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Quantitative structural characterization of local N-glycan microheterogeneity in therapeutic antibodies by energy-resolved oxonium ion monitoring.

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Abbreviations

The abbreviations used are: IgG, immunoglobulin G; FDA, United States Food and Drug Administration; Hex, hexose; Man, mannose; HexNAc, N-acetylhexosamine; GlcNAc, N-acetylglucosamine; Fuc, fucose; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; MRM, multiple reaction monitoring; 2-AP, 2-aminopyridine; TFA, trifluoroacetic acid; CE, collision energy;

Abstract

Site-specific characterization of glycoform heterogeneity currently requires glycan structure assignment and glycopeptide quantification in two independent experiments. We present here a new method combining multiple reaction monitoring mass spectrometry with energy-resolved structural analysis, which we termed “Energy-resolved oxonium ion monitoring”. We demonstrated that monitoring the yields of oligosaccharide-derived fragment ions (oxonium ions) over a wide range of CID energy applied to a glycopeptide precursor exhibits a glycan structure-unique fragmentation pattern. In the analysis of purified immunoglobulin glycopeptides, the energy-resolved oxonium ion profile was shown to clearly distinguish between isomeric glycopeptides. Moreover, LOD of glycopeptide detection was 30 attomole injection and quantitative dynamic range spanned 4 orders magnitude. Therefore both quantification of glycopeptides and assignment of their glycan structures was achieved by a simple analysis procedure. We assessed the utility of this method for characterizing site-specific N-glycan microheterogeneity on therapeutic antibodies, including validation of lot-to-lot glycoform variability. A significant change in the degree of terminal galactosylation was observed in different production lots of trastuzumab and bevacizumab. Cetuximab Fab glycosylation, previously known to cause anaphylaxis, was also analyzed and several causative antigens including Lewis X motifs were quantitatively detected. The data suggests that energy-resolved oxonium ion monitoring could fulfill the regulatory requirement on the routine quality control analysis of forthcoming biosimilar therapeutics.

Introduction

Foreseeing the patent expiry of several therapeutic antibodies that sold more than \$30 billion worldwide in 2009,¹ there is growing interest over how biosimilar substitutes could win FDA-approval in the near future.² The first draft guidance on the evaluation of biosimilarity was published by FDA in Feb 2012,³ in which emphasis was placed on the importance of evaluating minor structural differences that can significantly affect the potency and safety of biopharmaceuticals, with specific reference to glycosylation patterns. This implicates the need for precise and routine structural characterization of biopharmaceuticals. With regard to therapeutic antibodies which have a common N-glycosylation consensus sequence at Asn₂₉₇ in the conserved (Fc) region of heavy chain, some specific features of N-glycosylation have been characterized to affect their potency and safety. For example, the absence of core fucosylation showed enhanced antibody-dependent cellular cytotoxicity by 10-fold,⁴ Moreover, recent studies revealed that non-human oligosaccharide motifs such as glycolylneuraminic acid (Neu5Gc)⁵ and galactose- α 1,3-galactose (α -Gal epitope)^{6,7} are immunogenic and can cause anaphylaxis in patients expressing specific IgE.⁸ Further characterization elucidated more specifically that immunogenicity of α -Gal epitope was primarily attributed to an extra N-glycan occurring within the antigen-binding (Fab) region.⁹ These findings have raised the issue of antibody glycosylation to the level that global picture of its heterogeneity and biological impact is urgently needed.

Therefore we developed and describe herein a novel mass spectrometric (MS) method for analyzing the local microheterogeneity of antibody glycosylation. The method involves tryptic digestion of the antibody followed directly by MS analysis at the glycopeptides level, which enables simultaneous and independent

characterization of multiple glycosylation sites. The common drawbacks experienced in MS analysis of glycopeptides are the lack of sensitivity of glycopeptides in mixture with non-glycosylated peptides¹⁰ and, importantly, the difficulty in verifying the glycan structures by MS/MS analysis of glycopeptides, which in most cases can only determine the oligosaccharide composition. To address these problems we modified the methodology of multiple reaction monitoring (MRM) using triple-quadrupole mass spectrometer into a novel concept which we termed “energy-resolved oxonium ion profiling”. Oxonium ions are a series of oligosaccharide-derived fragment ions generated by collision induced dissociation (CID),^{11,12} We iteratively scanned oxonium ions at various collision energy and plotted the ion yield against the collision energy. This “energy-resolved” approach is known to strengthen structural interpretation of analytes based on differential activation energy of fragmentation,¹³⁻¹⁵ with potential application to characterization of oligosaccharide structures.^{16,17} Hence we examined whether the pattern of energy-resolved oxonium ion profile might be unique to the glycan structure of glycopeptides by acquiring the oxonium ion profiles of well-characterized standard glycopeptides. Next we showed the lot-to-lot heterogeneity in trastuzumab (Herceptin) and bevacizumab (Avastin) as a worked example of routine analysis of therapeutic antibodies for quality control. Moreover energy-resolved oxonium ion monitoring enabled simultaneous characterization of cetuximab glycoforms at both Fc and Fab glycosylation sites, demonstrating putative anaphylaxis-inducing glycan structures of non-human origin.

EXPERIMENTAL SECTION

Reagents – Trastuzumab (Herceptin) and bevacizumab (Avastin) were purchased from Chugai Seiyaku (Osaka, Japan). Cetuximab (Erbix) was purchased from Merck Serono (Tokyo, Japan). Human IgG, iodoacetamide, iodoacetic acid and ammonium bicarbonate were purchased from Sigma-Aldrich (Saint Louis, MO). Dithiothreitol, urea and PD-Miditrap desalting column was purchased from GE Healthcare (Buckinghamshire, UK). Trypsin Gold and ProteaseMAX was supplied by Promega (Madison, WI). All other unspecified reagents were products of Wako Pure Chemicals (Osaka, Japan).

Preparation of therapeutic immunoglobulin – 80 µg of trastuzumab, bevacizumab or cetuximab was dissolved in 33 µL of 8 M urea and dithiothreitol and ammonium bicarbonate was added to final concentration of 5 mM and 50 mM, respectively. After 30-minute incubation in 55 °C, reduced cysteine residues were alkylated in 50 mM iodoacetic acid at room temperature for 30 minutes. The product was diluted 10 times in 2.5 mM dithiothreitol 25 mM ammonium bicarbonate before addition of 4 µg mass spectrometric grade endoproteinase Lys-C (Wako Pure Chemicals) for digestion at 37 °C for 2 hours. The Lys-C digest was further digested with addition of 4 µg Trypsin Gold and allowed to react for additional 4 hours. The digest was desalted with OASIS HLB cartridge, eluting with 15% acetonitrile, and the eluate was diluted 5 times with 0.1% acetic acid for analysis by nanoHPLC-MRM.

Preparation of glycopeptide standards – For the preparation of glycopeptide standards, lyophilized powder of IgG was dissolved at 1.5 mg/mL in 50 mM Tris-HCl pH 7.5, 8 M urea. The solution was reduced and alkylated prior to trypsin digestion by 10 mM dithiothreitol followed by 50 mM iodoacetamide. The mixture was desalted by PD-Miditrap desalting column to 100 mM ammonium bicarbonate. 20 µg of

Trypsin Gold was added to every 1 mg of IgG and the mixture was incubated at 37 °C for 12 hours. Tryptic digest was aliquoted after desalting by OASIS HLB cartridge (Waters Corporation, Milford, MA) and the aliquots were dried by SpeedVac for long-term storage. Homogeneous glycopeptides were prepared by two-dimensional HPLC fractionation using Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan). The first dimension was performed with 4.6 mm x 500 mm Cadenza CD-C18 column (Imtakt Corporation, Kyoto, Japan). Using 0.2% TFA (Tokyo Chemical Industry, Tokyo, Japan) as solvent A and 75% acetonitrile 0.1% TFA as solvent B, IgG tryptic digest was separated by 5-20% solvent B gradient in 90 minutes at a flow rate of 0.6 mL/min. Collected fractions were dried, reconstituted in 0.1% TFA and further fractionated by 150 mm x 4.6 mm SunShell C18 column (Chromanik Corporation, Osaka, Japan). Solvent A was 6% acetonitrile with 0.1% TFA and Solvent B was 12% acetonitrile with 0.1% TFA and separation was performed over 10-90% solution B gradient.

Fluorescent HPLC – Portion of each glycopeptide fraction was treated with 0.5 U of N-glycosidase F (Roche Diagnostics, Basel, Switzerland) dissolved in 0.01% ProteaseMAX solution and incubated at 37 °C for 8 hours. Released glycans were dried in SpeedVac and derivatized with 2-aminopyridine (2-AP) by reductive amination as described elsewhere. 2-AP labelled oligosaccharides were purified by MassPREP HILIC μ Elution plate (Waters Corporation) according to the manufacturer's protocols but using 90% acetonitrile 100 mM triethylammonium bicarbonate for washing and 25% acetonitrile 100 mM triethylammonium bicarbonate for elution. The separation of 2-AP labeled oligosaccharides was carried out by RP-HPLC with Shimpack CLC-ODS 0.6 x 150 mm column (Shimadzu Corporation). Solvent A was 10 mM sodium phosphate buffer pH 3.8 and Solvent B was same

buffer containing 0.5% 1-butanol. After equilibrating the column with 20% solvent B, the oligosaccharides were eluted by raising the concentration of solvent B to 50% in 75 minutes. 2-AP labelled oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. The retention times were normalized and expressed as glucose units by using PA-glucose oligomer (Takara Bio Inc., Shiga, Japan) and glycan structures were verified according to the HPLC mapping data as described elsewhere.¹⁸

Quantification of glycopeptide standards – Glycopeptide fractions which were successfully purified at high yield and structural homogeneity were next subjected to quantification by UV absorbance following HPLC separation. The accuracy of quantification was guaranteed by using lyophilized synthetic peptide EEQYNSTYR as the standard for calibration curve. Two species of glycopeptides were successfully quantified and were used as calibration standards for assessing the quantification by oxonium ion monitoring (Figure 2b). Standard concentration was adjusted to 100, 33.3, 11.1, 3.70, 1.23, 0.41, 0.14, 0.045, 0.015 and 0.005 fmol/ μ L with 0.1% TFA containing 50 fmol/ μ L (final concentration) of EEQYNSTYR as the internal standard and analyzed by nanoHPLC-MRM as described in the next subsection.

Multiple reaction monitoring of oxonium ions – The energy-resolved oxonium ion profiling and glycopeptide quantification by m/z 138 oxonium ion monitoring was performed using 4000QTRAP triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) with Agilent 1200 nano-HPLC system (Agilent Technologies, Palo Alto, CA). Glycopeptide samples were separated by a 75 μ m x 200 mm ESI sprayer tip packed with 3 μ m C18 resin (Nikkyo Technos, Tokyo, Japan). The compositions of solvent A and B were 0.1% formic acid and 70% acetonitrile 0.1% formic acid,

respectively, and the flow rate was 250 nL/min. The column was equilibrated with 20% solvent B and glycopeptides were separated isocratically for 3 minutes, after which the remainder was eluted by 5 minutes gradient from 20% to 95% solvent B. The fixed instrumental settings were as follows: 2200 V ionization spray voltage; 12 psi curtain gas (N_2); CAD = 4; 70 V declustering potential; 10 V entrance potential; Q1 resolution, HIGH; Q3 resolution, LOW; and 2 ms pause in between. For energy-resolved data acquisition, analysis transitions were designed such that there were several transitions for the same Q1 and Q3 mass combination, differing only by Collision Energy settings (in steps of 3 eV or 5 eV). Dwell times were adjusted to the total cycle time of 1- 1.5 seconds. For relative quantification of glycopeptide microheterogeneity, precursor ion m/z were derived in-silico, covering all possible glycan compositions, and a single quantitative transition was monitored for each precursor ion (Q3 m/z = 138, CE = predicted optimum collision energy, as described in text). Only for generating the calibration curves for determining the sensitivity and dynamic range of detection (Figure 2b), synthetic peptide EEQYNSTYR was co-injected and the following internal standard transitions were monitored for normalization: Q1 m/z = 595.4, Q3 m/z = 640.4, CE = 38; Q1 m/z = 595.4, Q3 m/z = 526.4, CE = 30.

Data analysis – The acquired raw data was processed with MultiQuant version 2.02 (AB Sciex, Foster City, CA). The mass chromatogram of each transition was integrated after Gaussian Smoothing (σ = 1 point) and quantified by peak area. For energy-resolved oxonium ion profiling, the dataset for each glycopeptide was normalized with respect to the highest intensity of Q3 m/z=138 and expressed as percentage. Analyses were performed in triplicates and average normalized percentage was computed. Optimum collision energy was defined as the voltage

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setting to achieve the maximum percentage, unless when series of 98-100% was observed as a plateau, in which case the average collision energy was regarded as optimum. For relative quantification of glycopeptide microheterogeneity, the percentage composition of each component was calculated with respect to the sum of all components combined. The data for plotting the calibration curves (Figure 2b) was generated by normalizing the glycopeptide peak area by the average of two internal standard transitions.

RESULTS AND DISCUSSION

Energy-resolved oxonium ion profile

Figure 1a shows the scheme to acquire energy-resolved yield curve of oxonium ions using a triple quadrupole mass spectrometer. Briefly, the collision energy applied in the Q2 cell was changed in stepwise fashion in repetitive measurements of short time intervals for each mass filter settings. We termed simultaneous acquisition of oxonium ion yield curves as energy-resolved oxonium ion monitoring technology and examined whether this profile could be utilized for determination of glycan structures on a particular glycopeptide. We first prepared a series of glycopeptides from human IgG by chromatographic separation. The isolated glycopeptides shared the same amino acid sequence EEQYNSTYR but bore different glycan structures. For each glycopeptide, energy-resolved yield curves of 5 selected oxonium ions were simultaneously acquired and displayed on the same plot, namely m/z 138 (internal fragment of GlcNAc), m/z 204 (GlcNAc), m/z 366 (Hexose + GlcNAc), m/z 163 (Hexose) and m/z 274 (Neu5Ac - H₂O). The results as displayed in Figure 1b-f convey an interesting feature of the oxonium ion profiles, that they represent the glycan structure as a whole rather than simple summation of possible bond breakage events. The non-galactosylated glycoforms (Figure 1b) are characterized by explicitly high yield of m/z 204 (GlcNAc) than other glycoforms bearing the same number of GlcNAc. Moreover, the high yield of oxonium ion m/z 163, which is unique to the high-mannose type (Figure 1c) cannot be explained by the number of mannosyl linkages alone because hybrid type (Figure 1d) structures hardly produce m/z 163. A possible explanation of this is that mannose or galactose which is in proximity to GlcNAc lacks the labile proton needed for oxonium ion formation.

Most striking was the different profile acquired from independent analysis of isomeric glycopeptides with glycan composition 4[Hex]4[GlcNAc]1[Fuc] (structure validation by fluorescent HPLC supplied Figure S1, Supporting Information), differing by terminal galactose linked either to α 1-3 branch or α 1-6 branch (Figure 1f). These glycans share the same types of glycosidic linkages but exhibited distinct energy-resolved patterns, suggesting that galactose present on α 1-6 branch contributed to higher yield of m/z 366 oxonium ion at low collision energy than when present on α 1-3 branch. Previous examples of energy-resolved mass spectrometry suggested that the maximum of yield curve indicate the ease of CID event to generate the product ion.¹⁵ Extending this view, the different maxima observed in m/z 366 curves (Figure 1f) are indicative of one bond being more stable than the other, despite that the bonds responsible for m/z 366 production, GlcNAc β (1-2)Man, are locally identical in both structures.

From these results we conclude that bond stability in glycans is governed by the overall spatial arrangement, and the energy-resolved oxonium ion profile reflects the shape of the glycan rather than its composition. The notion of precise glycoform identification by relative quantification of fragment ions is not a novel one¹⁹ but its application has been limited to detached N-glycans. Here we showed that energy-resolved oxonium ion monitoring can identify glycoforms attached to peptides by focusing on low molecular weight oxonium ions, which occur at the same m/z for any glycopeptides, and differentiating between structures by collision energy-resolved profiles. Multiple reaction monitoring by triple-quadrupole mass spectrometer complies with this purpose because it does not exert a low-mass cutoff effect which otherwise alters the signal yield depending on the size of intact glycopeptides. Therefore we expect to observe the same energy-resolved oxonium ion profiles for

the same glycoform regardless of the peptide amino acid sequence. Furthermore, this energy-resolved approach can potentially be used to estimate the percentage composition of isomeric glycopeptides without requiring chromatographic separation. The energy-resolved yield curves of 1:1 and 1:2 mixtures of the two isomers showed curve shapes that fell between the two reference curves (Figure S2, Supporting Information).

High-sensitivity quantification of glycopeptides

A notable observation from various energy-resolved profiles was that the yield curve shapes of oxonium ion m/z 138 were similar among the tested glycan structures. We speculated that m/z 138 originates from the GlcNAc of the core structure which was conserved among all N-glycans and therefore oxonium ion m/z 138 under optimum collision energy would serve as an ideal reporter ion for quantifying glycopeptides of various glycan structures. To clarify this we plotted the collision energy at which m/z 138 was maximal against the m/z of various glycopeptides (Figure 2a). The result suggested a strong linear correlation ($R^2 = 0.842$), which was independent of the number of GlcNAc in the glycan composition or the peptide amino acid sequence. From these results it seemed adequate to assume that the collision energy to yield maximum m/z 138 signal can be predicted from the m/z of glycopeptide, and that m/z 138 signal acquired in this way would best represent the quantity of the glycopeptide. Furthermore, since the relative intensity of m/z 138 at its maximum was generally higher than other oxonium or peptide-derived fragment ions, it was expected that monitoring m/z 138 would enhance the sensitivity of glycopeptide detection. This was demonstrated by the standard curve of two glycopeptide standards, each composed of different and homogeneous glycan

structure and concentration determined by UV absorbance (Figure 2b). The linear quantitative range of the glycopeptides were 30 amol to 30 fmol injection, spanning 4 orders of magnitude. Surprisingly, the sensitivity of glycopeptide detection was about 10 times more than the synthetic non-glycopeptide EEQYNSTYR which was co-injected as an internal standard. This result is contrary to the common notion that glycopeptides have relatively low ionization efficiency,²⁰ and implicates the overwhelming capability of our technology in detecting glycopeptides at high sensitivity and quantitateness in mixture with non-glycopeptides. Moreover, the yield of m/z 138 is maximized at much higher collision energy than conventionally used for MRM.²¹ Under this optimum condition, production of other oxonium ions or peptide backbone fragmentation is suppressed, which effectively reduces the background signal and further contributes to selective detection of glycopeptides.

Lot-to-lot heterogeneity of trastuzumab and bevacizumab

We next evaluated the applicability of the oxonium ion monitoring technology, taking advantage of the highly quantitative detection of glycopeptides, to routine lot-to-lot quality control of therapeutic antibodies by analyzing four lots of trastuzumab (Herceptin) and bevacizumab (Avastin). The glycoform heterogeneity was quantified and the relative abundances of three most abundant glycoforms were presented on Figure 3 to illustrate the difference among the production lots. The quantification results of other glycoforms were given in Supplementary Table S1 and S2. Analysis was performed in three independent preparations for each lot, and statistical significance of the difference between two consecutive lots was evaluated by Student's t-test. The mean coefficient of variation of triplicate analysis was 4.7% for all measurements with more than 0.1% relative abundance, and the quantitative

linearity over 4 orders of magnitude enabled the detection of minor glycoforms of as low as 0.05% relative abundance. As a result, the frequency of galactosylation onto the biantennary GlcNAc was shown to be prone to significant lot-to-lot variation in both trastuzumab and bevacizumab (Figure 3a and Figure 3b, respectively, $P < 0.05$ by Student's t-test). Convincingly, the observed variations in glycoform composition were interrelated in the way that the decrease in galactosylated form was compensated by the increase in agalactosylated form. The smallest statistically significant variation observed here was 1.2-fold between Lot3 and Lot4 of bevacizumab in the abundance of di-galactosylated form (1.29% and 1.51%, respectively, $P < 0.05$ by Student's t-test). Surprisingly, we observed that bevacizumab contained a fraction of non-glycosylated form (EEQYNSTYR detected without glycosylation) and that this fraction also varied among lots. These results outline the continual lot-to-lot variation of glycoform heterogeneity in both trastuzumab and bevacizumab, and demonstrate that m/z 138 oxonium ion monitoring can accurately evaluate whether the glycoforms of therapeutic antibodies between lots are identical.

Analysis of immunogenic glycoforms on Fc and Fab of cetuximab

We next applied energy-resolved oxonium ion monitoring for site-specific glycoform characterization of therapeutic antibody cetuximab for identification of potentially immunogenic glycoforms. Figure 4a shows the MRM chromatogram of cetuximab digest, monitoring m/z 138 for the quantification of all possible glycoforms. A series of glycopeptides derived from Fc region (EEQYN₂₉₇STYR) or Fab region (MNSLQSN₈₈DTAIYYCAR) were eluted at 13 - 14 minutes or 17 - 19 minutes, respectively. Glycoforms on both sites were thus characterized simultaneously in a

single analysis. The most major glycoform in Fc glycan was agalactosylated biantennary structure, and relatively high level of hybrid and high-mannose structures were also detected (results summarized in Figure S3, Supporting Information). Focusing on the characterization of its Fab glycan, which has been reported as the primary cause of anaphylaxis by cetuximab,⁹ we quantified all theoretically available combinations of glycoforms with potentially immunogenic glycan motifs and verified their structures by energy-resolved oxonium ion profiling. As a result, we discovered that a Lewis X antigen was also present on cetuximab along with previously reported α -Gal and Neu5Gc antigens (Figure 4b). The Lewis X motif was verified by the energy-resolved yield curves including the structure-specific oxonium ion of m/z 512 (Gal-GlcNAc-Fuc), which clearly discriminated the two almost isomeric glycopeptides with Lewis X or Neu5Gc motifs. The result of quantification was summarized in Table 1, showing the panel of biantennary and triantennary glycan structures with Lewis X, α -Gal and/or Neu5Gc modification. Since the total number of modification never exceeded the number of antennae, it is most likely that addition of fucose to antennal GlcNAc (Lewis X), addition of galactose to terminal galactose (α -Gal) and addition of Neu5Gc to terminal galactose (Neu5Gc) occur in a competitive fashion, resulting in the occurrence of all possible combinations of the antigenic motifs. Considering the fact that 1-2% of patients administered with cetuximab experience severe hypersensitivity reactions,²² and potentially at much higher rate,²³ it is important to fully elucidate the cause of this hypersensitivity and strictly regulate it to minimize the risk. The implications of the variety of immunogenic glycoforms which has previously been overlooked would require revisiting.

Conclusions

We developed the new technology that combined multiple reaction monitoring mass spectrometry and energy-resolved structural analysis. It focuses on how the yields of commonly observed fragment ion signals change under various fragmentation conditions and uses this information as a new cue to replace the conventional notion of finding structure-specific signature ions for glycoform identification.^{24,25} The advantage of this technology over other methods is twofold. First, the collision energy-resolved oxonium ion profiles uniquely represent glycan structures on tryptic glycopeptides. It is therefore suited for site-specific analyses for characterizing glycoproteins with multiple glycosylation sites. Moreover, the depth of structural information covers differentiation of isomeric structures, which currently requires glycan detachment. By accumulating more reference data of glycopeptides with validated glycan structures, it should be possible in near future to program a matching system to assign unknown data with the glycan structure. Second, detection of glycopeptides by monitoring oxonium ion m/z 138 at optimum collision energy showed superior sensitivity and quantitative range to all currently available methods. This feature enables analysis using minute sample quantity or detection of very minor glycoforms to unravel glycoform microheterogeneity, as demonstrated herein by the analysis of therapeutic antibodies. The energy-resolved oxonium ion monitoring technology thus shows novelty in both qualitative and quantitative aspects of glycoprotein analysis. It can potentially alleviate a burden on multiple, orthogonal analyses for site-specific characterization of glycan microheterogeneity.

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Primarily it is applicable to evaluation of biosimilar pharmaceuticals at a routine basis, however, it can potentially be applied to any glycoproteins, with or without prior enrichment of the target.

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Competing interests – A.T. and T.A.S. are employees of Shimadzu Corporation.

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Supporting Information – This article contains Supplementary Figures S1 to S3 and Supplementary Tables S1 and S2.

Figure Legends

Figure 1. The energy-resolved oxonium ion monitoring and profiling. (a) An overview of glycoform analysis by multiple reaction monitoring (MRM) mass spectrometry. Glycoprotein sample (immunoglobulin) is digested by trypsin into peptide/glycopeptide mixture and is directly subjected to MS analysis. In the mass spectrometer, the first quadrupole (Q1) isolates the glycopeptide ions with unique intact mass, which are subsequently guided into the second quadrupole (Q2) where they undergo collision-induced dissociation. The kinetic energy of the precursor ions, which governs the energy and rate of dissociation, can be controlled by changing the collision energy applied in the Q2 cell. The third quadrupole (Q3) filters the product ions to selectively detect oligosaccharide-derived oxonium ions (inset) at high-sensitivity. (b-f) The energy-resolved oxonium ion profiles of representative glycopeptides displayed as yield curves. Similar profiles are observed for glycoforms sharing same motifs, such as non-galactosylated type (b), high-mannose type (c), hybrid type (d), and sialylated type (e). The mono-galactosylated glycopeptide isomers (f) have been purified to > 90% by HPLC. Blue square, GlcNAc; green circle, Man; yellow circle, Gal; red triangle, Fuc; purple diamond, Neu5Ac. The peptide portion EEQYNSTYR has been omitted from structure display for simplicity.

Figure 2. Monitoring oxonium ion m/z 138 for high-sensitivity quantification of glycopeptides. (a) The energy-resolved oxonium ion profiles were acquired for 28 glycopeptides and the collision energy that yielded highest signal intensity of m/z 138 were plotted against the m/z of precursor glycopeptide. Blue circle, glycopeptide containing 3 GlcNAc; red square, containing 4 GlcNAc; green triangle, containing 5 GlcNAc; filled points, peptide sequence EEQYNSTYR; open points, peptide

sequence MNSLQSNDAIYYCAR. Correlation coefficient R^2 is 0.842 and all the points fall within 95% confidence interval (broken lines). (b) Two purified homogeneous glycopeptides, 4[Hex]4[HexNAc]1[Fuc] and 5[Hex]4[HexNAc]1[Fuc], were serially diluted and subjected to sensitivity determination. m/z 138 was monitored at the optimum collision energy. Signal was normalized to 100 fmol of internal standard EEQYNSTYR (synthetic peptide with no glycosylation). The quantitative range of detection was determined to be 30 amol to 30 fmol injection with $R^2 = 0.9989$ and 0.9951 .

Figure 3. Evaluation of lot-to-lot heterogeneity of trastuzumab and bevacizumab. The glycoform heterogeneity of four lots of each product was quantified by m/z 138 oxonium ion monitoring and the relative abundances of agalactosylated, mono-galactosylated and di-galactosylated structures are displayed for (a) trastuzumab and (b) bevacizumab. The lots are numbered in chronological order of production and the time-course variation was evaluated by comparing two consecutive lots. Error bars are standard deviation of triplicate runs. Asterisks represent where the difference between consecutive lots was statistically significant ($p < 0.05$ by Student's T-test, $n = 3$).

Figure 4. Characterization of glycoform heterogeneity of cetuximab. (a) Overlaid MRM chromatogram showing the quantification of glycoform heterogeneity in cetuximab digest by monitoring product ion m/z 138 for 40 different glycan structures. Fc glycopeptides elute at 14 min and Fab glycopeptides at 18 min, with minor variation in retention time by glycoforms. (b) The energy-resolved oxonium ion profiles of glycopeptides with m/z 996.90 and m/z 996.65 were acquired for

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verification of their structures, represented by solid lines and broken lines, respectively. The deduced structures are shown in inset. Blue square, GlcNAc; green circle, Man; yellow circle, Gal; red triangle, Fuc; blue diamond, Neu5Gc. The peptide portion MNSLQSNDTAIYYCAR is omitted for simplicity.

Tables

Table 1 - Combinations of potentially immunogenic glycan motifs found on cetuximab. “Composition” indicates the number of α-Gal, Lewis X and Neu5Gc motifs in the glycan structure.

	Composition			% Abundance	
	α-Gal	Lewis X	Neu5Gc	Bi-antennary	Tri-antennary
Antenna	1	-	-	6.4	1.1
Occupancy	-	1	-	0.7	0.2
= 1	-	-	1	7.1	0.9
Antenna	2	-	-	25.9	1.7
Occupancy	1	1	-	2.5	1.0
= 2	1	-	1	18.5	1.3
	-	2	-	0.2	0.1
	-	1	1	1.0	0.2
	-	-	2	3.3	0.3
Antenna	3	-	-	-	2.5
Occupancy	2	1	-	-	1.0
= 3	2	-	1	-	1.8
	1	2	-	-	0.2
	1	1	1	-	0.5
	1	-	2	-	0.6
	-	3	-	-	0.0
	-	2	1	-	0.1
	-	1	2	-	0.2
	-	-	3	-	N.D.

N.D.: no signal detected.

Figures

Figure 1.

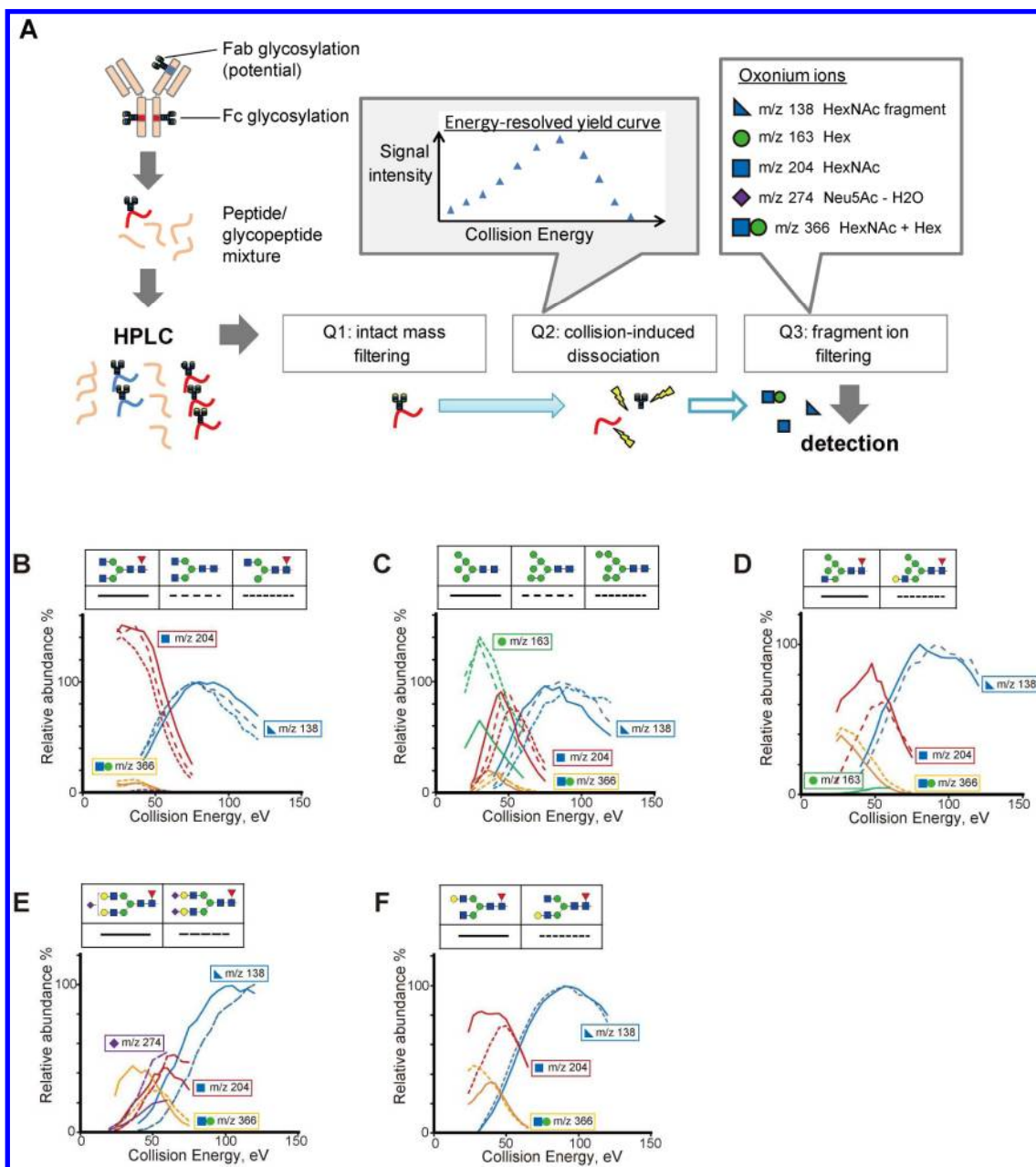


Figure 2.

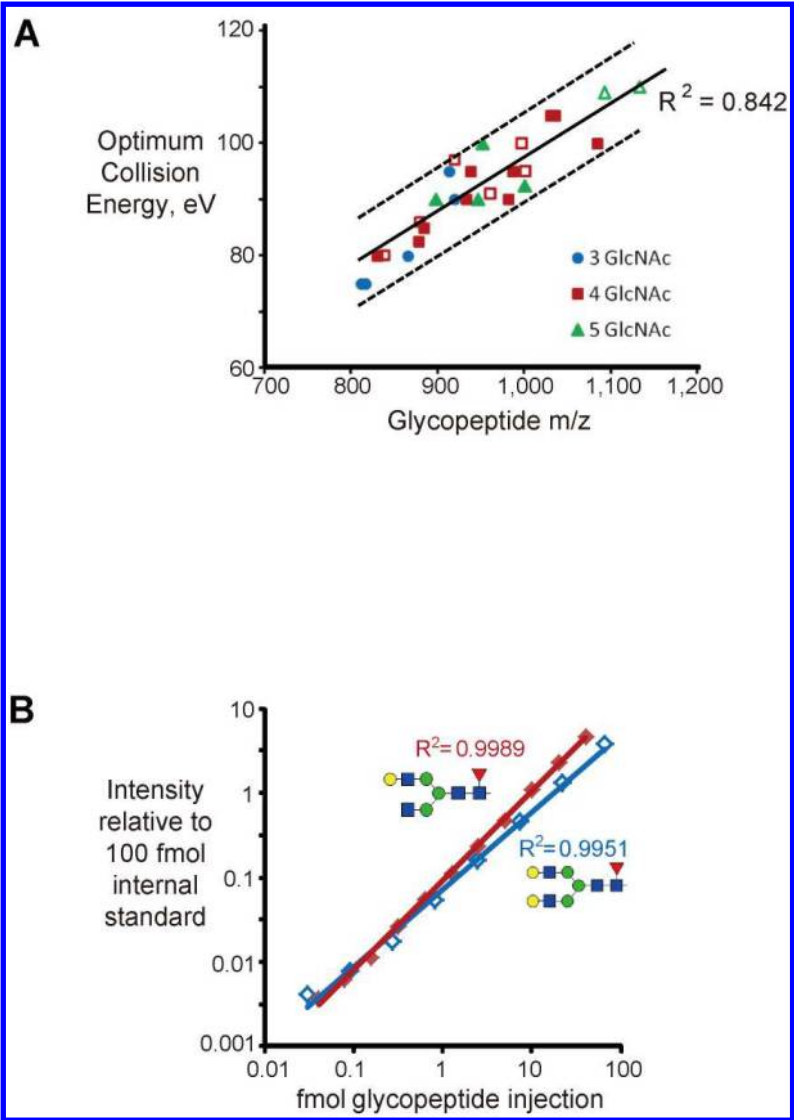


Figure 3.

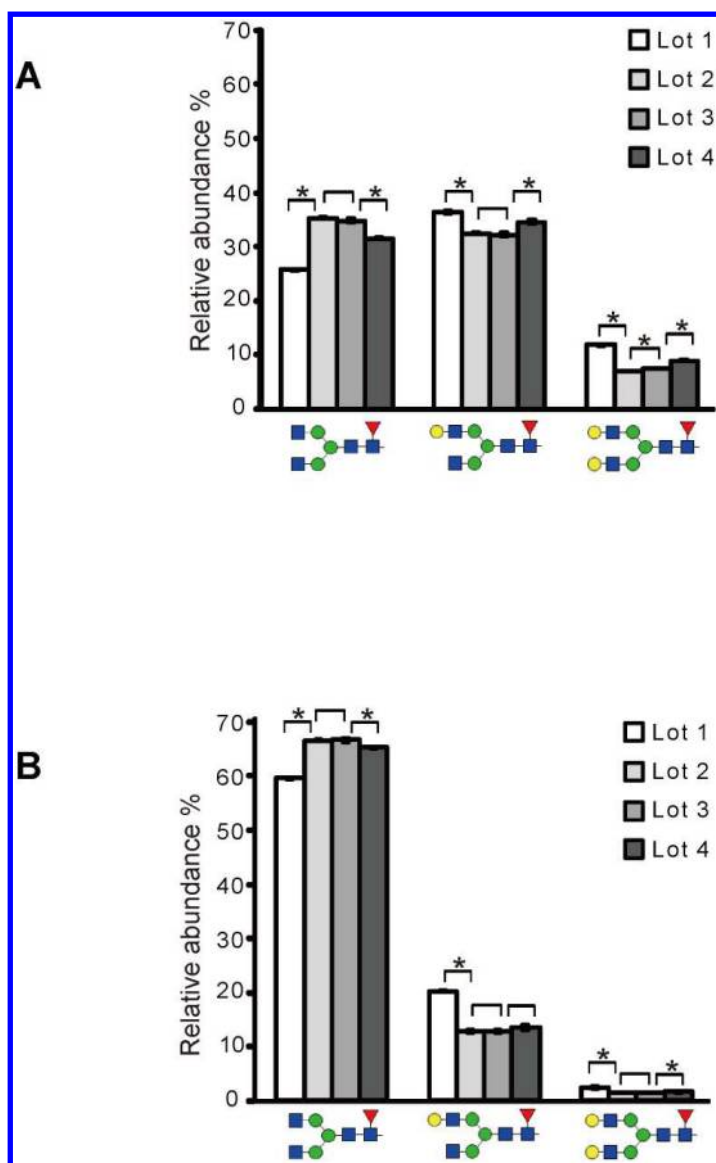
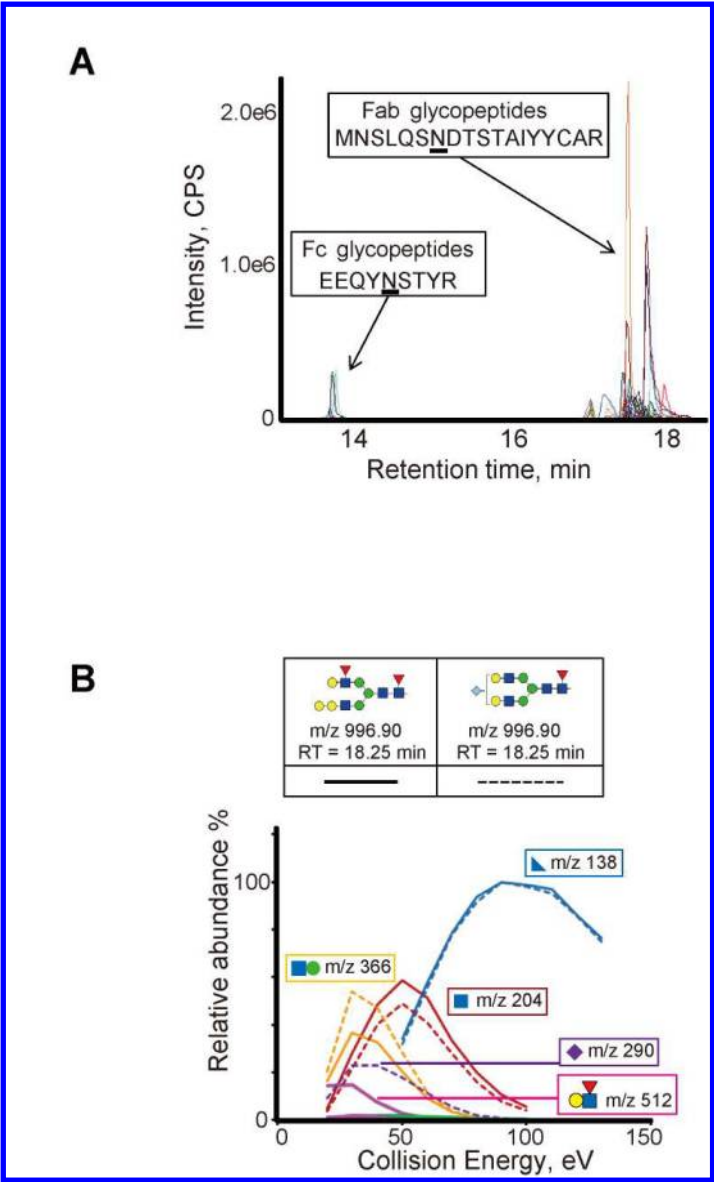


Figure 4.



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