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Site specific conjugation of fluoroprobes to the remodeled Fc N-glycans of monoclonal antibodies using mutant glycosyltransferases: Application for cell surface antigen detection

Elizabeth Boeggeman^{†,‡}, Boopathy Ramakrishnan^{†,‡}, Marta Pasek[†], Maria Manzoni[†], Anu Puri[¶], Kristin H. Loomis[¶], Timothy J. Waybright^{§,‡}, and Pradman K. Qasba^{†,*}

[†]Structural Glycobiology Section, CCR-Nanobiology Program

[¶]Membrane Structure and Function Section, CCR-Nanobiology Program

[‡]Basic Research Program, SAIC-Frederick, Inc.

[§]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc.

Abstract

The Fc N-glycan chains of four therapeutic monoclonal antibodies (mAbs), namely, Avastin, Rituxan, Remicade, and Herceptin, released by PNGase F, show by MALDI analysis that these biantennary N-glycans are a mixture of G0, G1, and G2 glycoforms. The G0 glycoform has no galactose on the terminal GlcNAc residues, and the G1 and G2 glycoforms have one or two terminal galactose residues, respectively, while no N-glycan with terminal sialic acid residue is observed. We show here that under native conditions we can convert the N-glycans of these mAbs to a homogeneous population of G0 glycoform using β 1,4 galactosidase from *Streptococcus pneumoniae*. The G0 glycoforms of mAbs can be galactosylated with a modified galactose having a chemical handle at the C2 position, such as ketone or azide, using a mutant β 1,4 galactosyltransferase (β 1,4Gal-T1-Y289L). The addition of the modified galactose at a specific glycan residue of a mAb permits the coupling of a biomolecule that carries an orthogonal reactive group. The linking of a biotinylated or a fluorescent dye carrying derivatives selectively occurs with the modified galactose, C2-keto-Gal, at the heavy chain of these mAbs, without altering their antigen binding activities, as shown by indirect Enzyme Linked Immunosorbent Assay (ELISA) and Fluorescence Activated Cell Sorting (FACS) methods. Our results demonstrate that the linking of cargo molecules to mAbs via glycans could prove to be an invaluable tool for potential drug targeting by immunotherapeutic methods.

INTRODUCTION

Monoclonal antibodies are increasingly becoming important therapeutic agents to target drugs to the tissue in need of treatment, leaving the normal tissue unharmed (1). They are commonly used in combination with chemotherapeutic agents for cancer treatment (2). The concept of making of antibody-drug conjugates (ADC) by which the antibody is used as a vehicle to deliver a cargo molecule to the tumor cell is gaining immense interest (3, 4, 5). Generally, the ADCs are antibodies conjugated to cytotoxic substances, such as drugs, toxins, and radioisotopes. The conjugated antibody recognizes and binds to cell surface

*Corresponding Author. Structural Glycobiology Section, CCR-Nanobiology Program, NCI-Frederick, Building 469, Room 221, Frederick, Maryland 21702; qasba@helix.nih.gov Phone: (301) 846-1934; Fax: (301) 846-7149.

antigen(s). At this point the cargo molecules must be internalized into the target cells and released from the antibodies for activation of the drug.

All licensed therapeutic antibodies are of the IgG class; these are complex glycoproteins with two main functions, including: a multivalent antigen binding through the Fab variable domains and effector functions through the Fc constant domain. IgG molecules are N-glycosylated in the CH2 domain of the Fc fragment at the conserved Asn 297. In human IgG1, which is the main subtype used for therapeutics, the majority of the Fc glycans are complex biantennary structures with variable galactosylation; 0, 1, or 2 terminal galactoses corresponding to G0, G1, and G2 glycoforms, respectively, and < 10–14% are sialylated (6, 7). Only 15–20% of human IgG1 are also glycosylated in the Fab region of the molecule (7, 8). Recent in vitro studies have established the importance of the Fc glycosylation profile of the IgG molecule on its effector functions: complement-dependent, cellmediated cytotoxicity (CDC) and antibody-dependent, cell-mediated cytotoxicity (ADCC), such that removal of terminal galactose from the IgG can reduce the CDC but not affect ADCC activity (9). However, the use of rat hybridoma YB2/0 cells has permitted the production of mAbs that lack fucose in the conserved core oligosaccharide increasing the ADCC activity of the IgG, allowing the antibody to be effective at lower doses (10). Thus, glycosylation of recombinant monoclonal antibodies has been the focus of attention of the pharmaceutical industry to produce a homogeneous human-type glycosylation product, irrespective of the system/means by which the antibodies are produced.

After years of work to produce successful monoclonal antibodies as targeted drug therapeutics, researchers have developed 6 un-conjugated and 3 antibody-drug conjugates that are currently approved for cancer treatment (11). Generally, the labeling of antibodies with the cargo molecules involves chemical methods which are difficult to control because the chemical reactions occur at tyrosine, lysine, aspartic, and glutamic acid residues that are distributed randomly on the surface of the protein. The result is the production of proteins with compromised activities (12). In order to maintain the biological activity of the protein and to optimize the production of homogeneous proteins, several methods have been developed for site-specific modification of the carriers. In the monoclonal antibodies this site should be selected such that it is away from the antigen binding site. The oligosaccharide moieties of immunoglobulins are not involved in the process of antigen binding and are N-linked to Asn297 on the heavy chain in the Fc domain of the immunoglobulin far from the antigen binding site. The conjugation of monoclonal antibodies to the cargo molecules via the oligosaccharides has been carried out by chemical or enzymatic methods, resulting in the formation of aldehydes (13, 14). Generally, the chemistry used for the modification of the sugar moieties present on glycoproteins such as the immunoglobulin's is mild periodate oxidation of sialic acid residues, resulting in the generation of aldehyde groups, which can then be used for coupling (15, 16). However, less than 10–14% of mAbs are sialylated (6, 7). An alternative method for the generation of aldehyde groups on the oligosaccharide moieties of glycoproteins is the use of enzymes (16, 17, 18). However, the maximum number of aldehyde groups is dictated by the structure/linkage of the sugar; thus, the heterogeneous nature of the sugar moieties on the monoclonal antibodies can result in poor conjugation.

Recently, new chemoenzymatic methods for conjugation have been developed for sitespecific conjugation (19, 20, 21). We have shown the utility of the galactosyltransferase mutant to transfer a sugar residue with a chemically reactive functional group (e.g., C2-keto-Gal or GalNAz) from their UDP-derivatives to the N-acetylglucosamine residue of glycoproteins or glycopeptides (20, 21). Using this method, we have shown that the N-glycan moiety of the IgG molecule can be used as the substrate for the transfer of C2-keto-Gal sugar by the mutant β 1,4Gal-T1-Y289L (20). After the transfer of the modified sugar

residue, the chemical handle is used for selective conjugation with a biomolecule that has an orthogonal reactive group, such as an aminoxy linked to biotin (19, 20, 21).

Here we show that one can take advantage of the unique heterogeneous oligosaccharide linked at Asn297 in the mAbs that can be made homogeneous by a simple enzymatic procedure and obtain a population of mAbs having a set of glycans of the G0 glycoform that are subsequently fully galactosylated to a G2 glycoform, with the modified C2-keto-Gal as monitored by MALDI-TOF analysis of the PNGase F-treated samples. The transferred C2-keto-Gal is coupled to an aminoxybiotin, which was previously detected by chemiluminescence techniques (20, 22) and here by MALDI-TOF analysis of the N-glycans. Using ELISA methodology, we show that the coupling of aminoxy-biotin to C2-keto-Gal does not compromise the antigen (Ag) binding site. Furthermore, Herceptin with C2-keto-Gal modified N-glycans coupled to Alexa Fluor 488 C₅-aminoxyacetamide binds to HER2-receptor-expressing cells, as shown by FACS methodology. Therefore, our conjugation technology presents a viable method to quantify the cargo molecules to be used for detection and targeting therapies.

EXPERIMENTAL PROCEDURES

REAGENTS

Rituxan (Rituximab) and Remicade (Infliximab) are recombinant chimeric monoclonal antibodies (mAbs), and Avastin (Bevacizumab) and Herceptin (Trastuzumab) are recombinant humanized mAbs (Genentech, Inc., South San Francisco, CA), purchased via the NIH pharmacy (Bethesda, MD). FITC-conjugated goat anti-human IgG (whole molecule) was from Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488 C₅-aminoxyacetamide, bis (triethylammonium) salt (Alexa Fluor® 488 hydroxylamine) was from Invitrogen (Eugene, OR). Recombinant β1,4 galactosidase from *Streptococcus pneumoniae* was from Calbiochem (San Diego, CA). Peptide N-glycoside F (PNGase F) was from New England Biolabs (Ipswich, MA). Microcon Ultracel YM-50 centrifugation devices came from Millipore Corporation, (Bedford, MA). Protein A-Sepharose 4B Conjugate was from Invitrogen (Eugene, OR). 2,5-Dihydrobenzoic Acid (DHB) came from Sigma-Aldrich (St. Louis, MO). Micro bio-spin chromatography columns P-30 were from Bio-Rad (Hercules, CA). Recombinant Human Vascular Endothelial Growth Factor (VEGF) was from Thermo Scientific and p-nitrophenyl phosphate from Sigma-Aldrich (St. Louis, MO). UDP-C2-keto-Gal was synthesized as described previously (19).

β1,4 Galactosyltransferase Expression in *E. coli* and in vitro Folding of Inclusion Bodies

The enzymes β1,4Gal-T1 and β1,4Gal-T1-Y289L used in this study have been previously described (23, 24). Inclusion bodies were purified from the bacterial pellet as described earlier (23, 24). The in vitro folding of the enzymes was carried out in a way similar to that of β1,4Gal-T1 (23), with a few modifications. Typically, 100 mg of sulphonated protein were folded for 48 hours in 1 L of folding solution that contained oxido-shuffling reagents and 550 mM arginine-HCl (23). The presence of arginine in the folding solution enhances the folding efficiency of β1,4Gal-T1-Y289L.

Degalactosylation of Monoclonal Antibodies

Avastin, Rituxan, Remicade, or Herceptin were washed with 50 mM sodium phosphate pH 6.0, using a Microcon Ultracel YM-50 centrifugation device. The samples at 8 mg mL⁻¹ were incubated with 100 mU of recombinant *Streptococcus pneumoniae* β1,4 galactosidase for 24 h at 37 °C. Removal of terminal galactose residues was confirmed by analysis of the N-glycans released after PNGase F treatment by MALDI TOF spectrometry. Approximately 3 µg of mAbs were incubated in the presence or absence of PNGase F (2500

Units), 16 h at 37 °C in 10 µl of G7 buffer. Samples were then purified on micro-spin charcoal columns (Harvard Apparatus, MA). Samples were eluted with 30% acetonitrile and analyzed by mass spectrometry. Degalactosylated monoclonal antibodies were then purified by protein A affinity chromatography.

Protein A Affinity Chromatography of mAbs

Degalactosylated samples were diluted 1:1 with 1 × PBS, pH 7.4 (binding buffer) and then added to the protein A columns (Invitrogen). The columns were washed several times with binding buffer and the mAbs were eluted with 100 mM glycine-HCl, pH 2.7. The eluted mAbs were neutralized with 1 M Tris-HCl buffer pH 8.0; concentrated and washed with 1 × PBS, pH 7.4, using the Microcon Ultracel YM-50 centrifugation device. Protein amounts were determined using the Bio-Rad Protein Assay kit based on the method of Bradford (BIO-RAD), and the purity of all mAbs further verified by SDS-PAGE electrophoresis.

Transfer of C-2 keto Galactose from Its UDP-derivative to Free GlcNAc Residues on mAbs using the Mutant β1,4Gal-T1-Y289L and Biotinylation of the mAbs

Monoclonal antibodies (12 µg) were incubated with 2 mM UDP-C2 keto-Gal and 12 µg of the mutant β1,4Gal-T1-Y289L in a 25-µl final incubation mixture containing 10 mM MnCl₂ and 25 mM Tris-HCl (pH 8.0). Reactions were incubated at 30 °C for 12 h. The ketone-labeled proteins were subsequently diluted to 30 µl in a mixture containing 50 mM NaOAc (pH 3.9) and 3 mM N-Aminooxy-methylcarbonylhydrazino-D-biotin (AOB), purchased from Dojindo Laboratories. The biotinylation reactions were incubated with gentle shaking for 12–16 h at 25 °C. A portion of the sample was treated with PNGase F for the MALDI-TOF analysis of the glycan chain, and a portion was boiled in Tris-Glycine-SDS sample buffer containing β-mercaptoethanol and analyzed by Western blot analysis as described (19–21).

SDS-PAGE Analysis of mAbs and Western Blotting of IgGs

The SDS-PAGE analyses of native or biotinylated mAbs respectively, before and after PNGase F treatment, were performed in 14% Tris-glycine gels (Invitrogen). For Western blotting analysis, the proteins from the gels were then transferred by electrophoresis to nitrocellulose paper (0.45 µm pore size) for two hours at 25 V and biotinylated protein bands identified as described previously (20).

Fluorescent Labeling of Herceptin

Fluorescent labeling reactions were carried out by treating 10 µg of C2-keto-Gal labeled Herceptin with 400 µM of Alexa Fluor 488 C₅- aminooxyacetamide in 27 µl containing 166 mM NaOAc (pH 4.9). The reactions were incubated for 2–6 h at 37 °C. The un-reacted fluorescent dye was removed by purification on micro bio-spin chromatography columns P-30 from Bio-Rad (Hercules, CA), as per manufacturer's directions. Purified samples were separated by SDS-PAGE and fluorescence was detected using a multi view FMBIO II scanner (Hitachi).

Indirect ELISA

The binding activity of Avastin to recombinant human vascular endothelial growth factor (VEGF) was determined in a solid-phase binding assay using soluble Avastin and coated VEGF. Binding of antibodies was then detected by a specific secondary antibody in an enzyme-linked immunosorbent assay. Briefly, flat-bottom 96-well microtiter plates (Costar 3690, Corning Inc., NY) were coated with the appropriate amounts of VEGF overnight at 4 °C and were washed three times with phosphate buffer saline (PBS) pH 7.4 containing 0.05% Tween-20 and 0.1% sodium azide. The plates were blocked with 3% bovine serum

albumin (BSA) in PBS with 0.05% Tween-20 for 1 h at room temperature. A serial dilution of native or modified Avastin was added to the VEGF coated plate and incubated for 1 h at room temperature. The detection antibody conjugate (anti-human-Fcphosphatase) (Thermo Scientific) was then applied for 1 h at room temperature. The detection of alkaline phosphatase was performed with p-nitrophenyl phosphate (Sigma). Samples were measured at 405 nm with a plate reader.

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

Typically 1–2 μ l of sample were mixed with an equal volume of 2,5-Dihydrobenzoic Acid (DHB) matrix solution prepared by dissolving 100 mg of DHB (Sigma Chemicals) in 1 ml of a 1:1 solution of water and acetonitrile. In some instances a matrix solution of a mixture of DHB / DMA (N, N-dimethylaniline) was used which was prepared by adding 30 μ l of distilled DMA to the DHB matrix (25). Mixtures were spotted onto a stainless steel plate after mixing the samples and matrix solutions (1 μ l each). The samples were dried by evaporation at room temperature. A hybrid triple-quad time-of-flight (QqTOF) mass spectrometer (QSTAR XL, Applied Biosystems, Inc., Framingham, MA) was configured for matrix-assisted laser desorption/ionization (MALDI). Mass spectrometry (MS) data was obtained using a laser intensity = 21000 μ J, pulse rate = 20 Hz, Collision Gas (CAD) = 3 (High Purity Argon, Airgas, Inc., Frederick, MD), Focusing Potential (FP) = 35 V, Declustering Potential (DP) = 0 V, Declustering Potential 2 (DP2) = 20 V, and Ion Energy (IE1) = 0.8 V. All samples were analyzed in the positive mode with a three-minute accumulation time over an *m/z* range of 150–3000 amu.

Cell Surface Immunostaining of HER2 Receptor by FACS Analysis

To determine if the modifications in Herceptin influenced its ability to bind to the cellular HER2 receptor, we used either indirect immunostaining (when using modified Herceptin) or direct immunostaining of cells when using Alexa-conjugated-Herceptin. HER2 receptor expressing human breast adenocarcinoma cells (SKBR-3) and HER2 receptor negative human breast adenocarcinoma cells (MDA-MB-468) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM/F12 medium supplemented with 10% (v/v) heatinactivated fetal bovine serum (FBS) and penicillin/streptomycin as antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. Other culture reagents were bought from Invitrogen (Carlsbad, CA). For immunostaining experiments, cells were harvested using a PBS-based, enzyme free cell dissociation buffer, and suspended to a concentration of 10⁷ cells per ml in PBS containing 5% FBS. The cells were further incubated for 15 minutes at room temperature, centrifuged and washed twice with cold PBS supplemented with 0.1% BSA (PBS-BSA), and resuspended in PBSFBS at a concentration of 10⁷ per ml. For incubation with antibodies, 100 μ l cell suspension (containing 10⁶ cells) were placed in 1.5 ml eppendorf tubes. Native Herceptin, modified Herceptin or Alexa-conjugated Herceptin (4 μ g each) were added to individual samples and incubations were continued for 1 hour at 4°C in the dark with constant shaking. At the end of incubations, samples were washed twice with cold PBS-BSA, and resuspended in 100 μ l PBS-FBS. The samples incubated with Alexa-conjugated Heceptin were analyzed by FACS. The cells incubated with Native Herceptin or modified Herceptin were incubated with a 1:32 dilution of FITC-conjugated goat anti-human IgG for 1 hour at 4°C in the dark with constant shaking. Cells were washed twice with PBS-BSA, and resuspended in PBS-BSA (1 ml per sample). Control samples were incubated without any primary antibody, with only the FITC-conjugated goat anti-human IgG. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Each sample was analyzed for 10,000 counts of viable cells and all samples were run at least in duplicate.

RESULTS

Glycoforms of Therapeutic Monoclonal Antibodies; MS Analysis of Native and β -galactosidase-treated IgGs

The human IgGs are N-glycosylated in the CH2 domain of the Fc fragment and only ~15–20% are also glycosylated in the Fab domain (7, 8). SDS-PAGE analysis of mAbs, Avastin, Rituxan, Remicade, and Herceptin, before and after the PNGase F treatment, shows the mobility difference only in the heavy chain protein band, and not in the light chain, indicating that the oligosaccharide chain is released only from the IgG heavy chain, and not from the light chain (Figure 1).

The degree of heterogeneity of the oligosaccharides in the native mAbs was determined by carrying out the MALDI-TOF analysis of the oligosaccharide released after PNGase F treatment of mAbs. MS analysis of oligosaccharides released after PNGase F treatment of native forms of Rituxan and Remicade show that they carry oligosaccharides of G0, G1, and G2 glycoforms (Figure 2A and 2B), corresponding to 0, 1, and 2 galactose residues, respectively, while Avastin and Herceptin have only G0 and G1 glycoforms (Figure 2C and 2D). None had sialylated structures as observed with some mAbs (6). To selectively remodel the oligosaccharide of the monoclonal antibodies at Asn 297 in the Fc domain and to obtain a homogeneous population of glycoforms, the IgGs were degalactosylated by β 1,4-galactosidase from *S. pneumoniae* for 24 h. After galactosidase treatment, the degree to which the mAbs glycans were de-galactosylated was confirmed by MALDI-TOF analysis of the N-glycans after PNGase F treatment. Twenty-four-hour incubation of 8 mg/mL⁻¹ of mAbs with 100 mU units of galactosidase in 50- μ l incubation mixture completely converts the IgG in G0 glycoform (Figure 2A'–2D').

Re-glycosylation of G0 Glycoform of mAbs using either UDP-Gal or UDP-GalNAc as Sugar Donors

The mutant β 1,4Gal-T1-Y289L enzyme, in contrast to the wild-type enzyme β 1,4Gal-T1, exhibits both β 1,4Gal transferase and β 1,4GalNAc transferase activities (24). Using chemoenzymatic methods, it was shown that the mutant enzyme can transfer the C2-keto-Gal from its UDP-derivative to the GlcNAc residue on the N-glycan chain of an asialo-agalacto IgG molecule (20). In the present studies, we investigated the transfer of UDP-sugars to the protein A purified G0 glycoform of Herceptin by the wild-type and a mutant β 1,4-galactosyltransferase. Figure 3 shows the MALDI-TOF profile of the oligosaccharide after the transfer of galactose by the wild-type β 1,4Gal-T1 (Figure 3A) and the transfer of N-acetylgalactosamine (Figure 3B) by the mutant enzyme β 1,4Gal-T1-Y289L to the free GlcNAc residues on the G0 glycoforms of Herceptin (Figure 2D'). Nearly 100% galactosylation of both arms of the N-glycan of the IgG-G0 glycoform (Figure 2D') was observed when 80 pmoles of IgG (12 μ g) were incubated at 30 °C for 12 h with 120 pmoles of β 1,4Gal-T1 enzyme (4 μ g) at 1.5 mM concentration of the sugar donor substrate UDP-Gal (Figure 3A'). A peak occurred at m/z 1810.2, corresponding to the G2 form, and a very small peak at m/z 1648.1, corresponding to the G1 form where galactosylation is on either the 1,3 or 1,6 arm of the N-glycan. The transfer of GalNAc (Figure 3B') by the mutant enzyme β 1,4Gal-T1-Y289L to both arms of the G0 glycoform (Figure 2 D') is observed as a main peak at m/z 1892.3 corresponding to the G2 glycoform of the mAb. Minor peaks at m/z 1486 and 1689 correspond to the G0 and G1 glycoforms, respectively. Under the conditions described here, the transfer of galactose and of GalNAc to both GlcNAc residues on each arm of the N-linked structure is observed.

The Transfer of C2-keto-Gal to the Free GlcNAc Residues on the N-glycan Chains of mAbs by the Mutant Enzyme β 1,4Gal-T1-Y289L

In a previous study we showed that the transfer of C2-keto-Gal to the N-linked oligosaccharides of the IgG by the mutant β 1,4Gal-T1-Y289L (20) can be detected by the chemoenzymatic method. In this study, we followed the transfer of C2-keto-Gal to mAbs by the MALDI-TOF of the PNGase F-released oligosaccharides. There is nearly full conversion of 80 pmoles of the G0 form of IgG (12 μ g) to the G2 form with either C2-GalNAz (Figure 4A–D) or C2-keto-Gal (Figure 4E–G) using 2 mM concentration of sugar donor substrate UDP-C2-GalNAz or UDP-2-keto-Gal, respectively, with ~360 pmoles of β 1,4Gal-T1-Y289L mutant enzyme (12 μ g) in a 25- μ l incubation mixture. The appearance of a major peak at m/z 1973.5 after the transfer of C2-GalNAz (Figure 4A–D), and at m/z 1890.0 after the transfer of C2-keto-Gal (Figure 4E–G), shows that the transfer occurs under these conditions on both arms of the G0 glycoform.

Biotinylation of the Glycosylated IgG

The transfer of C2-keto-Gal has been followed by coupling the biotinylated aminoxy biotin to the ketone group at the C2 position of the galactose (Figure 5A) and detected by a sensitive chemiluminescence assay (19–21). MALDI-TOF analysis of the oligosaccharides released after PNGase F treatment of the C2-keto-galactosylated mAbs showed a major peak at m/z 2519, corresponding to the G2 glycoform of the antibodies (Figure 5B) having 4 molecules of C2-keto-Gal transferred per IgG molecule. The conjugated proteins were also analyzed by SDS-PAGE, followed by Western blotting and detection by the streptavidin-HRP technique, as previously described (19–21). The lack of chemiluminescence band in the Western blot analysis after PNGase F treatment of the mAbs (Figure 5C) supports the conclusion that the biotinylated aminoxy ligand is linked only to the C2-keto-Gal modified N-glycan chain of the IgG heavy chain.

Comparing the Binding of Native and Modified Avastin to VEGF by the ELISA Method

We used an indirect ELISA method described previously (26) to analyze the binding of native and modified Avastin to VEGF immobilized on a microtiter plate surface. Remodeling of the Fc N-glycans of Avastin did not change the binding to VEGF, when compared to the binding of the native mAb (Figure 6).

Herceptin Labeling with Alexa Fluor 488 C₅-Aminoxyacetamide

Since C2-keto galactosylated mAbs could be successfully coupled to aminoxy containing ligands (Figure 5B and C) without affecting the antigen binding, as observed by the ELISA method (Figure 6), we linked the C2-keto-Gal modified Herceptin (Figure 4H) to the Alexa Fluor 488 C₅-aminoxyacetamide and analyzed the protein by fluorescence imaging of the SDS-PAGE (Figure 7A), and subsequent staining of the gel with Coomassie blue (Figure 7B). Results from the fluorescence imaging indicate that there is a selective labeling of the heavy chain of the mAb, and this signal is almost lost following PNGase F treatment of the fluorescent-tagged antibody, which removes the N-linked sugars in the Fc domain of the IgG (Figure 7A). No fluorescence was detected at other protein bands seen after Coomassie blue staining of the SDS-PAGE gel (Figure 7B). Conjugation of Alexa Fluor 488 to enzyme-modified Herceptin was further confirmed using a spectrofluorometer (Model FluoroMax 3, HORIBA Jobin Yvon Inc., NJ) to measure fluorescence at Ex/Em 494/518 nm (not shown).

Effect of Herceptin Modification(s) on its Binding to HER2 Receptor

To evaluate the effect of carbohydrate modifications in Herceptin on its biological activity, we examined its binding to HER2 receptor using a human breast adenocarcinoma cancer cell line (SKBR-3) that over expresses this receptor. MDA-MB-468 cells, that do not express the

HER2 receptor, were used as controls. The immunostaining with Alexa Fluor 488 C₅-aminoxyacetamideconjugated Herceptin or modified Herceptin was performed as described in the Methods section. The results are presented in Figures 8 and 9, respectively.

Alexa-conjugated Herceptin showed binding only to SKBR-3 cells (receptor expressing) (Figure 8B). In contrast, under identical conditions, we did not observe any binding above background for MDA-MB-468 cells (receptor negative) (Figure 8A). Therefore, the conjugation of Alexa Fluor 488 and Herceptin did not have any effect on its receptor-binding activity, suggesting that these conjugates may serve as suitable tools for imaging.

Next, we examined the effect of carbohydrate modifications in Herceptin, other than Alexa Fluor 488, on the binding to the HER2 receptor expressing cells. We performed cell sorting experiments using indirect immunostaining (using the FITC-conjugated goat anti-human IgG, see Methods). The results are presented in Figure 9. SKBR-3 (receptor positive) cells bound to native and to modified Herceptin-conjugates with similar efficiency (Figure 9F–H). In contrast, FITC-conjugated goat anti-human IgG alone did not show any significant binding above background (Figure 9E). Our data also show that MDA-MB-468 cells (receptor negative) did not bind to Herceptin (or modified Herceptin) above background (Figure 9, A–D). Therefore, carbohydrate modifications and/or subsequent conjugation via the introduced functional groups do not alter their receptor binding activity.

DISCUSSION

The mAbs are used as powerful therapeutic agents for the treatment of various diseases. They can be used “naked” with their inherent effector functions or as targeting vehicles. Here we used an enzymatic method (19–21) for the production of the conjugated targeting vehicle of mAbs that has the advantage (I) of being site-specific and accessible for conjugation; (II) the cargo does not interfere with or modify the Fab domain; (III) the conjugation of a cargo molecule to a mAb makes it possible to direct the cargo to the desired site; (IV) mAb conjugated with fluoroprobes via glycan chains are suitable probes for imaging and clinical applications.

We have previously shown that the mutant enzyme β 1, 4Gal-T1-Y289L can transfer GalNAc and galactose from its UDP-derivatives to GlcNAc residues present at the non-reducing end of proteins (24). We have also shown that this mutant can transfer modified sugars such as 2-keto-Gal from their UDP-derivatives for detection of O-GlcNAc modifications in proteins (19) or detection of free GlcNAc residues at the non-reducing end of the N-glycans of proteins (20). Our findings led us to propose that two glycoproteins having modified sugars with unique chemical handles could be conjugated via linkers having orthogonal chemical reactive groups, allowing the design of novel mAb-drug conjugates and imaging agents (27).

The present study extends our previous work by showing: (1) Fc N-glycans of native therapeutic antibodies are highly heterogeneous; (2) it is possible to modify the therapeutic monoclonal antibodies from a heterogeneous to a homogeneous glycoform population; (3) the modification at the N-linked oligosaccharide occurs in a site-specific manner without compromising the antigen binding site, and (4) we have been able to produce mAb-fluoroprobes conjugates for imaging applications.

The glycosylation profile of recombinant mAbs has been well characterized and is known to be highly heterogeneous with respect to core fucosylation, terminal sialylation, and galactosylation (28). The difference between the observed glycoform types depends on the enzymes/host cell type, substrates, and culture conditions utilized for their production. The MALDI-TOF analysis of PNGase F-released oligosaccharides of the mAbs used in our

study concur with the published data (28). These N-glycans are core-fucosylated with differences in the terminal sugars having a mixture of G0, G1 and G2 glycoforms. None of the mAb used here had terminal sialic acids. Previously we showed that the C2-keto-Gal from its UDP-derivative was transferred by β 1,4-Gal-T1-Y289L to free GlcNAc residues on the N-linked glycan chains of a heterogeneous population of IgG molecules (20). An important issue in this study was the transfer of the modified sugar to each available GlcNAc present in a homogeneous mAb glycoform population. In order to obtain a population of mAb having a single G0 glycoform with 2 mol of GlcNAc available for transfer to each IgG heavy chain, we tested galactosidases from various sources to remove the non-reducing end galactose residues in the G1 and G2 glycoforms. Only after using β 1,4 galactosidase from *Streptococcus pneumoniae* (9), we were able to show, by MALDI-TOF analysis of the PNGase F-released carbohydrates, a single-type G0 glycoform. The β 1,4 galactosidase from other sources did not yield 100% fully de-galactosylated glycoforms. Having demonstrated the production of a single glycoform, the G0 form, by enzymatic degalactosylation, we explored the transfer of the modified sugar and the coupling of the ketone handle to aminoxy-biotin, which was then detected by MALDI-TOF analysis of N-glycan chains or by chemiluminescence (20), or coupling to aminoxy Alexa Fluor 488, which was detected by a fluorescence technique after SDS-PAGE and FACS analysis. Both chemiluminescence and fluorescence analysis showed that the method of coupling described here is highly specific; almost all the chemiluminescence and fluorescence from the antibody conjugates were associated with binding to the heavy chain. Thus, the cargo is linked in a site-directed manner and only occurs where the sugar binds in the Fc domain of the IgG.

This study also shows that the linking of the target agent, either biotin or Alexa Fluor 488, to the mAb via the N-linked carbohydrates does not modify the antibody affinity for the antigen. One important goal in using this conjugation method is to generate a delivering agent: an antibody-cargo conjugate that will not interfere in its binding to the target cells. The results from ELISA assays using native mAb showed no detectable loss of antigen (Ag) binding activity. Moreover, all antibodies described in this study, obtained either by removal or by addition of sugars, when conjugated via glycan chains, did not show a loss of Ag binding activity. The Ag binding activity of the Fc-N-glycan modified antibodies was comparable to the results obtained with the native mAb. Also, the results obtained by FACS are consistent with the ELISA assay in that the binding of mAb-conjugate to HER-2-expressing cells was not affected by the modification.

In conclusion, the present study describes an enzymatic method for conjugation of monoclonal antibodies at a unique site, away from the Ag binding domain. This method using modified sugars for conjugation provides a specific labeling with 4 molecules of fluorescent dyes if the IgG molecule has free GlcNAc residues available on the Fc N-glycan chains. To our knowledge, this is the first report on the site-specific conjugation of fluoroprobes to the N-linked glycans of intact mAb using an enzymatic method. Other laboratories have reports on the use of molecular imaging probes using mAbs; however, they use chemical conjugation methods, which lead to random labeling (29, 30). Thus, this conjugated fluoroprobe may be used as a probe for imaging and clinical applications.

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ABBREVIATIONS

| | |
|-------------------------------------|--|
| β1,4-Gal-T1 | β 1,4-galactosyltransferase |
| ELISA | Enzyme linked immunosorbent assay |
| FACS | Fluorescence Activated Cell Sorting |
| PNGase F | Peptide N-glycosidase F |
| DHB | 2,5-Dihydrobenzoic Acid |
| DMA | N, N-dimethylaniline |
| Gal | galactose |
| GlcNAc | N-acetylglucosamine |
| GalNAz | N-acetyl-azido-galactosamine (Gal-2-NHCO-CH ₂ -N ₃) |
| UDP-Gal | uridine 5'-diphosphogalactose |

| | |
|-----------------------|--|
| UDP-2-keto-Gal | Uridine 5'-diphospho-2-acetonyl-2-deoxy-galactose |
| UDP-GalNAc | Uridine 5'-diphospho-N-acetylgalactosamine |
| UDP-GalNAz | Uridine 5'-diphospho-N-acetyl-azido-galactosamine |
| MALDI | Matrix-Assisted Laser Desorption Ionization |
| MS | Mass Spectra |
| AOB | <i>N</i> -aminoxy-methylcarbonylhydrazino-D-biotin |
| HRP | horseradish peroxidase |
| FBS | Fetal Bovine Serum |
| BSA | Bovine Serum Albumin |
| PBS | Phosphate Buffer Saline. |

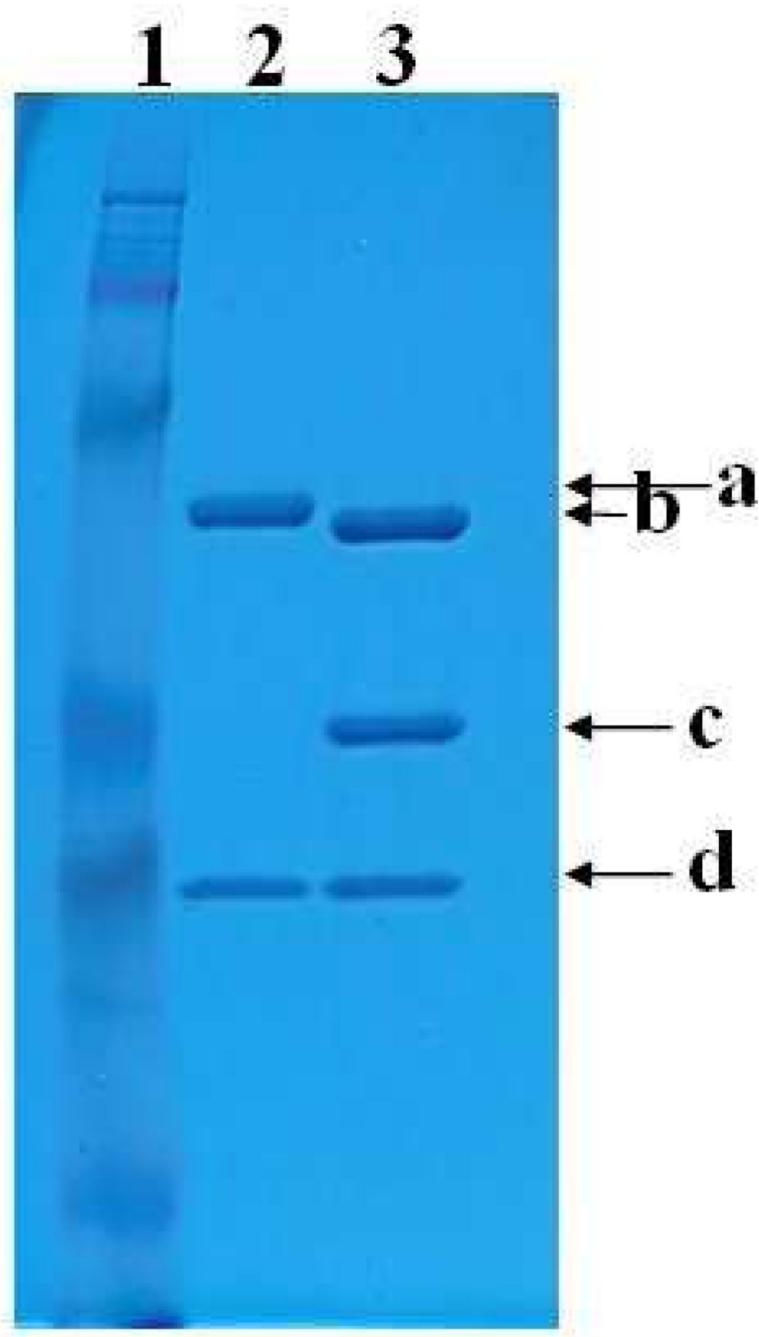
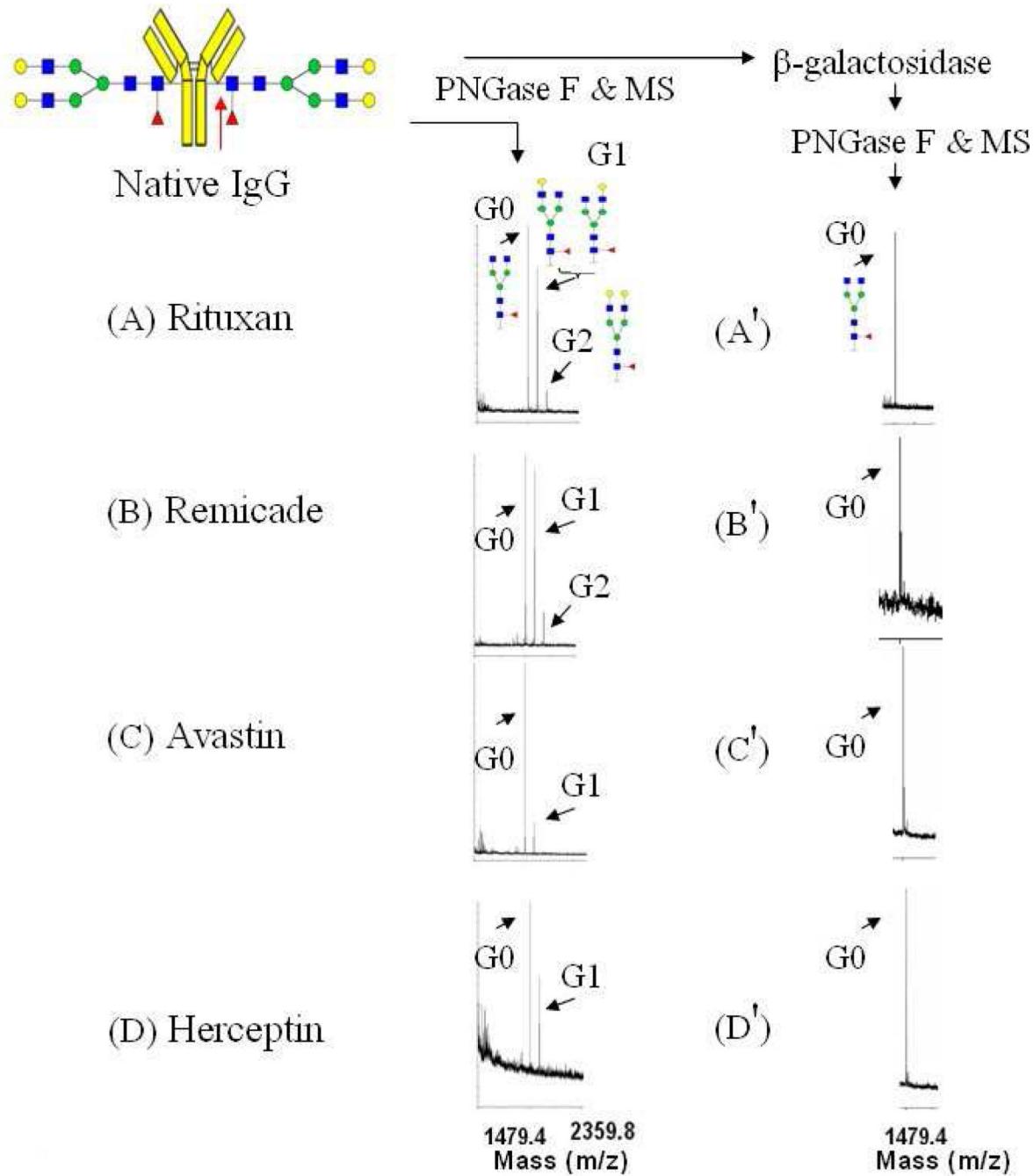
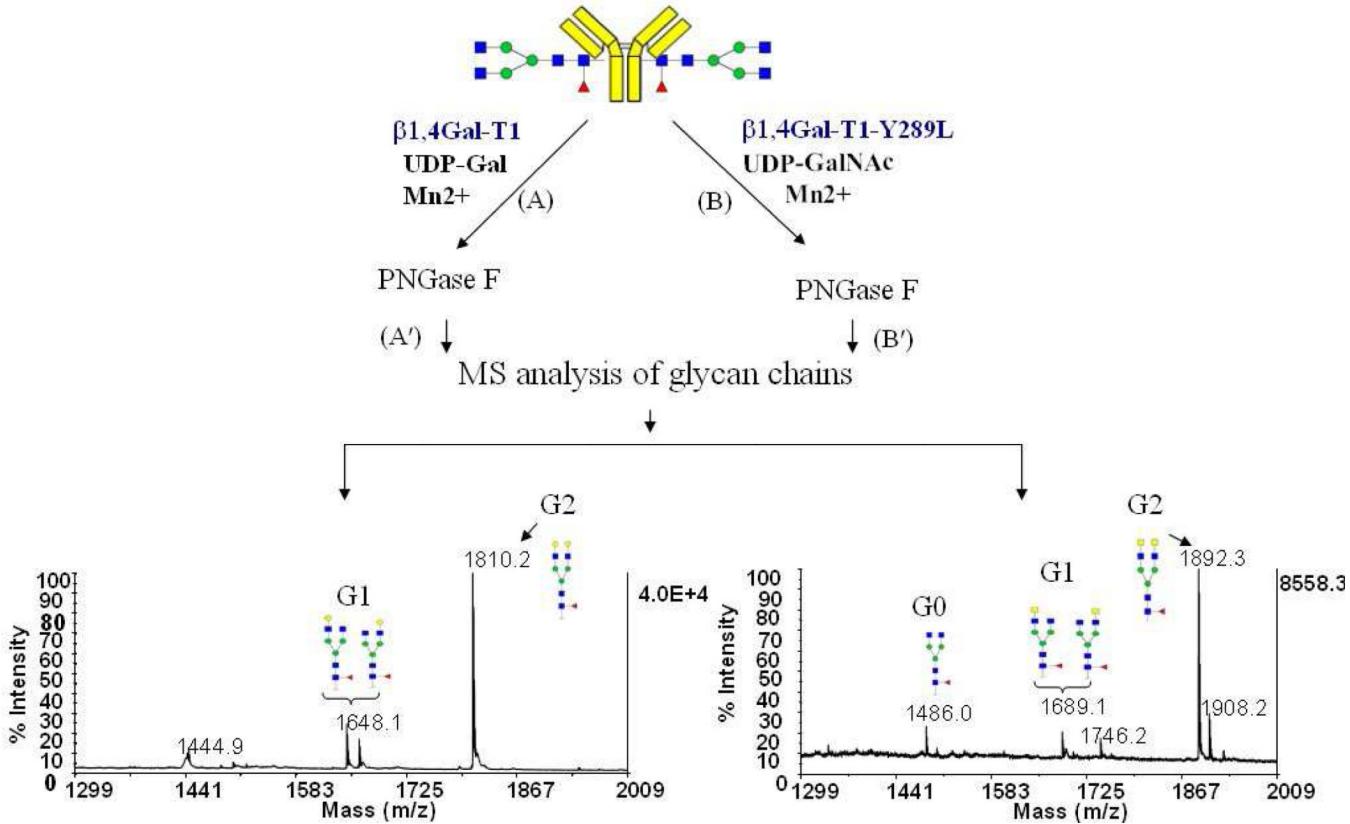


Figure 1.
SDS-PAGE analysis of monoclonal antibody before and after PNGase F treatment. (1) Kaleidoscope Pre-stained standards; (2) the native IgG; (3) IgG after treatment with PNGase F. Arrows show from top to bottom: (a) heavy chain of native IgG, (b) de-glycosylated heavy chain of IgG, (c) PNGase F used for de-glycosylation, and (d) light chain of IgG.

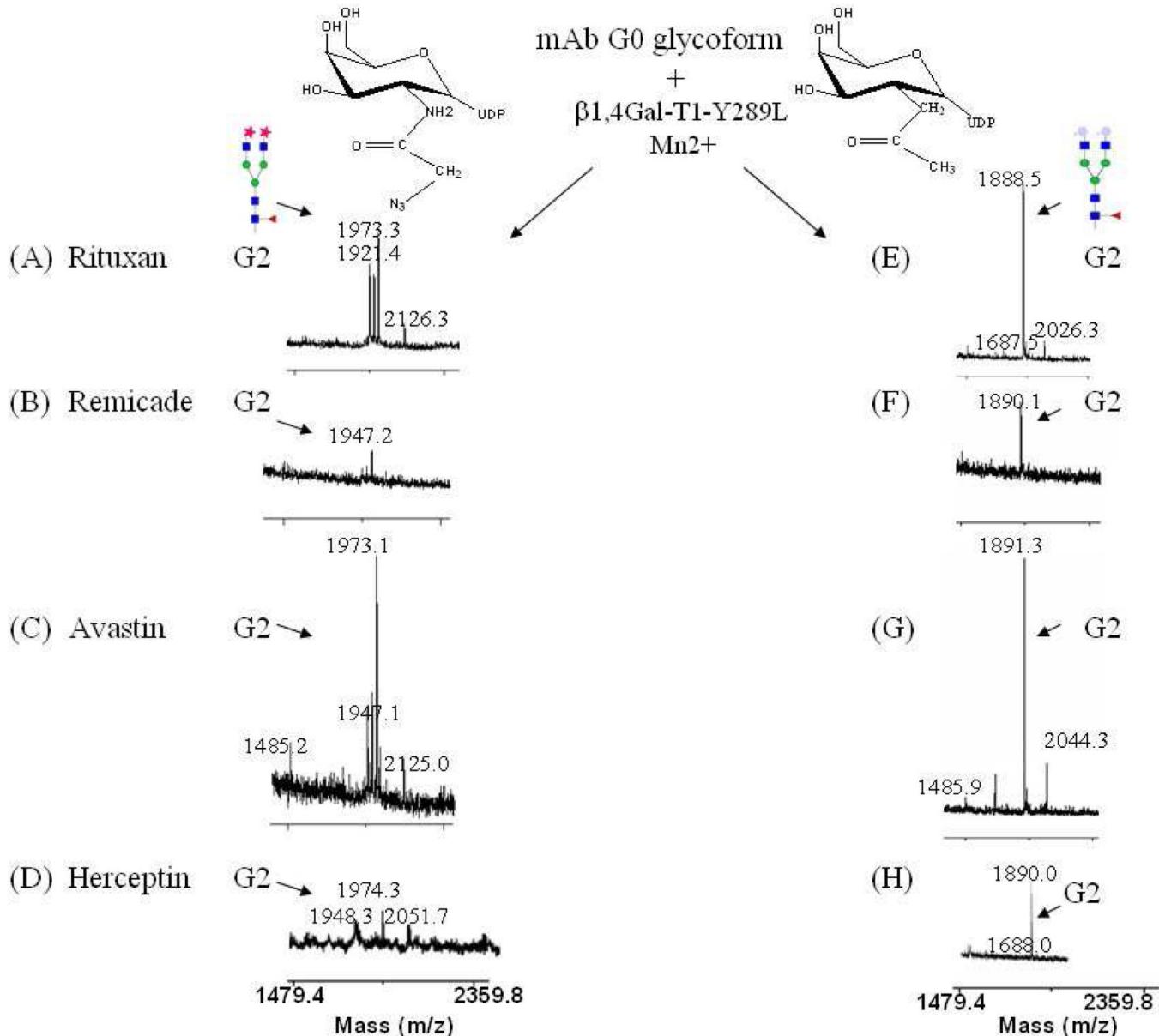
**Figure 2.**

Glycoforms of therapeutic monoclonal antibodies; MS analysis of N-glycans after β -galactosidase treatment of IgG's. (A–D) MALDI-TOF MS analysis of N-glycans released by PNGase F treatment (Red arrow shows the cleavage site) of native Rituxan (A), Remicade (B), Avastin (C), and Herceptin (D). G0 glycoform with a peak at 1485.6 m/z, the G1 glycoform with a peak at 1647.6 m/z and G2 glycoform with a peak at 1809.6 m/z. mAbs were treated with β -galactosidase to obtain a homogeneous G0 glycoform population (A'–D') MALDI-TOF analysis of N-glycans released by PNGase F treatment of β -galactosidase treated Rituxan (A'), Remicade (B'), Avastin (C') and Herceptin (D'), respectively. Only the G0 glycoform with a peak at 1485.6 m/z is seen after treatment.

Major peaks are annotated with the carbohydrate structure shown in the symbols for monosaccharides, according to the nomenclature adopted by the consortium for functional glycomics: GlcNAc (blue squares), mannose (green spheres), galactose (yellow spheres), and fucose (red triangles). The symbols were drawn using the GlycoWorkbench program found in the Eurocarb database <http://www.Eurocarb.org/>.

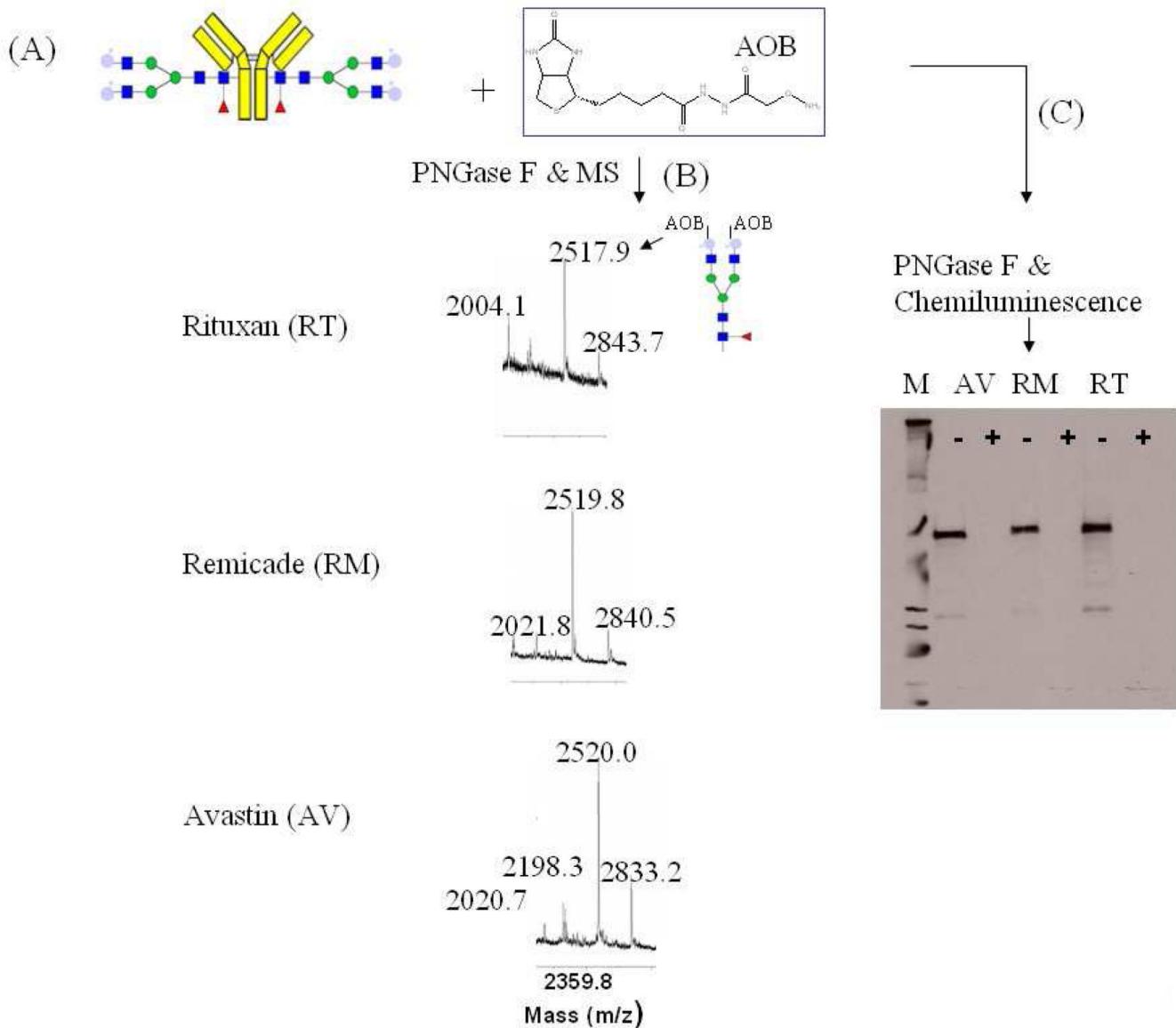
**Figure 3.**

Re-glycosylation of β -galactosidase treated mAbs using natural UDP-sugar donors. To the de-galactosylated G0 glycoform of the IgG, (A) galactose moiety was transferred from UDP-Galactose using the wild-type enzyme β 1,4Gal-T1 or (B) GalNAc moiety was transferred from UDP-GalNAc using the mutant β 1,4Gal-T1-Y289L. After overnight incubation, mixtures were treated with PNGase F prior to MS analysis. (A') After galactose transfer, ions at 1648.1 m/z and 1810.2 are assigned to G1 and G2 glycoforms, respectively, and (B') after GalNAc transfer, ions at 1486.0 m/z , 1689.1 m/z and 1892.3 m/z are assigned to G0, G1, and G2 glycoforms, respectively.

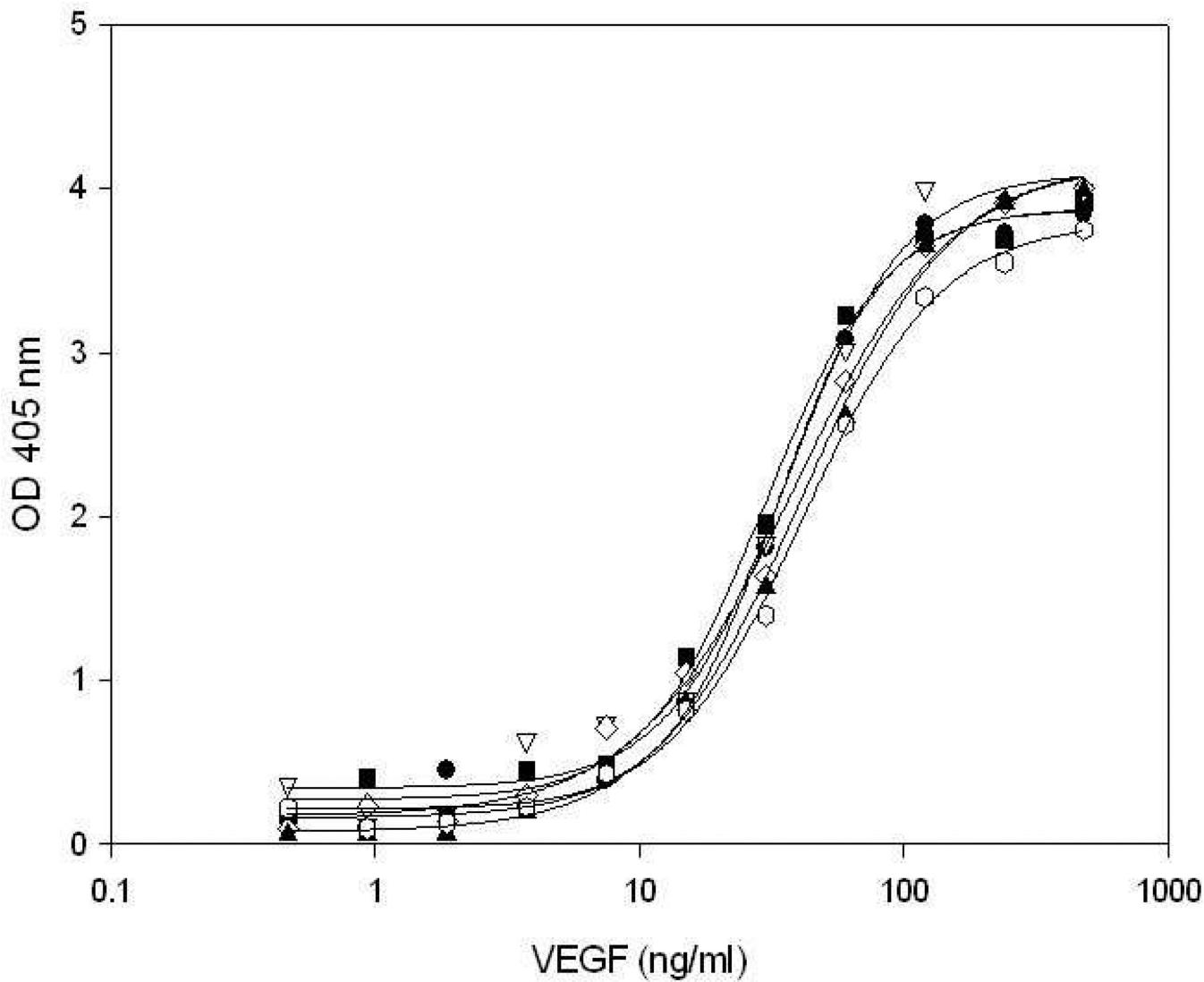
**Figure 4.**

Re-glycosylation of G0 glycoform using C-2 modified sugar donors: The mutant enzyme β 1,4Gal-T1-Y289L was used to transfer the C-2 modified sugars from their respective UDP-sugar nucleotides to the de-galactosylated mAbs; UDP-2-keto-Gal (right panel) and UDP-2-azido-Gal (left panel). MALDI mass analysis after treatment of N-glycans with a mixture of sialyldase and beta-galactosidase (not shown), gave the same G0 glycoform profile, upon PNGase F treatment, as did the beta-galactosidase treatment alone. The transfer of the modified sugar nucleotide was analyzed by mass spectrometry of the modified N-glycans released after PNGase F treatment. β -galactosidase treated mAbs having a G0 glycoform are fully galactosylated to the G2 glycoform after transfer of GalNaz moiety to the terminal GlcNAc residues showing a peak of 1973 m/z (left panel A, C and D). The G2 glycoform peak at 1947 m/z in (B) is due to partial fragmentation of the azido group that can take place during mass spectrometry analysis. The peak at 2126 m/z corresponds to the matrix DHB adduct. Right panel (E-H) shows the transfer of C2-keto-Gal moiety to the terminal GlcNAc

residues in the G0 glycoform (middle panel). A peak at ~1891 m/z corresponds to the G2 glycoform and peaks at 2026 m/z and 2044 m/z correspond to the DHB adduct. GalNAz (pink stars) and C2-keto-Gal (purple circles).

**Figure 5.**

Detection by chemiluminescence technique of the transfer of the C2-keto-Gal to free GlcNAc residues on the N-glycan chains of mAbs. (A) The C2-keto-Gal has been transferred to the free GlcNAc residues in the N-linked carbohydrate of IgG. (B) Detection of the transferred C2-keto-Gal is accomplished by linking to aminoxy biotin followed by PNGase F treatment and MS analysis of the N-glycans. The peaks at 2517.9 m/z , 2519.8 m/z , and 2520.0 m/z correspond to AOB being transferred to both arms of N-glycans carrying C2-keto-Gal in Rituxan (RT), Remicade (RM) and Avatin (AV), respectively. The peaks at 2843.7 m/z , 2840.5 m/z , and 2833.2 m/z correspond to AOB transferred to the sugars at the reducing end. (C) The linked AOB-C2-keto-Gal of AV, RM and RT is also detected on Western blots by chemiluminescence. In contrast to the samples that were not treated with PNGase F (-), the samples treated with PNGase F (+) showed no chemiluminescence. C2-keto-Gal (purple circles).

**Figure 6.**

VEGF ELISA comparing the binding of modified Avastin. Avastin, native or modified, was bound to VEGF immobilized on a microtiter plate surface, and was detected with an anti-human Fc antibody –conjugated to alkaline phosphatase. The transfer of various substrates by the mutant β 1,4Gal-T1-Y289L, namely, GalNAc and C2-keto-Gal to Avastin did not perturb the binding to VEGF. Aminooxy biotin is also linked to the modified C2-keto-Gal without perturbing the Fab domain. (●) Avastin native. (∇) de-galactosylated Avastin. (\square) de-galactosylated Avastin+ GalNAc (\diamond) de-galactosylated Avastin+ no UDP-GalNAc + β 1,4Gal-T1-Y289L. (\square) de-galactosylated Avastin+ C2-keto-Gal. (\circ) de-galactosylated Avastin+ C2-keto-Gal +AOB.

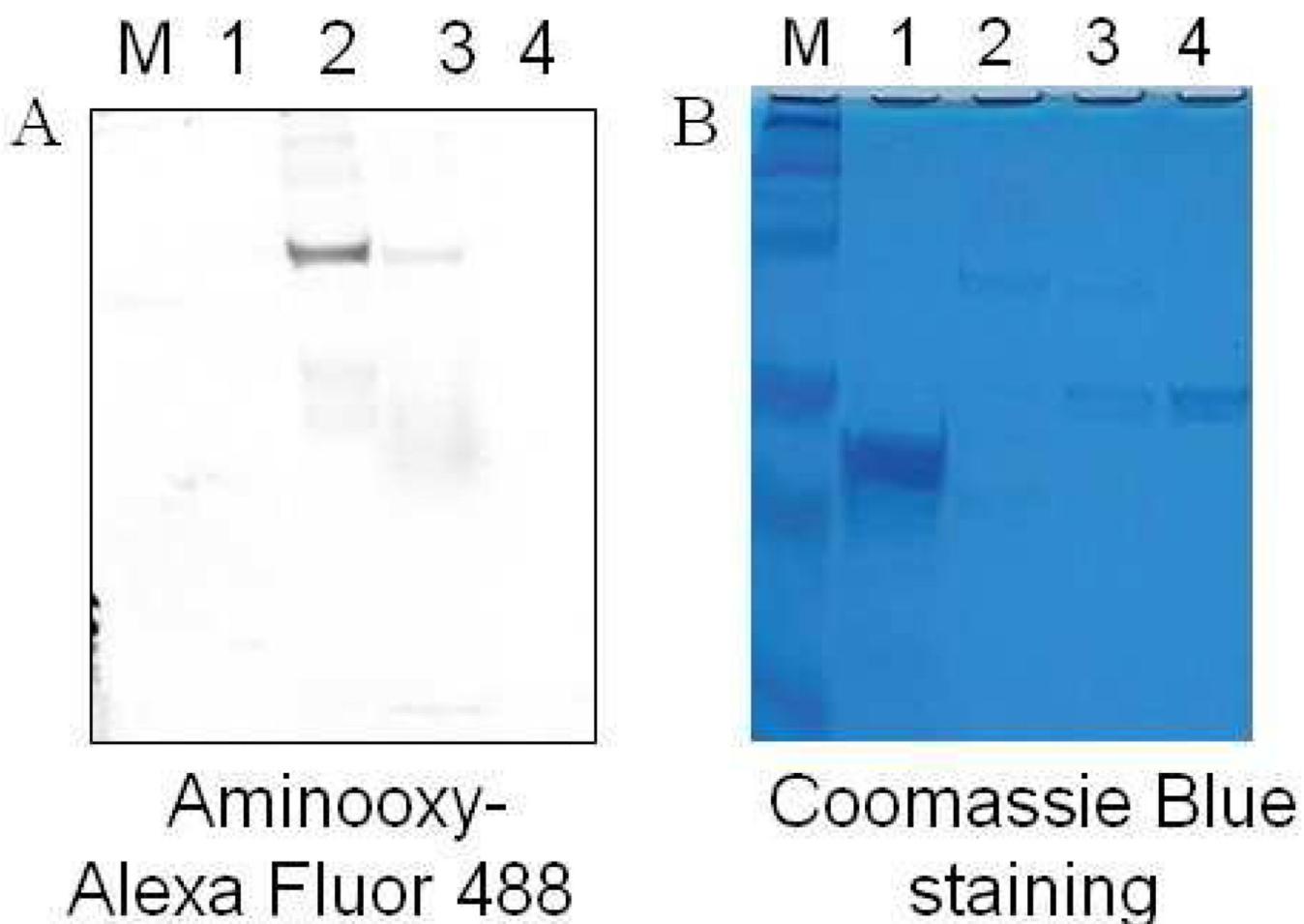
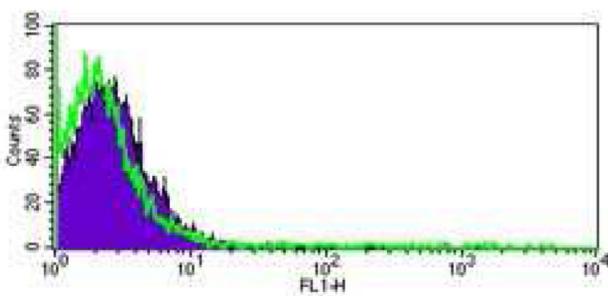


Figure 7.

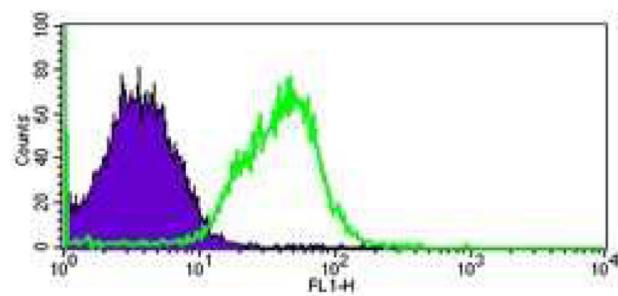
Herceptin labeling with Alexa Fluor 488 C₅-aminooxy acetamide. C₂-keto-Gal modified Herceptin was conjugated with Alexa Fluor 488 C₅-aminooxyacetamide and analyzed by SDS-PAGE (see Materials and Methods). (A) Fluorescence imaging of the SDS-PAGE. (B) The gel was subsequently stained with Coomassie blue. (M) Kaleidoscope standard, (1) β 1,4Gal-T1-Y289L, (2) C₂-keto-Gal modified Herceptin conjugated to Alexa Fluor 488 C₅-aminooxy acetamide, (3) C₂-keto-Gal modified Herceptin conjugated to Alexa Fluor 488 C₅-aminooxyacetamide digested with PNGase F and (4) PNGase F.

(A)



MDA-MB-468 cells
(HER2 receptor
negative)

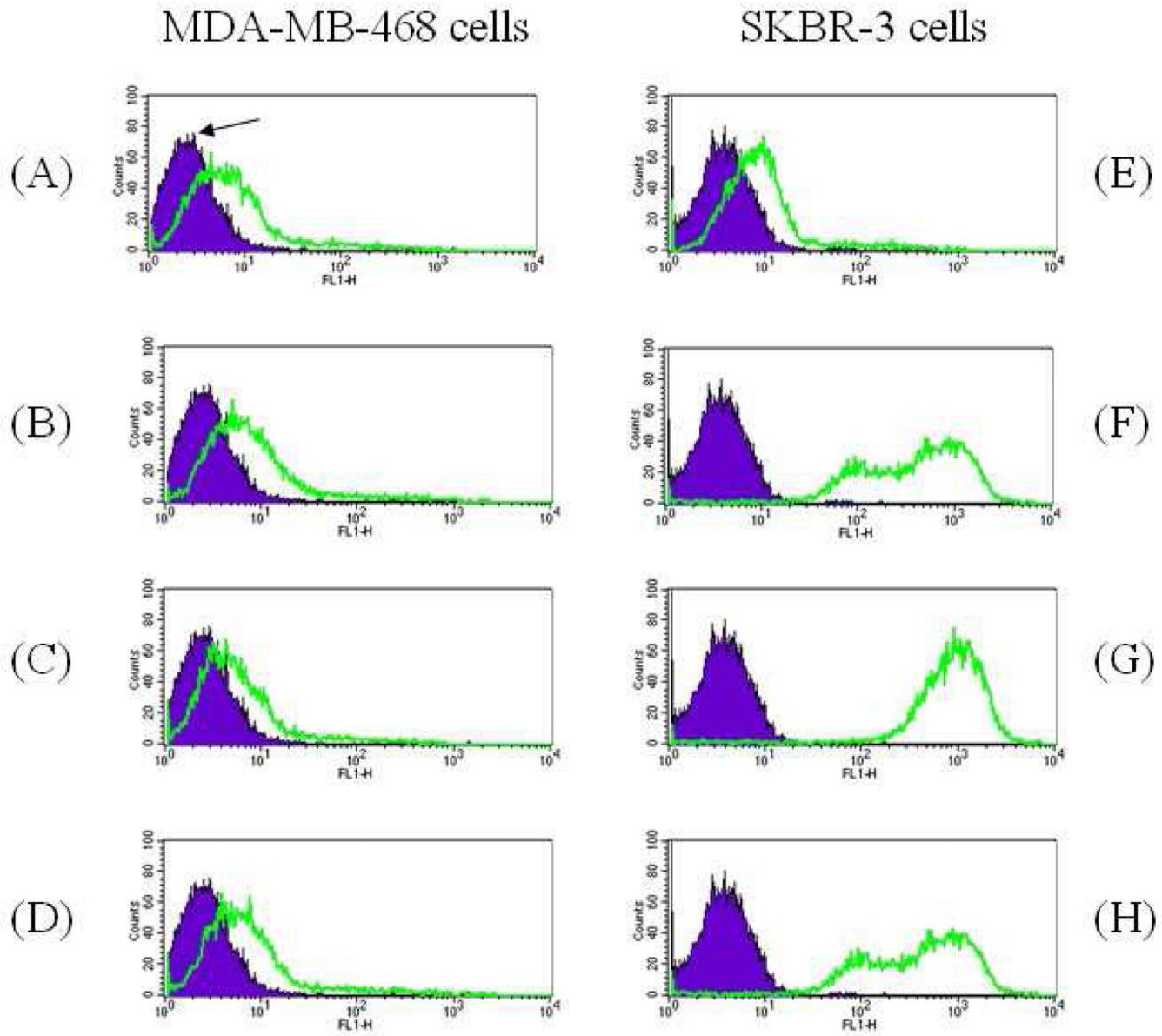
(B)



SKBR-3 cells
(HER2 receptor
positive)

Figure 8.

Binding of Alexa Fluor 488 conjugated Herceptin to HER2-receptor expressing cells. Herceptin was enzymatically modified with C2-keto-Gal and conjugated with Alexa Fluor 488 C₅-aminoxyacetamide. Alexa-conjugated Herceptin (4 µg) was incubated with 10⁶ cells/100 µl PBS-FBS for 60 minutes at 4 °C while shaking in the dark. The cells were washed twice with PBS-BSA and resuspended in 1 ml PBS-BSA. Samples were analyzed by FACS (see Materials and Methods). (A) MDA-MB-468 control cells and (B) SKBR-3 cells. The purple area indicates the background value. The empty area defines the fluorescence of Herceptin conjugated Alexa Fluor 488 C₅-aminoxyacetamide (green line).

**Figure 9.**

Effect of carbohydrate modification of Herceptin on its binding to HER2-receptor expressing cells. Herceptin was subjected to various enzyme treatments (see Materials and Methods). Native and modified Herceptin molecules were incubated with MDA-MB-468 cells or SKBR-3, followed by incubation with FITC-conjugated goat anti-human IgG. The samples were analyzed by FACS (see Methods section). (A & E) secondary antibody alone; (B & F) Herceptin un-treated; (C & G) Herceptin treated with β -galactosidase; (D & H) Herceptin treated with β -galactosidase + C2-keto-Gal. The arrow indicates background fluorescence of cells in the absence of antibodies.