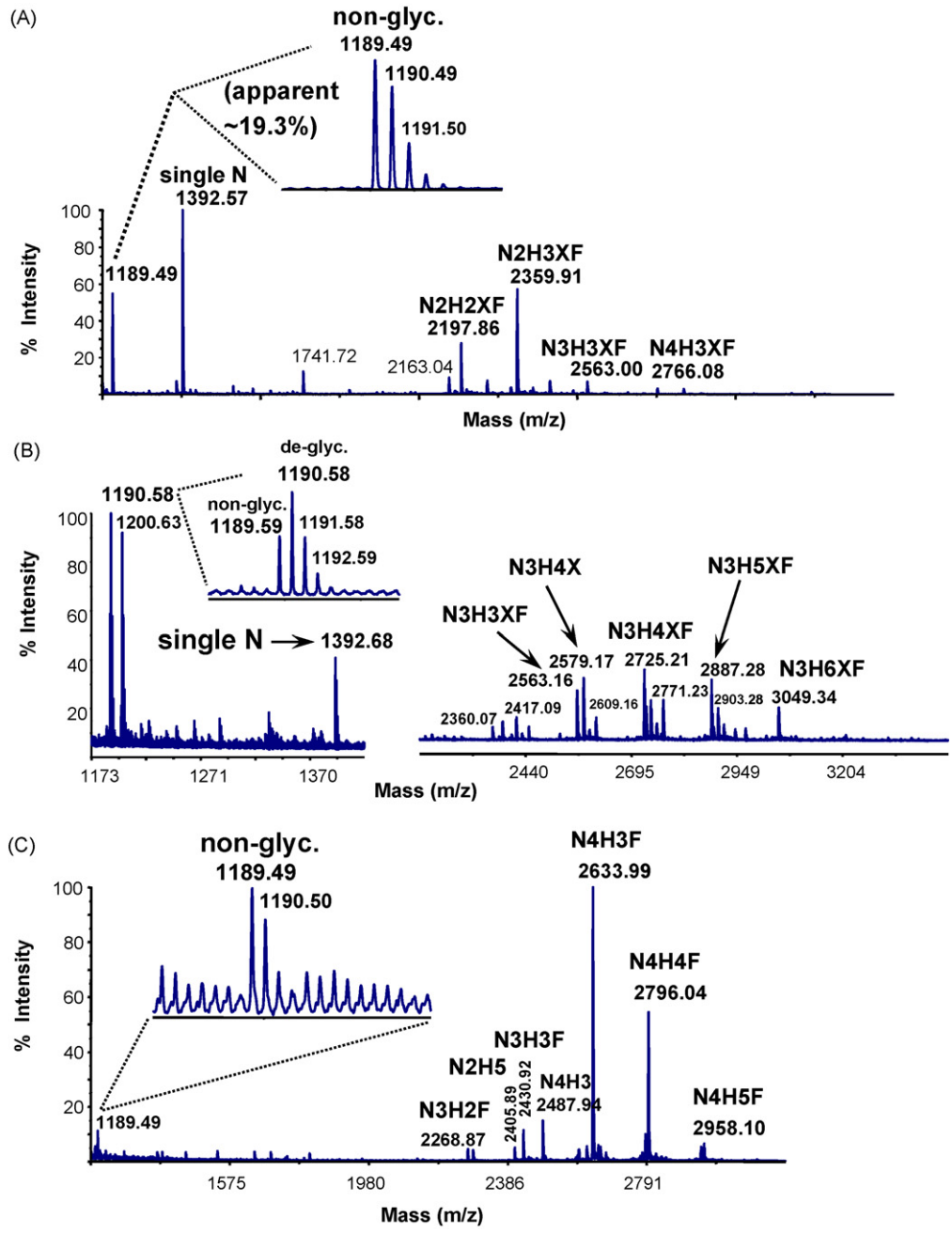

Fig. 1. Examples of MALDI MS glycosylation profiles of IgG1 antibodies. (A) Maize-expressed IgG1 "X" sample. An apparent high abundance of non-glycosylated species, as well as species carrying single GlcNAc residue is observed. (B) Rice-expressed IgG1 "Y" sample. An apparent high abundance of de- and non-glycosylated species, as well as species carrying single GlcNAc residue is observed. (C) Mammalian cell culture (CHO) expressed Herceptin IgG1 (Genentech) control. Only low apparent abundance of non-glycosylated species is detected (∼4.5 (±4)% of sum of MALDI MS intensities) (Karnoup, unpublished).

their signal intensity in MALDI MS was linear and whether the MALDI MS intensity of aglycosylated species relative to that of glycosylated species actually reflected their concentration ratio. The use of MALDI MS profiles of tryptic glycopeptides to estimate amounts of non- and de-glycosylated fragments relative to glycosylated fragments appears to be challenging because of (a) possibly different ionization efficiencies of non-glycosylated, de-glycosylated, and glycosylated species, and (b) overlap of isotopic clusters for non- and de-glycosylated fragments (see Section 3.2).

To answer the above questions, the following model glycoproteins were examined by MALDI MS and UPLC–UV–MS: Herceptin IgG1 expressed in CHO cell culture (Genentech), and human polyclonal IgG (pooled from human serum, Sigma ("Sigma IgG")). The corresponding MALDI MS profile of *N*-linked glycosylation for Herceptin IgG1 is given in Table 1 ("no spike" column). The MALDI MS profile of Sigma IgG *N*-glycopeptide sample (before spiking with synthetic peptides) included 0% non- and de-glycosylated species, ∼21.7% peptide carrying N4H3F glycan (N = HexNAc, H = Hex, F = Fuc),

Table 1
MALDI MS profiles of Herceptin IgG1 tryptic glycopeptides unspiked and spiked with various amounts of synthetic "**N**" (EEQY**N**STYR) and "**D**" (EEQY**D**STYR) peptides

N-Glycan[a]	*m/z* theoretical	*m/z* observed (represen-tative)	% of sum of intensities in MALDI MS (glycopeptide)										
			No spike[b] (0% "N", 0% "D" by UPLC–UV–MS[c])	7.96 ng "N" (6.83% "N", 0.00% "D" by UPLC–UV–MS)	31.85 ng "N" (22.09% "N", 0.00% "D" by UPLC–UV–MS)	63.69 ng "N" (36.38% "N", 0.00% "D" by UPLC–UV–MS)	7.16 ng "D" (0.00% "N", 7.05% "D" by UPLC–UV–MS)	26.65 ng "D" (0.00% "N", 22.53% "D" by UPLC–UV–MS)	57.31 ng "D" (0.00% "N", 36.66% "D" by UPLC–UV–MS)	7.96 ng "N",7.16 ng "D" (7.53% "N", 6.77% "D" by UPLC–UV–MS)	15.92 ng "N",14.33 ng "D" (12.56% "N", 12.03% "D" by UPLC–UV–MS)	31.85 ng "N",28.65 ng "D" (19.53% "N", 19.97% "D" by UPLC–UV–MS)	63.19 ng "N",57.31 ng "D" (27.50% "N", 28.91% "D" by UPLC–UV–MS)
No glycan, non-glycosylated	1189.51	1189.40	**0.4**	**25.8**	**43.6**	**53.6**	0.0	0.0	0.0	18.5	27.6	27.3	34.1
No glycan, de-glycosylated	1190.50	1190.57	0.0	0.0	0.0	0.0	24.1	44.4	57.6	34.2	29.6	58.2	48.9
Single N	1392.59	1392.44	0.5	0.3	0.2	Trace	Trace	0.0	0.2	0.1	0.3	0.0	0.0
N_3H_2	2122.85	2122.60	0.4	0.2	0.2	0.1	Trace	Trace	Trace	0.1	0.1	0.0	0.0
N_3H_2F	2268.91	2268.64	2.1	1.0	0.7	0.7	1.0	0.7	0.7	0.7	0.6	0.2	0.2
N_3H_3	2284.90	2284.62	2.0	1.4	0.9	0.8	1.5	1.0	0.7	0.9	0.8	0.4	0.3
N_2H_5	2405.92	2405.63	2.3	1.7	1.1	0.9	1.8	1.4	0.8	1.1	1.0	0.5	0.4
N_3H_3F	2430.96	2430.66	5.5	3.3	2.5	1.7	3.1	2.1	2.0	1.9	1.8	0.6	0.6
N_3H_4	2446.95	2446.67	0.8	0.5	0.4	0.3	0.6	0.4	0.3	0.3	0.3	0.0	0.1
N_4H_3	2487.98	2487.68	**7.3**	**5.5**	**4.2**	**3.4**	**5.5**	**3.8**	**2.9**	**3.4**	**3.3**	**1.1**	**1.2**
N_3H_4F	2593.01	2592.71	1.9	1.2	1.1	0.8	1.2	1.1	0.7	0.8	0.7	0.3	0.3
N_4H_3F	2634.04	2633.71	47.0	35.5	27.1	22.8	36.8	26.8	20.9	23.1	20.7	6.9	8.4
N_4H_4	2650.02	2649.72	2.4	1.6	1.5	1.1	1.7	1.3	1.0	1.0	1.0	0.5	0.4
N_5H_3	2691.09	2691.71	Trace	0.4	0.3	0.2	Trace	0.5	0.2	0.2	0.1	0.0	0.0
N_4H_4F	2796.09	2795.75	**24.7**	**19.3**	**14.6**	**12.3**	**20.2**	**14.2**	**10.9**	**12.2**	**10.9**	**3.6**	**4.5**
N_4H_5F	2958.14	2957.80	2.7	2.2	1.7	1.4	2.5	1.8	1.1	1.5	1.3	0.4	0.5
Total (%)			100.0	99.9	100.1	100.1	100.0	99.5	100.0	100.0	100.1	100.0	99.9

Content (%) of synthetic peptides was determined by UPLC–UV. A representative glycosylation profile before spiking is given in "no spike" column.

[a] *N*-Gycan composition: N = HexNac (e.g. GlcNAc), H = Hex (e.g. Man, Gal), X = Xyl, F = Fuc. *N*-Glycans are arranged according to their size (or *m/z*). Values for aglycosylated peptides and peptides carrying most abundant *N*-Glycans are highlighted in bold.

[b] Amount of spike (ng) with synthetic peptide(s).

[c] Content of synthetic peptide(s) (%) determined by UPLC–UV.

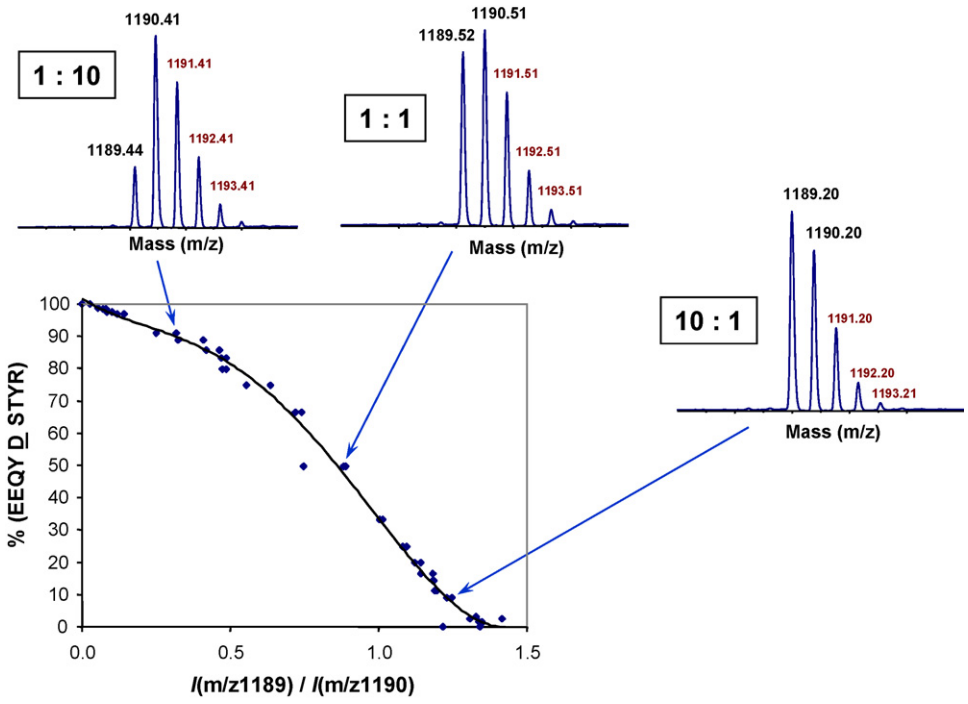

Fig. 2. Dependence of % of EEQY**D**STYR ("D") peptide content in the binary sample mixture consisting of EEQYNSTYR and EEQYDSTYR peptides on the measured ratio of peak intensities at *m/z* 1189.5 and 1190.5. Inserts show MALDI mass-spectra for overlapping isotopic clusters of peptides EEQYNSTYR ("N") (theoretical monoisotopic *m/z* (MH$^+$) = 1189.51) and EEQYDSTYR ("D") (theoretical monoisotopic *m/z* (MH$^+$) = 1190.50) corresponding to EEQYDSTYR ("D") content equal to 90, 50, and 10%. The data are best fit with a polynomial function $y = 95.919x^4 - 229.05x^3 + 120.23x^2 - 55.026x + 101.51$, where y = % EEQYDSTYR ("D") in binary EEQYNSTYR:EEQYDSTYR mixture, and x is the ratio of intensities at *m/z* 1189.5 and 1190.5.

~31.8% peptide carrying N4H4F glycan, ~17.73% peptide carrying N4H5F glycan, ~7.0% peptide carrying N5H4F glycan, and low amounts of other glycoforms (some of them containing fucosylated glycans) (data not shown). The analysis of these glycoprotein samples is discussed below (Section 3.3).

3.2. Use of synthetic peptide standards in glycopeptide MALDI MS

In MALDI MS profiles of IgG1 tryptic glycopeptides, the isotopic cluster of de-glycosylated Fc-region fragment EEQYDSTYR (*m/z* (MH$^+$) = 1190.50) overlaps with the cor-

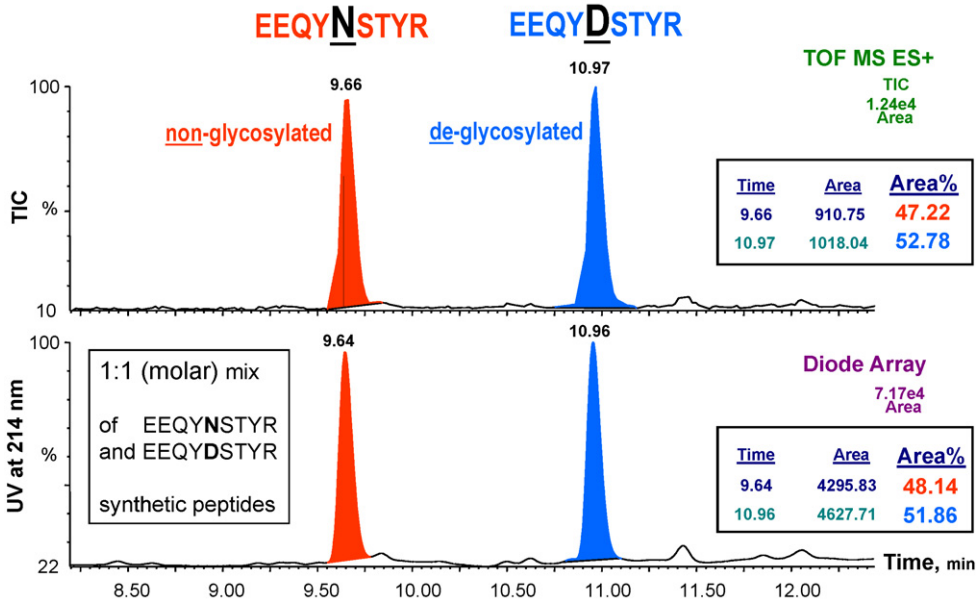

Fig. 3. UPLC–MS TIC (upper panel) and UPLC–UV (detection at 214 nm) (lower panel) of an approximately 1:1 mixture of EEQY**N**STYR ("N") and EEQY**D**STYR ("D") synthetic peptides. Peak area ratio correctly reflects ~1:1 concentration ratio of the two synthetic peptides in the mixture by using both TIC and UV detection.

responding non-glycosylated fragment EEQYNSTYR (m/z (MH^+) = 1189.51). However, it was noticed that the relative intensities of the m/z 1189.5 and 1190.5 peaks changed from sample to sample, apparently as the relative content of EEQYNSTYR and EEQYDSTYR peptides in the samples changed.

Initially, this dependence of relative peak intensities on the relative content of EEQYNSTYR and EEQYDSTYR peptides was investigated. The peptides EEQYNSTYR ("N") and EEQYDSTYR ("D") were obtained as pure compounds via solid-phase peptide synthesis. Mixtures with various molar ratios of the two peptides were prepared and the corresponding MALDI mass-spectra were obtained. Fig. 2 shows the measured dependence of the % of EEQY**D**STYR peptide content in the binary sample mixture consisting of EEQY**N**STYR and EEQY**D**STYR peptides on the measured ratio of peak intensities at m/z 1189.5 and 1190.5. The data can be best fit to a polynomial equation ($r^2 = 0.9956$):

$$y = 95.919x^4 - 229.05x^3 + 120.23x^2 - 55.026x + 101.51 \quad (1)$$

where y = % EEQY**D**STYR in binary EEQY**N**STYR: EEQY**D**STYR mixture, and x is the ratio of intensities at m/z 1189.5 and 1190.5 peaks in MALDI MS.

The central linear portion of the plot (x between 0.4 and 1.3) could also be fit reasonably well with a linear equation ($r^2 = 0.9851$):

$$y = -97.07x + 130.1 \quad (2)$$

The dependence in Fig. 2 is not linear because the first isotopic peak for EEQY**D**STYR peptide overlaps second isotopic peak for EEQY**N**STYR peptide, and the higher the relative content of EEQY**N**STYR is, the greater is the intensity contribution of its second isotopic peak at m/z 1190.5. In addition, the ionization efficiency (in positive ionization mode) of EEQY**D**STYR peptide is likely to be different from that of EEQY**N**STYR peptide due to the presence of an additional negative charge located on Asp (D) residue in EEQY**D**STYR peptide.

Table 2
LOD and LOQ values determined for the synthetic "**N**" (EEQY**N**STYR) and "**D**" (EEQY**D**STYR) peptides by UPLC with three different detection techniques

EEQYNSTYR peptide ("N")	
UV214 detection	
LOD (ng)	0.69 (20 μL of 3.08×10^{-5} mg/mL)
LOQ (ng)[a]	2.06
Linearity range (ng)	0.69 to ~14,000
TIC detection	
LOD (ng)	0.69 (20 μL of 3.08×10^{-5} mg/mL)
LOQ (ng)[a]	2.06
XIC detection	
LOD (ng)	0.34 (20 μL of 1.54×10^{-5} mg/mL)
LOQ (ng)[a]	1.02
EEQYDSTYR peptide ("D")	
UV214 detection	
LOD (ng)	0.62 (20 μL of 3.08×10^{-5} mg/mL)
LOQ (ng)[a]	1.85
Linearity range (ng)	0.62 to ~13,000
TIC detection	
LOD (ng)	0.31 (20 μL of 1.54×10^{-5} mg/mL)
LOQ (ng)[a]	0.93
XIC detection	
LOD (ng)	0.31 (20 μL of 1.54×10^{-5} mg/mL)
LOQ (ng)[a]	0.93

[a] LOQ = 3× LOD.

It was examined whether it was possible to more accurately estimate the abundance of EEQY**D**STYR fragment relative to its precursor, EEQY**N**STYR fragment, in the fractionated tryptic digests of human IgG1, using the dependence in Fig. 2 and Eq. (1). In this approach, it was assumed that the intensity at m/z 1189.5 should reflect the relative abundance of non-glycosylated fragment EEQY**N**STYR in the full glycosylation profiles of IgG1 antibodies, because control samples (e.g. CHO-expressed Herceptin IgG1) with near-zero or low content of non-glycosylated species exhibited low abundance of m/z 1189.5 peak and no m/z 1190.5 peak in their MALDI mass-spectra (see

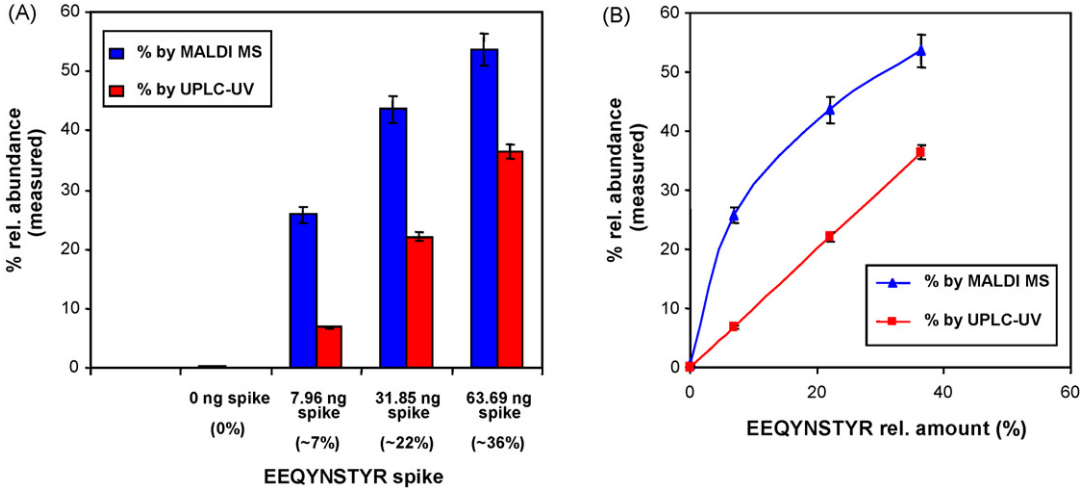

Fig. 4. Comparison of relative quantities of "N" (EEQYNSTYR) peptide in glycosylation profiles of Herceptin IgG1 (spiked with "N" peptide only), obtained by UPLC–UV and MALDI MS. (A) Bar graph representation. (B) Line chart representation. See Tables 1 and 3 for corresponding numerical data.

Table 3
Comparison of quantitative glycopeptide profiling by UPLC and MALDI MS

Description[a]	Average amount (from two reps) of "N", ng (calculated from UPLC–UV)	Known amount of spiked "N", ng	Deviation from known amount of spiked "N", % (UPLC–UV)	Average amount (from two reps) of "D", ng (calculated from UPLC–UV)	Known amount of spiked "D", ng	Deviation from known amount of spiked "D", % (UPLC–UV)	ng "D" (calculated) based on XIC (*m/z* 595.5) peak area. Average of two reps	Deviation from known amount of spiked "D", % (UPLC–XIC)	% total glycosylated peptides (by UPLC–UV peak area)	% non-glycosylated ("N") (by UPLC–UV peak area)	% de-glycosylated ("D") (by UPLC–UV peak area)	% Total glycosylated peptides (by MALDI MS intensity; sum for all glycoforms)	% non-glycosylated ("N") (by MALDI MS)	% de-glycosylated ("D") (by MALDI MS)	Additional comment
Herceptin IgG1, no spike	0.00	0.00	x	0.00	0.00	x	x	x	100.00	0.00	0.00	99.10	0.40	0.00	0.5% of single GlcNAc detected by MALDI MS
Spiked (N + D) Herceptin IgG1	8.11	7.96	1.8%	6.19	7.16	13.5%	8.30	15.91	85.70	7.53	6.77	47.30	18.50	34.20	0.1% of single GlcNAc detected by MALDI MS
Spiked (N + D) Herceptin IgG1	12.96	13.47	3.8%	12.08	12.12	0.33%	12.53	3.42	73.70	13.10	13.19	n/d	n/d	n/d	
Spiked (N + D) Herceptin IgG1	16.23	15.92	1.9%	12.52	14.33	12.6%	11.25	21.52	75.42	12.56	12.03	42.50	27.60	29.60	0.3% of single GlcNAc detected by MALDI MS
Spiked (N + D) Herceptin IgG1	28.95	31.85	9.1%	25.88	26.65	9.7%	21.79	18.23	60.50	19.53	19.97	14.50	27.30	58.20	0% of single GlcNAc detected by MALDI MS
Spiked (N + D) Herceptin IgG1	59.37	63.69	6.8%	51.76	57.31	9.7%	45.02	21.44	43.58	27.50	28.91	17.00	34.10	48.90	0% of single GlcNAc detected by MALDI MS
Spiked (D) Herceptin IgG1	0.00	0.00	x	6.29	7.16	12.2%	8.02	12.05	92.95	0.00	7.05	75.90	0.00	24.10	0% of single GlcNAc detected by MALDI MS
Spiked (D) Herceptin IgG1	0.00	0.00	x	25.07	26.65	5.9%	22.15	16.87	77.47	0.00	22.53	55.60	0.00	44.40	0% of single GlcNAc detected by MALDI MS
Spiked (D) Herceptin IgG1	0.00	0.00	x	51.41	57.31	10.3%	43.12	24.75	63.34	0.00	36.66	42.20	0.00	57.60	0.2% of single GlcNAc detected by MALDI MS
Spiked (N) Herceptin IgG1	7.92	7.96	0.5%	0.00	0.00	x	x	x	93.17	6.83	0.00	73.90	25.80	0.00	0.3% of single GlcNAc detected by MALDI MS
Spiked (N) Herceptin IgG1	30.26	31.85	5.0%	0.00	0.00	x	x	x	77.91	22.09	0.00	56.20	43.60	0.00	0.2% of single GlcNAc detected by MALDI MS
Spiked (N) Herceptin IgG1	60.70	63.69	4.7%	0.00	0.00	x	x	x	63.62	36.38	0.00	46.40	53.60	0.00	0% of single GlcNAc detected by MALDI MS

Spiking Herceptin IgG1 glycopeptide isolate with known amounts of synthetic "**N**" and "**D**" peptides. "**N**" is EEQY**N**STYR peptide spike, "**D**" is EEQY**D**STYR peptide spike. x = not applicable (n/a), n/d = not determined.

[a] Samples are isolates of glycopeptides ("glyco-isolates") from tryptic digests by RP-HPLC.

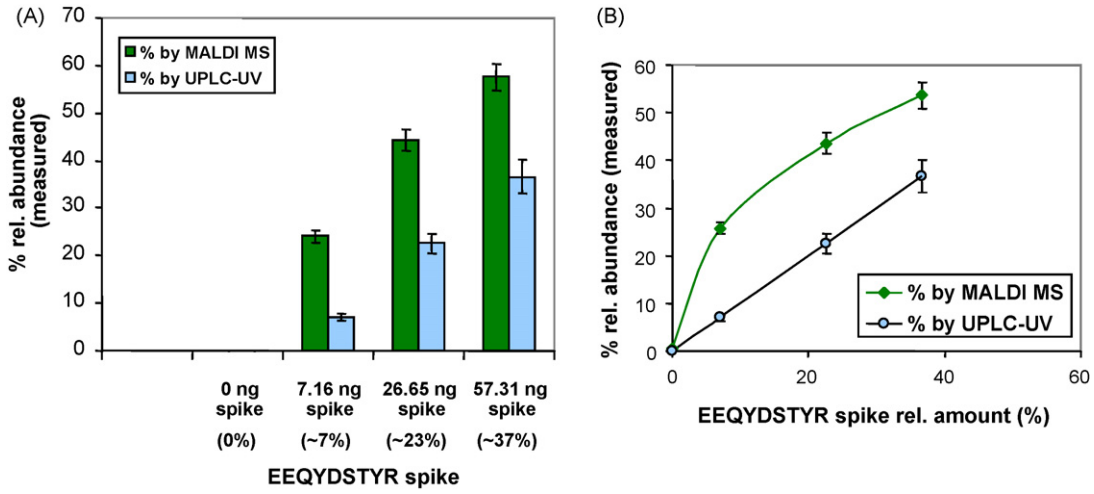

Fig. 5. Comparison of relative quantities of "D" (EEQYDSTYR) peptide in glycosylation profiles of Herceptin IgG1 (spiked with "D" peptide only), obtained by UPLC–UV and MALDI MS. (A) Bar graph representation. (B) Line chart representation. See Tables 1 and 3 for corresponding numerical data.

example in Fig. 1C and Table 1 ("no spike" column)). It was hypothesized that it could be possible to more correctly estimate relative abundances of all variants in the glycosylation heterogeneity, aglycosylated and glycosylated, after a correction for "true" abundance of de-glycosylated EEQY**D**STYR fragment. Such corrections were attempted (Karnoup, unpublished data), but it was still not clear if they were valid because it was not known if the MALDI ionization efficiency of aglycosylated fragments was different from that of glycosylated fragments. Therefore, further experiments were conducted to establish if one could meaningfully relate MALDI signal intensities of aglycosylated fragments ("N" and "D") to signal intensities of the glycosylated fragments in a total MALDI MS glycosylation profile.

3.3. Quantitative UPLC–UV–MS of glycopeptides

A separation-based methodology was employed, using high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC system ("UPLC" further in the text), with the aim of developing a method for measuring relative and absolute contents of non-glycosylated, de-glycosylated, and total glycosylated protein in IgG1 samples and to compare the UPLC-based results to those obtained by MALDI MS. HPLC with the use of a conventional reversed-phase column (C18, 15 cm × 2 mm, 4 μm particle size) did not produce desired resolution for the "N" and "D" peptides and their glycoforms, whereas an analogous "UPLC" method (C18, 15 cm × 2.1 mm, 1.7 μm particle size) did (data not shown). Although in our method the UPLC system was not operated in the truly high-pressure mode, we were able to take advantage of the smaller particle size to increase chromatographic resolution.

3.3.1. Optimization of UPLC method using binary mixture of non- and de-glycosylated peptides: calibration curves

Prior to experiments with IgG antibodies, we tested the mixtures of the synthetic "N" (EEQY**N**STYR) and "D" (EEQY**D**STYR) peptides by UPLC–UV–MS (Fig. 3). The UPLC method was optimized to yield maximum resolution for the "N" and "D" peptides. UPLC peak area ratios (using both TIC and UV detection) correctly reflected the relative concentrations of the peptides in the binary mixtures. The following relationships (calibration curves) relating peptide concentrations to UPLC peak areas (using UV, TIC, and XIC detection) were obtained. For EEQYNSTYR peptide by UPLC–UV (detection at 214 nm): $y = 85.799x$ ($R^2 = 0.9985$), where x is ng of EEQYNSTYR peptide (up to ~1400 ng), and y is UPLC–UV peak area. For EEQYDSTYR peptide by UPLC–UV (detection at 214 nm): $y = 103.42x$ ($R^2 = 0.9998$), where x is ng of EEQYDSTYR peptide (up to ~1300 ng), and y is UPLC–UV peak area. A representative curve for EEQYNSTYR peptide by

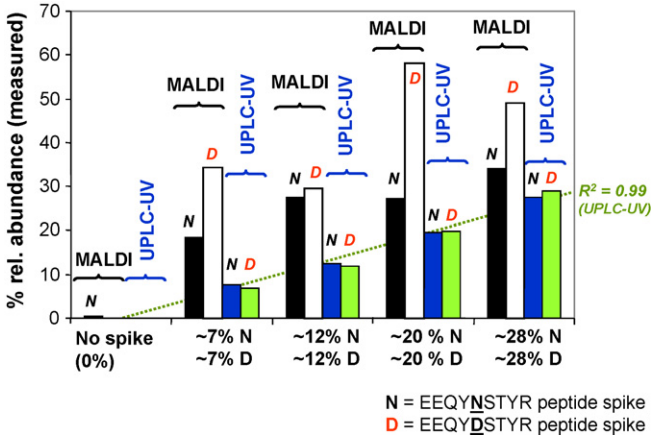

Fig. 6. Comparison of relative quantities of "N" and "D" peptides in glycosylation profiles of Herceptin IgG1 (spiked with both "N" and "D" peptides) obtained by UPLC–UV and MALDI MS. Only "N" and "D" peptide relative quantities are shown on the graph. Total quantity of glycosylated species is 100% − [quantity of "N", %] − [quantity of "D", %]. Note that when both "N" and "D" peptides are present in a sample, error in determination of relative amount of "D" peptide from its peak intensity in MALDI profiles is much greater than that in Fig. 5 due to close overlap of isotopic envelopes for the "N" and "D" peptides (there is only a +1 Da difference in the corresponding monoisotopic m/z values; even deconvolution of the mixed isotopic cluster using theoretical natural isotope abundances does not completely remediate such increased error).

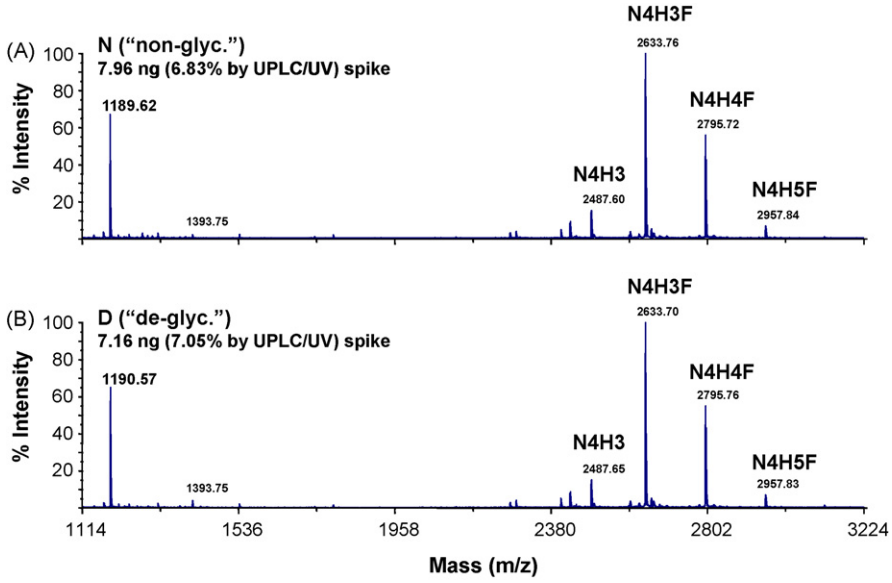

Fig. 7. Representative MALDI mass-spectra of Herceptin IgG1 "glyco-isolate" (isolated tryptic glycopeptides) spiked with low amounts of N (EEQYNSTYR) and D (EEQYDSTYR) synthetic peptides. (A) Sample was spiked with 7.96 ng of N peptide (6.83% content as determined by UPLC/UV). (B) Sample was spiked with 7.16 ng of D peptide (7.05% content as determined by UPLC/UV). See Table 1 for more detail.

UPLC–TIC: $y = 8E{-}06x^2 + 0.1246x$ ($R^2 = 0.9991$), where x is UPLC–TIC peak area, and y is ng of EEQYNSTYR peptide (up to ~1400 ng). A representative curve for EEQYDSTYR peptide by UPLC–TIC: $y = 4E{-}06x^2 + 0.1151x$ ($R^2 = 0.9899$), where x is UPLC–TIC peak area, and y is ng of EEQYDSTYR peptide (up to ~1300 ng). A representative curve for EEQYNSTYR peptide by UPLC–XIC (detection of a doubly charged ion at m/z 595.4): $y = 5E{-}05x^2 + 0.2961x$ ($R^2 = 0.9973$), where x is UPLC–XIC peak area, and y is ng of EEQYNSTYR peptide (up to ~1400 ng). A representative curve for EEQYDSTYR peptide by UPLC–XIC (detection of a doubly charged ion at m/z 595.8): $y = 4E{-}05x^2 + 0.2611x$ ($R^2 = 0.9889$), where x is UPLC–XIC peak area, and y is ng of EEQYNSTYR peptide (up to ~1300ng). UV-based calibration curves were stable (could be used for accurate quantitation purposes) for several weeks, whereas TIC- and XIC-based calibration curves were unstable and it was imperative that TIC- and XIC-based calibration curves be obtained immediately prior to measurements on actual samples.

3.3.2. LOD and LOQ for non- and de-glycosylated peptides

The limit-of-detection (LOD) and limit-of-quantitation (LOQ) values determined for the synthetic "N" and "D" peptides in water by UPLC with various detection techniques (UV214, TIC, and XIC) are summarized in Table 2. Briefly, as low as ~0.6–0.7 ng of "N" or "D" peptide could be detected by UPLC–UV (at 214 nm), as low as ~0.7 ng of "N" peptide and ~0.3 ng of "D" peptide could be detected by UPLC–TIC, and as low as ~0.3 ng of "N" or "D" peptide could be detected by UPLC–XIC.

3.3.3. Quantitation by UPLC–UV–MS: comparison to MALDI MS profiling

To investigate quantitative aspects of the UPLC-based method, a series of spiking experiments were conducted first (before using PNGase A to release *N*-glycans), in which Herceptin IgG1 glycopeptide isolate ("glyco-isolate"; tryptic glycopeptides isolated by RP-HPLC) was spiked with known amounts of synthetic "N" and "D" peptides and examined by UPLC with various detection techniques (UV at 214 nm (UV214), TIC (total ion current), and XIC (peak areas from extracted ion chromatogram)). The determined relative quantities were compared to those obtained by MALDI MS. The results of the experiments are summarized in Table 3 and Figs. 4–6. Representative MALDI MS spectra of Herceptin IgG1 glycopeptide heterogeneity spiked with low amounts (~7%) of synthetic "N" and "D" peptides are shown in Fig. 7 (see Table 1 for numerical data ("no glycan" rows)). These experiments showed that MALDI MS method significantly overestimated the relative amounts of non- and de-glycosylated peptides in the samples of isolated glycopeptides as compared to UPLC–UV method. Again, as was mentioned earlier, the dependence of relative intensity *versus* concentration of aglycosylated peptide was not linear in MALDI MS method.

3.3.4. Strategy for quantitation of glycosylated species in glycoproteins (IgG1) by UPLC–UV–MS

The following strategy for analysis was employed (schematically outlined in Fig. 8). An aliquot of an isolated pooled

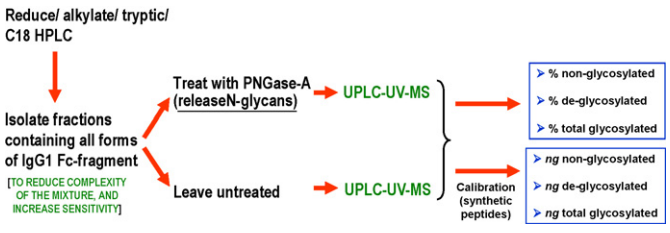

Fig. 8. General sample preparation and analysis scheme of the UPLC–UV–MS approach.

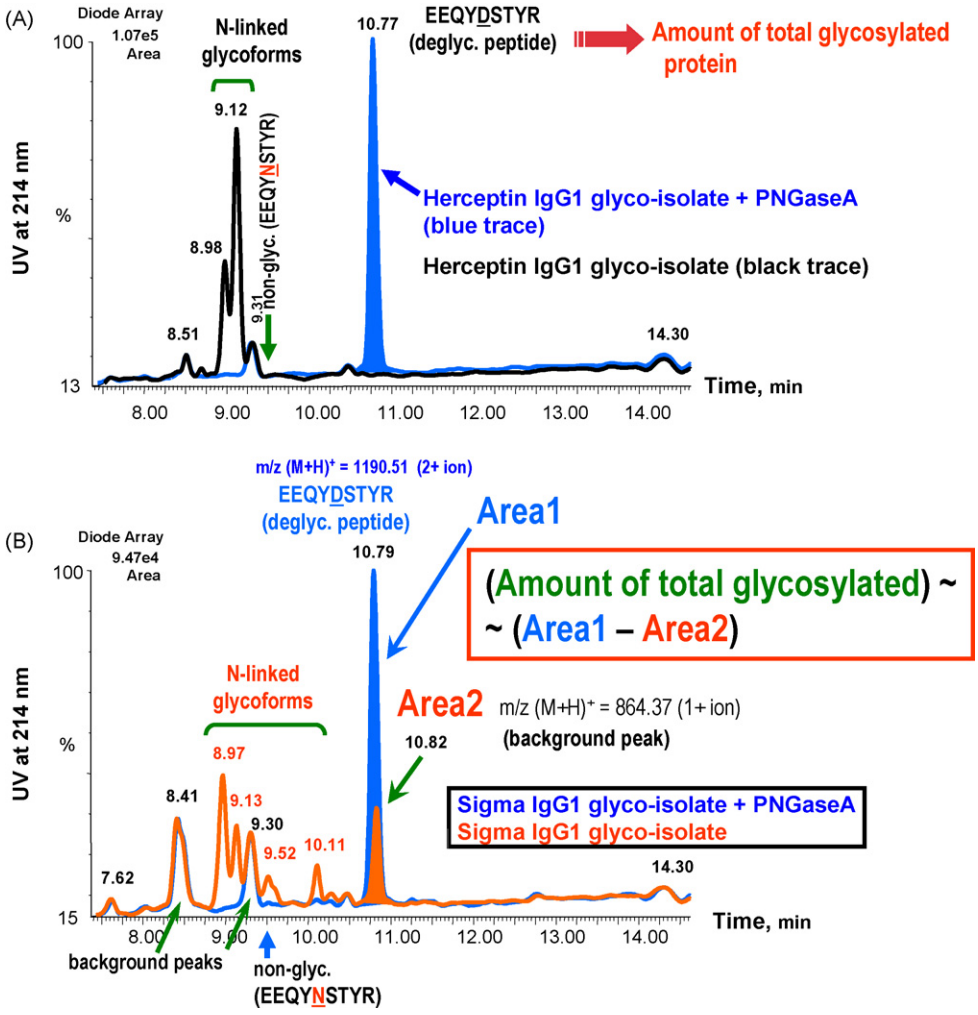

Fig. 9. (A) A pair of representative UPLC–UV chromatograms for a sample of tryptic "glyco-isolate" of Herceptin IgG1 (no spike) with and without treatment with PNGase-A. (B) A pair of representative UPLC–UV chromatograms for a sample of tryptic "glyco-isolate" of Sigma IgG (no spike) with and without treatment with PNGase-A. The results illustrate the approach outlined in Fig. 8.

fraction containing IgG1 Fc-region fragment variants (including non- and de-glycosylated species) was run by UPLC. An identical aliquot was treated with PNGase A enzyme to release *N*-linked glycans, and also run by UPLC, along with a "blank" chromatogram. The UPLC peak areas in these two chromatograms were normalized to that of non-glycosylated EEQYNSTYR ("N") fragment. The first chromatogram contained peaks for non-glycosylated fragment and de-glycosylated fragment that was due to glycoprotein degradation. The second chromatogram (after release of *N*-glycans) contained peaks for non-glycosylated fragment and de-glycosylated fragment that was due to both release of *N*-glycans from all of the present glycoforms and the glycoprotein degradation. Under appropriate conditions, it was therefore possible to determine the relative % of non-glycosylated, de-glycosylated (due to degradation), and total glycosylated variants based on these chromatograms. Absolute amounts of "N" and "D" fragments were obtained from the calibration curves (described earlier in the text). Fig. 9 illustrates using this approach for Herceptin IgG1 and Sigma IgG (IgG1 tryptic "glyco-isolates"). When UPLC has enough resolving power, it is possible to inject the whole tryptic digest on the UPLC column directly, without isolating glycopeptides first, as shown in Fig. 10(C and D). However, isolating the glycopeptides first (off a semi-preparative C18 column) allowed us to reduce the overall complexity of the samples and increase the glycopeptide load onto UPLC.

3.3.5. *Interferences during UPLC–UV–MS analysis*

Because no interferences with "N" (non-glycosylated) and "D" (de-glycosylated) peptides in the UPLC–UV background were observed for Herceptin IgG1 (*i.e.* no other compounds co-eluted with "N" and "D" peptides), the data analysis for this sample was straightforward. The UPLC–UV peak area corresponding to the amount of generated de-glycosylated "D" peptide (via de-glycosylation reaction) allowed a direct determination of the amount of total glycosylated peptides (Fig. 9A) using the UPLC–UV calibration curve for EEQYDSTYR peptide. In the case of Sigma IgG1, there was an interference of another peptide co-eluting with the de-glycosylated ("D", EEQY**D**STYR) peptide (Fig. 9B). In the absence of de-glycosylated species in the original ("before PNGase A") sample, the amount of total glycosylated peptides (protein)

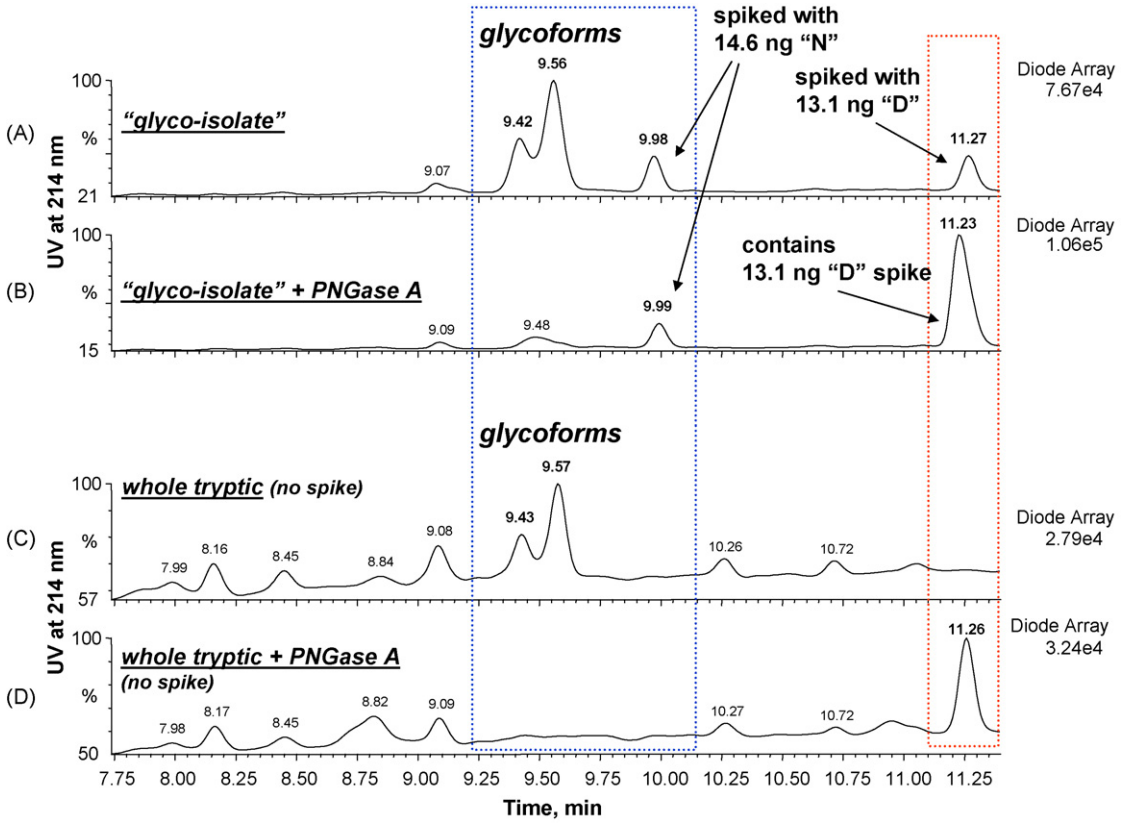

Fig. 10. Representative UPLC chromatograms of Herceptin IgG1 glycopeptide heterogeneity. (A) A sample of trypric glycopeptides isolated by RP-HPLC was spiked with 14.6 ng of "N" (EEQYNSTYR) and 13.1 ng of "D" (EEQYDSTYR) peptides. (B) Sample in (A) treated with PNGase A to release *N*-glycans and convert previously glycosylated peptide to "D" peptide. (C) Unspiked whole tryptic digest of Herceptin IgG1 before PNGase A treatment. (D) Unspiked whole tryptic digest of Herceptin IgG1 after PNGase A treatment.

is directly derived from the UPLC–UV peak area difference between "with PNGase A" and "without PNGase A" sample injections (using the calibration curve for EEQY**D**STYR peptide). The deviations of determined absolute amounts from the known amounts (spiked in) were between 2 and 9% (typical ~5%) for the "N" peptide and between 0.3 and 14% (typical ~10%) for the "D" peptide when determined using UPLC–UV. When UPLC–XIC was used, the corresponding deviation was 3–51% for the "D" peptide and slightly less for the "N" peptide. Such inaccuracy of the XIC-based quantitation (without using internal standards) is attributed to instability of the XIC calibration curves and the fact that XIC calibration curves obtained for pure peptides in water may not be directly applicable to peptides in complex mixtures/ matrices. Although XIC-based quantitation of unlabeled peptides has been used to obtain rough estimates of protein abundance (typically within a factor of 3–5 of the true value) in proteomic experiments [13], more accurate measurements are required in analytical characterization of biopharmaceutical substances, such as therapeutic MAbs. Therefore, although the XIC-based quantitation is specific (only signal from a particular ion is taken into account), it is far less accurate than the UV-based quantitation. In both cases, the areas of the background chromatographic peaks did not noticeably change between injections of "with PNGase A" and "without PNGase A" samples.

3.3.6. UPLC–UV–MS analysis of samples containing aglycosylated protein

Glycoprotein samples represented in Fig. 9 were fully glycosylated and originally did not contain appreciable amounts of non- or de-glycosylated species. This made the quantitative analysis relatively straightforward. To simulate a situation in which both non-glycosylated ("N", EEQY**N**STYR) and de-glycosylated ("D", EEQY**D**STYR) fragments are present in the original sample along with the glycosylated species, Herceptin IgG1 and Sigma IgG1 tryptic glycopeptide isolates were spiked with known amounts of synthetic "N" and "D" peptides and analyzed with UPLC–UV and UPLC–MS before and after treatment with PNGase A as described above. The results are summarized in Table 4. It can be seen from Table 4 that addition of PNGase A did not have any effect on quantitation of "N" and "D" peptides (PNGase A was added to a solution with known amounts of N and D and analyzed by UPLC–UV). Again, quantitation was relatively straightforward when there were no chromatographic interferences (producing signal in UV at 214 nm and in TIC) with the de-glycosylated ("D") and non-glycosylated peptides, such as in the case of spiked Herceptin IgG1 samples. In the cases when such interferences occurred (in spiked Sigma IgG1 samples), it was impossible to perform all calculations based solely on UV214 peak areas, and we had to resort to using XIC (at *m/z* 595.8 for the "D" peptide) to isolate the signal specifically

Table 4
Examples of determination of amounts of non-glycosylated, de-glycosylated, and total glycosylated protein in spiked IgG1 samples

Sample description[a]	"N" UPLC–UV peak area (average)	ng "N" from UPLC–UV calibration	% deviation from known amount (13.47 ng "N")	Total "D" UPLC–UV peak area (average)	Coelution with "D" peptide?	ng total "D" from UPLC–UV calibration	% deviation from known amount (12.12 ng "D") (only before de-glycosylation)	ng total (average) "D" from calibration by XIC (*m/z* 595.8)	% deviation of XIC-determined amount from known amount (12.12 ng "D") (before de-glycosylation)	Total glycosylated UPLC peak area = Δde-glycosylation[b]	Sum of UPLC peak areas for non-glycosylated ("N"), de-glycosylated ("D"), and total glycosylated ("100%")	% total glycosylated in original sample (calculated)	% non-glycosylated ("N") in original sample	% de-glycosylated ("D") in original sample	Comment
Herceptin IgG1 + N + D	1204.48	12.96	3.79	1212.95	No	12.08	0.33	12.53	3.42	x	8987.88	**73.10**	**13.40**	**13.50**	Original (spiked) sample
Herceptin IgG1 + N + D + PNGaseA	1190.16	12.81	4.90	6226.06	No	62.01	x	91.46	x	6570.45	x	x	x	x	De-glycosylation reaction
Herceptin IgG1 + N + D	1290.71	13.82	5.32(14.6 ng spiked)	1357.56	No	13.13	0.02(13.1 ng spiked)	6.47	50.71	x	10005.62	**73.53**	**12.90**	**13.57**	Original (spiked) sample
Herceptin IgG1 + N + D + PNGaseA	1305.86	14.31	1.96	7573.18	No	73.23	x	62.38	x	7357.35	x	x	x	x	De-glycosylation reaction
Herceptin IgG1 whole tryptic (no spike)	0.00	0.00	x	0.00	No	0.00	x	x	x	x	977.27	**100.00**	**0.00**	**0.00**	Original sample
Herceptin IgG1 whole tryptic (no spike) + PNGaseA	0.00	0.00	x	977.27	x	9.45	x	6.02	x	977.27	x	x	x	x	De-glycosylation reaction
Sigma IgG1 + N + D + PNGaseA	1316.60	14.17	5.20	5336.50	*m/z* 864.5 (*M* + 2H) co-elutes with "D" peak	51.75	x	78.10	x	x	x	x	x	x	De-glycosylation reaction
Sigma IgG1 + N + D	1190.03	12.81	4.90	1152.33	*m/z* 864.5 (*M* + 2H) co-elutes with "D" peak	11.18	7.76	14.07	16.13	x	6526.53	**64.11**	**18.23**	**17.66**	Original (spiked) sample
N + D + PNGaseA	1192.81	12.84	4.68	1214.42	x	11.77	2.89	14.38	18.66	x	x	x	x	x	Mix of two peptide standards + PNGaseA

"**N**" is EEQY**N**STYR peptide spike, "**D**" is EEQY**D**STYR peptide spike. x = not applicable (n/a).

[a] Samples are isolates of glycopeptides ("glyco-isolates") from tryptic digests by RP-HPLC.

[b] Signal normalized to "N" peptide peak area.

from the EEQY**D**STYR ("D") peptide to estimate its quantity. This approach introduced significant inaccuracies in the calculations. However, the rough estimates obtained in this way were still better than those obtained via MALDI MS glycopeptide profiling.

The developed methodology was successfully applied in our laboratory to analysis of a number of plant-expressed IgG1 antibodies (data not shown). In addition, the developed methodology was successfully applied to analysis of other plant-expressed glycoproteins that were not related to antibodies. In all cases, non- and de-glycosylated protein fragments were detected and quantified relative to total glycosylated protein fragments. In particular, using our methodology, a commercially available maize-expressed recombinant chicken avidin (Sigma) was found to be fully glycosylated.

4. Conclusions

The experiments showed that MALDI MS method significantly overestimated the relative amounts of non- and de-glycosylated peptides in the samples of isolated glycopeptides as compared to UPLC–UV method. In addition, the dependence of relative intensity *versus* concentration of aglycosylated peptide was not linear in MALDI MS method. MALDI MS glycosylation profiling can be used to obtain reasonable quantitative estimates within the pool of glycosylated peptide/protein (with neutral glycans), but agycosylated species cannot and should not be quantitatively included in such a profile. When a quantitative measurement or estimate of aglycosylated species is required, an HPLC-based method can be used.

Evaluation of the HPLC-based methodology was carried out in this work to quantitatively measure non-glycosylated, deglycosylated, and total glycosylated species for glycoproteins. The methodology has its advantages and limitations. Quantitation in an HPLC-based method can be achieved using various types of post-LC detection techniques, such as UV absorption, total ion current (TIC), or using extracted ion chromatogram (XIC). A Waters UPLC system and a small particle size (1.7 μm) column were used for HPLC separations. Although in our work the UPLC system was not operated in the truly high-pressure mode, we were able to take advantage of the smaller particle size to gain extra chromatographic resolution.

Quantitation by UPLC–UV was found to be accurate, sensitive, linear, reproducible, but often non-specific (in cases when co-elution of peptides to be quantitated is occurring). It can be successfully used when a UPLC method allows separation of all components of interest into individual LC peaks, without interferences. Quantitation by UPLC–TIC was found neither specific, nor reliable/reproducible, nor accurate. Quantitation by UPLC–XIC can be specific (often only signal from a particular ion can be isolated), but it is not nearly as accurate, reproducible/reliable, and linear as UPLC–UV. For accurate quantitation using a UPLC–MS-based method, isotopically substituted analogues of peptides of interest can be used as internal standards [14]. This is a promising avenue of development for quantitation of the glycosylation microheterogeniety of various glycoproteins, and such experiments are currently underway in our laboratory.

Acknowledgements

We greatly acknowledge Marsha Langhorst and Demetrius Dielman for technical assistance.

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