Isolation and Characterization of IgG1 with Asymmetrical Fc Glycosylation

Sha Ha, Yangsi Ou, Josef Vlasak, Yuan Li, Shiyi Wang, Kim Vo, Yi Du, Anna Mach, Yulin Fang, and Ningyan Zhang

Merck Research Laboratories, West Point, PA 19486

Key words: Asymmetrical glycosylation/Fc effector function/FcγR binding/therapeutic antibody

*Corresponding Author:
Ningyan Zhang, Ph. D.
Institute of Molecular Medicine
University of Texas Health Center at Houston
1825 Pressler Street
Houston, TX 77030
USA
Telephone: 713-500-3332
Fax: 713-500-2447
Email: ningyan.zhang@uth.tmc.edu

1Current address: Institute of Molecular Medicine, University of Texas Health Center at Houston, Houston, TX 77030

© The Author 2011. Published by Oxford University Press. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com
Abstract

N-glycosylation of immunoglobulin G (IgG) at asparagine residue 297 plays a critical role in antibody stability and immune cell mediated Fc effector function. Current understanding pertaining to Fc glycosylation is based on studies with IgGs that are either fully-glycosylated (both heavy chains glycosylated) or aglycosylated (neither heavy chain glycosylated). No study has been reported on the properties of hemi-glycosylated IgGs, antibodies with asymmetrical glycosylation in the Fc region such that one heavy chain is glycosylated and the other is aglycosylated. We report here for the first time a detailed study of how hemi-glycosylation affects the stability and functional activities of an IgG1 antibody, mAb-X, in comparison to its fully-glycosylated counterpart. Our results show that hemi-glycosylation does not impact Fab mediated antigen binding, nor does it impact FcRn receptor binding. Hemi-glycosylated mAb-X has slightly decreased thermal stability in the CH2 domain and a moderate decrease (about 20%) in C1q binding. More importantly, the hemi-glycosylated form shows significantly decreased binding affinities towards all Fc gamma receptors including the high affinity FcγRI, and the low affinity FcγRIIA, FcγRIIB, FcγRIIA, and FcγRIIB. The decreased binding affinities to Fc gamma receptors result in a 3.5-fold decrease in antibody dependent cell cytotoxicity (ADCC). As ADCC often plays an important role in therapeutic antibody efficacy, glycosylation status will not only affect the antibody quality but also may impact the biological function of the product.
Introduction

Immunoglobulin G (IgG) antibodies are composed of two heavy chains (HC) and two light chains (LC) that form the Fab (fragment antigen binding) and Fc regions of the antibody. Naturally occurring IgG is glycosylated in the Fc region at asparagine (Asn) residue 297. The N-glycan has predominantly the biantennary complex structure with the core heptasaccharide of GlcNAc$_2$-Man$_3$-GlcNAc$_2$ (Figure 1).

The impact of Fc glycosylation on antibody structure and effector function has been well studied (Jefferis, R. 2005, Jefferis, R. 2007, Okazaki, A., Shoji-Hosaka, E., et al. 2004, Shibata-Koyama, M., Iida, S., et al. 2009). It has been reported that glycosylation in the Fc region confers CH2 domain stability compared to the deglycosylated form of IgG1, while stepwise truncation of the N-glycan sugar moieties leads to progressive decrease in both thermal stability and biological function (Mimura, Y., Church, S., et al. 2000). Modern technologies, such as X-ray crystallography (Krapp, S., Mimura, Y., et al. 2003), NMR spectroscopy (Yamaguchi, Y., Nishimura, M., et al. 2006), and hydrogen/deuterium exchange mass spectrometry (Houde, D., Peng, Y., et al. 2010, Mimura, Y., Ashton, P.R., et al. 2007), have revealed the conformational changes caused by different Fc glycosylation. These technologies have linked the glycoform-dependent conformation to IgG stability and function.

Glycosylation in the antibody Fc region has been shown to modify antibody effector function through effects on the binding affinity to Fc gamma receptors (FcγRs) (Hodoniczky, J., Zheng, Y.Z., et al. 2005, Lifely, M.R., Hale, C., et al. 1995, Shinkawa, T., Nakamura, K., et al. 2003). FcγRs are a superfamily of receptors expressed on many types of immune effector cells, including lymphocytes and myeloid immune cells such as natural killer (NK) cells, dendritic cells, macrophages, monocytes, and neutrophils. FcγR binds to antibodies at their lower hinge
region and the CH2 domain (Sondermann, P., Huber, R., et al. 2000). Upon binding, the hinge rearranges in an asymmetrical manner that excludes the second molecule of FcγR from binding. As a result, the Fc region of the antibody interacts with the FcγR at a ratio of 1:1.

It is known that the Fc glycan composition strongly influences binding between Fc and FcγR. It has been shown that afucosylated IgG1 has up to a 50-fold higher FcγRIIIA binding affinity compared to its fucosylated counterpart (Shields, R.L., Lai, J., et al. 2002). Removing the terminal Gal-GlcNAc disaccharides (Figure 1, M3N2 structure) decreases FcγRIIIA binding by 2-3 fold (Houde, D., Peng, Y., et al. 2010, Yamaguchi, Y., Nishimura, M., et al. 2006), and decreases FcγRIIb binding by less than 2 fold (Mimura, Y., Sondermann, P., et al. 2001). Additional truncation of the mannose residues in both arms (Figure 1, MN2 structure) decreases FcγRIIb binding by more than 2 fold (Mimura, Y., Sondermann, P., et al. 2001). Removing the entire glycan on Fc (aglycosylated antibody) abolishes the binding to FcγRIIs and FcγRIIs, and significantly decreases FcγRI binding (Houde, D., Peng, Y., et al. 2010, Mimura, Y., Sondermann, P., et al. 2001, Sazinsky, S.L., Ott, R.G., et al. 2008, Yamaguchi, Y., Nishimura, M., et al. 2006)

These functional studies are in agreement with the proposed model structure (Krapp, S., Mimura, Y., et al. 2003, Sondermann, P., Huber, R., et al. 2000) that the fully galactosylated IgG adopts an "open" horseshoe-shaped conformation between the CH2-CH2 interface, allowing the optimal binding of FcγRs. Truncation and removal of the glycans lead to a relatively "closed" conformation that can compromise binding to Fc receptors and therefore, impact Fc effector function.

Glycosylation has also been shown to impact the ability of IgG to bind to the complement system and induce complement-mediated cytotoxicity (CDC). Like FcγR, the key binding motif
for C1q is also located at the antibody lower hinge and CH2 domain (Duncan, A.R. and Winter, G. 1988, Morgan, A., Jones, N.D., et al. 1995). C1q binding is impacted by terminal galactose content, as G2 glycoform shows about a 2-5 fold higher C1q binding and CDC activity compared to the G0 glycoform (the structure of G0 and G2 are illustrated in Figure 1) (Raju, T.S. 2008). The deglycosylated antibody is deficient in C1q binding and CDC activity (Boyd, P.N., Lines, A.C., et al. 1995, Mimura, Y., Church, S., et al. 2000, Wright, A. and Morrison, S.L. 1994).

Antibodies produced from both natural immune cells and the recombinant systems used in the manufacture of therapeutic products have complex glycosylation profiles. Although there are extensive studies on IgGs with different glycoforms, all of the studies have focused on antibodies produced either from glycoengineered host systems or generated through in vitro enzymatic conversion (Jefferis, R. 2005, Jefferis, R. 2007, Tao, M.H. and Morrison, S.L. 1989, Wright, A. and Morrison, S.L. 1997). Therefore, the reported studies so far include IgGs containing N-glycan at both heavy chains. Little is known of IgGs with asymmetrical glycosylation in the Fc region such that one heavy chain is glycosylated and the other is aglycocalydated. In this manuscript, we refer to this asymmetric form as hemi-glycosylated antibody.

Asymmetrical glycosylation of Fab was first reported in 1986 when Labeta et al. discovered that naturally existing IgGs could be asymmetically glycosylated in the Fab region (Labeta, M.O., Margni, R.A., et al. 1986). Due to the steric hindrance of the Fab glycosylation, IgGs that are asymmetically glycosylated in the Fab region lose their divalent binding capability and behave as univalent antibodies and cannot form large complexes through divalent binding to antigens. Since most therapeutic monoclonal antibodies contain small, but detectable amounts of aglycosylated heavy chain when analyzed using CE-SDS methods (Rustandi, R.R., Washabaugh,
M.W., et al. 2008), we hypothesize that asymmetrical glycosylation or hemi-glycosylation in Fc region could exist as well when the aglycosylated heavy chain pairs with glycosylated heavy chain to form the IgG. Because of the low abundance of this hemi-glycosylated population as well as the technical difficulty of separating hemi- from fully-glycosylated species using conventional analytical procedures, to our knowledge, there has been no reported study on the hemi-glycosylated IgG in comparison with fully-glycosylated IgG counterpart. In order to understand the impact of the hemi-glycosylated IgG on the potency and quality of therapeutic antibodies, it is important to isolate the hemi-glycosylated IgG and to characterize its biological function. Here we report the in vitro separation of hemi-glycosylated mAb-X, and the first full characterization of the hemi-glycosylated mAb-X in comparison with its fully-glycosylated counterpart in terms of its thermal stability, biological activity and Fc effector functions.

Results

Isolation of hemi-glycosylated mAb-X

Antibody mAb-X contained a mixture of fully-glycosylated, hemi-glycosylated, and aglycosylated species. Earlier studies in our group demonstrated that under mildly acidic condition, the hemi-glycosylated species could be separated from the fully-glycosylated and aglycosylated forms using cation exchange chromatography (CEX) without impacting antibody function (Wang, S., Ionescu, R., et al. 2010). At pH 4.9, the mixture of mAb-X glycovariants eluted in the order of fully-glycosylated, hemi-glycosylated, and aglycosylated IgGs (black solid line in Figure 2). Each peak of mAb-X was collected and re-analyzed using the same CEX method. The hemi-glycosylated (red break line, in Figure 2) and fully-glycosylated (black dotted line, in Figure 2) IgG fractions each showed one symmetric peak, indicating that both the fully-
and hemi-glycosylated IgG were highly enriched. Reduced CE-SDS was used to evaluate the purity of the collected fractions. A pure fully-glycosylated IgG is expected to contain 100% glycosylated HC, while a pure hemi-glycosylated IgG is expected to contain 50% aglycoylted and 50% glycosylated HC. As shown in Figure 3, the hemi-glycosylated mAb-X fraction contained about 60% glycosylated HC and 40% aglycosylated HC under CE-SDS reducing conditions (Trace 1 in Figure 3) and the estimated purity of the hemi-glycosylated mAb-X was 80% based on the assumption that all impurity came from the fully-glycosylated form. The enriched fully-glycosylated mAb-X fraction from CEX separation showed >95% glycosylated HC and <5% aglycosylated HCs (Trace 2 in Figure 3). As expected, mAb-X without CEX separation showed a mixture of glycosylated and aglycosylated HCs (trace 3 in Figure 3). LC-MS was used to confirm the molar mass of the hemi-glycosylated mAb-X (Figure 4). Under non-reducing conditions, each glycovariant contained covalently linked two LCs and two HCs but with different glycosylation patterns. The enriched hemi-glycosylated mAb-X fraction showed the main peak cluster with molecular weights of 146473.6, 146635.1, and 146796.5 Da, matching IgG with one aglycosylated heavy chain covalently linked to G0, G1, and G2 heavy chains (Figure 4A). The fully-glycosylated mAb-X fraction showed molecular weights of 147771.7, 147933.6, 148095.3, 148258.0, 148419.2 Da, matching heavy chain pairs of G0/G0, G0/G1, G1/G1, G1/G2 and G2/G2, respectively (Figure 4B). As expected, the mAb-X without separation on CEX showed mass peaks in two clusters corresponding to the fully- and hemi-glycosylated fractions (Figure 4C). Consistent with the CE-SDS results, the enriched hemi-glycosylated mAb-X also showed residual masses corresponding to the fully-glycosylated species. This is due to the impurity in the prepared fraction. The amount of impurity was estimated to be about 13% based on the LC-MS peak intensity.
**Thermal stability by differential scanning calorimetry (DSC)**

It is known that glycosylation in the Fc region impacts thermal stability of the CH2 domain (Mimura, Y., Church, S., et al. 2000). The Fc region exhibits two thermal transitions: the first transition peak (Tm1) represents the unfolding of the CH2 domain, while the second one (Tm2) reflects the unfolding of CH3 (Ionescu, R.M., Vlasak, J., et al. 2008). For mAb-X, the thermal unfolding profile for the Fab region overlaps with the CH3 unfolding. As shown in Figure 5, the fully-glycosylated and hemi-glycosylated mAb-X had the same Tm2 transition temperature of 81°C, suggesting that the differences in the Fc glycosylation had no effect on the thermal stability of CH3 domain or Fab. The CH2 domain transition temperatures (Tm1) showed a 1°C reduction from 71°C for the fully-glycosylated form to 70°C for the hemi-glycosylated form (Figure 5). An earlier onset of the CH2 domain unfolding was observed for the hemi-glycosylated mAb-X and it was manifested by a slightly broader Tm1 transition. The results indicate that hemi-glycosylation can introduce a mild destabilization in the CH2 domain.

**Effects of hemi-glycosylation on antibody/antigen engagement and downstream signaling**

Interaction of the antigen and antibody occurs in the Fab region, and the binding epitope of each antibody is determined by the complementarity determining regions (CDRs). In order to investigate if hemi-glycosylation in the Fc region has an impact on the Fab interaction with antigen, antigen binding affinities of the fully- and hemi-glycosylated mAbs were determined by ELISA. The hemi-glycosylated and fully-glycosylated mAb-X showed similar dose response curves and both yielded EC50 values of 14 ng/mL, where the EC50 is the effective antigen concentration required to obtain 50% binding.
The functional activity of the hemi-glycosylated mAb-X was studied by evaluating the inhibition of target receptor phosphorylation and cell proliferation in a breast cancer cell line, BT474.m1. The hemi-glycosylated antibody showed similar inhibitory activity when compared with the fully-glycosylated antibody (Figure 6), indicating that hemi-glycosylation had minimum impact on Fab target engagement.

**Effects of hemi-glycosylation on Fc mediated functions**

It is well established that glycosylation at Asn297 of the Fc region is important for Fcγ receptor binding. The binding affinity of both fully- and hemi-glycosylated mAb-X to high affinity FcγRI and low affinity FcγRII and FcγRIII were determined by surface plasmon resonance (SPR). The binding kinetics was monitored using seven individual Fcγ receptors at concentrations ranging from 0 to 1.6 μM. The results showed that hemi-glycosylated mAb-X had a 2-3 fold decrease in binding affinity to all Fcγ receptors including both activating (FcγRI, RIIA, RIIIA &RIIIB) and inhibitory (FcγRIIB) receptors (Table 1).

In order to study if hemi-glycosylation has an impact on the interaction of antibody Fc with complement systems, C1q binding was measured using an ELISA assay by capturing the same amount of each mAb-X glycovariant on an ELISA plate, which was pre-coated with antigen, and adding a fixed volume of C1q at a concentration of 100 μg/mL. There was a moderate but statistically significant (p<0.001) decrease (about 20%) in C1q binding for hemi-glycosylated mAb-X in comparison with fully-glycosylated antibody (Figure 7).

ADCC function has been implicated as the mechanism of action for several therapeutic antibodies. As hemi-glycosylated mAb-X showed decrease in binding affinities to both activating and inhibitory FcγRs, it was important to understand whether the ADCC function of
mAb-X was affected by the reduced binding to these two types of receptors. Two different effector immune cells were used to examine the ADCC activity: periphery blood mononuclear cells (PBMC) and monocytes. PBMC induces cell killing through the dominant natural killer (NK) cells. NK cells constitute about 25% of the PBMC population and express solely the activating FcγRIIIA. Monocytes express both the activating FcγRs and inhibitory FcγRIIB. As expected, ADCC mediated by PBMC was approximately 5-fold higher than that mediated by monocytes (Figure 8). When comparing fully- and hemi-glycosylated mAb-X, the hemi-glycosylated mAb-X showed about 3.5-fold lower ADCC activity independent of effector cells used, either PBMC (Figure 8A) or monocytes (Figure 8B).

**Discussion**

We have demonstrated that the hemi-glycosylated antibody has a slightly decreased thermal stability in the CH2 domain compared to the fully-glycosylated antibody counterpart. The results are in agreement with the published data (Mimura, Y., Church, S., et al. 2000) that both glycan structure and occupancy in the Fc region can impact the stability of an antibody. In contrast to the dramatic CH2 domain destabilization by complete deglycosylation, the impact on the CH2 domain by hemi-glycosylation is subtle with 1°C reduction in Tm. This implies that the presence of a single N-glycan chain in the Fc region provides significant stabilization to the CH2 domains and the two CH2 domains present a cooperative thermal unfolding. Future studies of X-ray crystallography or hydrogen/deuterium exchange mass spectrometry will be able to reveal the subtle conformational changes induced by hemi-glycosylation.

Because the Fab region is distant from the Fc glycosylation site, as expected, hemi-glycosylation has minimal impact on the Fab mediated antigen binding and downstream
signaling. FcRn binds to IgGs at the interface of CH2 and CH3 domain (Burmeister, W.P., Huber, A.H., et al. 1994, Martin, W.L., West, A.P., Jr., et al. 2001), and is not affected by glycosylation status in the Fc region (Simmons, L.C., Reilly, D., et al. 2002). Consistent with the reported data, there were no differences in binding affinities of FcRn to hemi-glycosylated and fully-glycosylated mAb-X. Different from the FcRn binding, all FcγRs interact with Fc near the lower hinge/CH2 domain, a region where Asn297 glycosylation has a significant impact on the conformation (Krapp, S., Mimura, Y., et al. 2003). Our results have shown that hemi-glycosylation significantly reduces the FcγRs binding affinity in comparison with the fully-glycosylated mAb-X. There is a 2-3 fold decrease in binding affinity for hemi-glycosylated mAb-X to all seven FcγRs studied, suggesting that the lower hinge/CH2 domain is significantly impacted by hemi-glycosylation of the Fc. As the C1q binding motif is located in the lower hinge region as well, it is not surprising that hemi-glycosylation also impacts C1q binding, even though the change is relatively small in comparison to the impact to FcγR binding. Since mAb-X does not show complement mediated cell cytotoxicity (CDC) in an in vitro assay, impact of the decreased C1q binding on CDC activity could not be tested in this study.

It is noted that the impact of hemi-glycosylation on FcγR binding is modest and less severe than the impact of aglycosylated antibody. While hemi-glycosylated mAb-X decreases FcγR binding by 2-3 fold, complete deglycosylation decreases the binding to high affinity FcγRI by approximately 10 folds, and completely abolishes the binding to all the low affinity FcγRs (Sazinsky, S.L., Ott, R.G., et al. 2008). It is reported that in the crystal structure of FcγRIII/Fc complex, while both CH2 domains in the Fc region are involved in the protein-protein interactions with FcγRIII, only one CH2 domain contributes a carbohydrate residue to a potential hydrogen bond with FcγRIII (Sondermann, P., Huber, R., et al. 2000). For the hemi-glycosylated
IgG1, we speculate that the glycosylated HC is able to provide the necessary contacts with FcγRIII including the critical carbohydrate/protein interaction, while the aglycosylated HC can engage in protein/protein interaction and provides the ‘proline sandwich’ for the asymmetrical interaction. Therefore, hemi-glycosylation has a less severe impact on FcγRIII binding in comparison to the completely deglycosylated IgG, which lacks this critical carbohydrate/protein interaction.

The decreased ADCC activity of hemi-glycosylated mAb-X is consistent with the decrease in FcγR binding affinities detected in this study. Although in vitro ADCC activity does not directly translate into in vivo efficacy, there are several clinical reports regarding FcγR polymorphism that indirectly links the in vitro ADCC to clinical efficacy (Nimmerjahn, F. and Ravetch, J.V. 2007, Peipp, M., Lammerts van Bueren, J.J., et al. 2008, Sazinsky, S.L., Ott, R.G., et al. 2008). For example, Musolino et al. have studied correlation of clinical efficacy of trastuzumab treatment with the FcγRIIIA genotypes and showed that the higher affinity FcγRIIIA -158 V/V genotype was associated with better response rate and progression-free survival than the lower affinity FcγRIIIA -158 F/F genotype (Musolino, A., Naldi, N., et al. 2008). Similar correlations between FcγRIIA polymorphism and clinical response were reported by Paiva et al. for non-Hodgkin lymphoma patients treated with rituximab (Paiva, M., Marques, H., et al. 2008). These reports underline the important correlation of in vitro ADCC and clinical efficacy of current therapeutic antibodies for treatment of various types of cancer. Hemi-glycosylation decreases the PBMC mediated ADCC function by 3.5-fold, consistent with the decrease in FcγRIIIA binding. While the NK cell mediated ADCC is widely acknowledged (Nimmerjahn, F. and Ravetch, J.V. 2007), the importance of monocytes as effector cells has also been demonstrated by Uchida et al. who reported that anti-CD20 antibody depleted B cells
mainly through monocyte mediated cell killing (Uchida, J., Hamaguchi, Y., et al. 2004). In order
to better understand the influence of the inhibitory FcγRIIB on ADCC activity, we have tested
monocytes as the effector cells. Hemi-glycosylated mAb-X had a 3.4-fold decrease in monocyte
mediated ADCC function in comparison to the fully-glycosylated mAb-X, suggesting that the
decreased FcγRIIB binding could not compensate for the decreased binding to FcγRIIIA. The
differences in the FcγR binding affinity and ADCC activity of hemi-glycosylated mAb-X
compared with the fully-glycosylated antibody provide strong evidence that N-glycan occupancy
directly impacts antibody Fc-mediated effector functions. N-glycan occupancy defines the extent
of heavy chain glycosylation. Incomplete N-glycan occupancy leads to either symmetrical
aglycosylated IgG which is deficient in ADCC (Shields, R.L., Namenuk, A.K., et al. 2001, Tao,
M.H. and Morrison, S.L. 1989), or asymmetrical hemi-glycosylated IgG which has impaired
FcγR binding and decreased ADCC function that is demonstrated in this study. For therapeutic
monoclonal antibody development, it is imperative to monitor the N-glycan occupancy in order
to achieve maximum functional activities and to ensure lot-to-lot consistency.

In summary, we have, for the first time, characterized the thermal stability and functional
activity of hemi-glycosylated IgG1 in comparison with the fully-glycosylated counterpart. Since
hemi-glycosylated IgG is often co-produced with the fully-glycosylated form, although at low
levels, it is important to understand how hemi-glycosylation impacts antibody thermal stability
and functional activity. The results in this study clearly indicate that hemi-glycosylation can
impact not only the thermal stability of CH2 domain but also significantly impair FcγRI,
FcγRIIA, FcγRIIB, FcγRIIIA, and FcγRIIIB bindings and decrease ADCC activity of the
antibody.
Materials and methods

Isolation of hemi-glycosylated mAb-X

The mAb-X was produced using a glycoengineered *Pichia* strain that was capable of producing human glycans (Li, H., Sethuraman, N., et al. 2006). Purification was conducted using protocols reported earlier (Jiang, Y., Li, F., et al. 2010, Jiang, Y., Li, F., et al. 2011, Wang, S., Ionescu, R., et al. 2010). In order to isolate the hemi-glycosylated IgG fraction, mAb-X was separated on a cation exchange chromatography (CEX) using a ProPac WCX-10 (4 x 250 mm) column (Dionex, Sunnyvale, CA) and the hemi-glycosylated fraction was eluted with a salt gradient at a mild acidic condition (mobile phase A: 20 mM sodium acetate, pH 4.9; mobile phase B: 20 mM sodium acetate, pH 4.9, 1.0 M NaCl). A constant flow rate of 1 mL/min was maintained. The NaCl gradient was increased 1% per minute, which was equivalent to 10 mM NaCl increase per minute. Two peaks corresponding to the fully- and hemi-glycosylated mAb-X, respectively, were collected, concentrated, and dialyzed into a buffer containing 3% sucrose, 50 mM histidine, 50 mM arginine, pH 6.0.

Purity analysis by CE-SDS

CE-SDS experiments were conducted on Beckman PA800 CE system with a UV diode-array detector. Each mAb-X glycoform was denatured in the presence of 0.5% SDS (Sigma-Aldrich) and reduced using 5% β-mercaptoethanol (Sigma-Aldrich). After heating for 10 minutes at 70°C, each sample was injected at 5 kV for 25 seconds onto bare-fuse silica capillary (L_D = 20.2 cm, L_T = 30.2 cm, inner diameter = 50 μM, outer diameter = 375 μM) filled with SDS gel matrix (Beckman Coulter), followed by separation at 15 kV for 30 minutes. The separated protein bands were detected by UV absorbance at 220 nm with background subtraction.
at 350 nm. The electropherogram of each sample was integrated using Beckman 32Karat software. The purity of the hemi-glycosylated fraction was estimated using the peak area (A) of the aglycosylated HC and glycosylated HC, based on the formula: Purity of hemi-glycosylation = 2x A_{aglycosylated HC} / A_{total HC}. The factor of 2 was applied to account for the fact that the hemi-glycosylated antibody contains one aglycosylated HC and one glycosylated HC.

**LC-MS analysis**

A 10 μL aliquot of each mAb-X glycovariant was diluted with 10 μL of 0.1% formic acid. Samples were analyzed by LC-MS with an Agilent 1100 capillary HPLC coupled to an Agilent MSD-TOF MS. A PS-DVB column (0.5 mm i.d. × 50 mm, LC-Packings) was run at 80°C with a flow rate of 20 μL/min. The samples were eluted with a gradient of acetonitrile (mobile phase A: 0.1% formic acid in H₂O; mobile phase B: 0.1% formic acid in acetonitrile). The masses of the analytes were determined from the deconvolution of the acquired spectra using the TOF Protein Confirmation software (Agilent).

**Thermal stability study using differential scanning calorimetry (DSC)**

DSC measurements were conducted on a VP-DSC Capillary Cell Microcalorimeter (MicroCal) with a scan rate of 1°C/min in the temperature range of 25 ºC to 85 ºC. Both hemi-glycosylated and fully-glycosylated mAb-X antibodies were diluted to 1 mg/mL in 3% sucrose, 50 mM histidine, 50 mM arginine, pH 6.0, before the DSC measurement. A buffer/buffer scan was collected and subtracted from the buffer/protein scan. The thermograms were processed using the Origin 7.0 software and normalized to the molar concentration of the protein. The final
excess heat capacity thermogram was obtained by interpolating a cubic baseline in the transition region.

**Antigen binding by ELISA**

High binding 96-well plates were coated overnight at 4°C with 2 µg/ml of antigen in phosphate buffered saline (PBS), pH 7.4. Plates were washed after each incubation step with PBS/0.05% Tween 20, pH 7.4, and incubations were performed at room temperature. After coating, the plates were blocked with 300 µL of assay buffer (1% BSA in PBS) for 1 hour, and incubated for 1 hour with series of antibody titrations. A 1:2000 dilution of anti-human IgG Fc peroxidase-conjugated antibody (SouthernBiotech) was added to the plates and allowed to incubate for 1 hour. Aliquots of 100 µL of TMB solution (Virolabs) were added and the absorbance at 450nm was measured using a plate reader (Victor 3, PerkinElmer).

**Inhibition of cell proliferation**

Exponentially growing BT474.m1 cells were harvested and plated onto 96-well plates (Costar 3603, Corning Inc.) at 5,000 cells/well with 100 µL of cell culture medium (RPMI media with 10% FBS). After 24 hours, a 3-fold serial dilution ranging from 33.3 to 0.03 nM, was added to the plates. After additional 96-hour incubation, 10 µL of alamarBlue (Invitrogen) was added to each well and the cells were cultured for additional 4 hours. Fluorescence signal was read at an Ex/Em of 535/590nm (Victor 3, Perkin-Elmer).
**Inhibition of target phosphorylation**

BT474.m1 breast cancer cells were seeded at $6 \times 10^5$ cells/ml in 96-well tissue culture plates containing 100 µL of RPMI media with 10% FBS. The cells were cultured at 37°C overnight in an incubator supplied with 5% CO2. Three-fold serial dilutions of each mAb-X antibody were prepared in complete media at concentrations starting at 300 nM. A 100 µL aliquot of each dilution was added to the plate and incubated for 2 hours at 37°C. The cell lysates were prepared using cell extraction buffer containing 1 mM fresh PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). The plates were incubated for 60 min with gentle rocking at 4°C. The cell lysates were centrifuged in 96-well plates at 3,000 rpm for 10 min to collect the supernatant. The target phosphorylation was measured using an MSD assay kit (Meso-Scale Discovery) based on the manufacture's recommended procedure. Briefly, Meso-Scale assay plates were pre-coated with capture antibodies for the target and the cell lysate was added into the blocked assay plate. The plates were incubated for 1 hour at 25 °C. Detection was performed by adding an antibody against the phosphorylated target that was pre-labeled with the MSD SULFO-TAG and detecting the complex by reading the electrochemiluminescence signal (MA2400).

**FcγR binding assays using surface plasmon resonance (SPR) method**

FcγRI protein was purchased from R&D systems and the binding of antibody to the high affinity FcγRI was determined by measuring the on and off rates. The FcγRIIs and FcγRIIIs were produced in a human embryonic kidney (HEK293) cell line using a mammalian expression vector system (Invitrogen). Antibody Fc binding to low affinity FcγRIIs and FcγRIIIs were measured using a steady state binding model. Briefly, Goat F(ab')2 anti-human Kappa was
immobilized on CM5 biosensor chips (GE Healthcare). The capture antibody was then added to
the chip at a concentration of 5 μg/mL. For affinity studies, individual FcγR at various
concentrations (1600, 800, 400, 200,100, 50, 25 and 0 nM) was injected into the cells at 60
μL/min for 2 min to ensure steady state binding was achieved. Dissociation of the antibody was
then monitored for a period of 10 min. The sensor surface was regenerated using Glycine-HCl
buffer pH 1.5. Data were fitted into a 1:1 binding model using the BIAcore T100 evaluation
software. For FcγRIIs and FcγRIIIa, the equilibrium constant (KD) was calculated using steady
state fitting model; for FcγRI, the dissociation constant K_D was calculated using k_off/k_on.

**C1q binding assay**

The binding of mAb-X to human C1q (Quidel, San Diego, CA) was assessed by an
ELISA binding assay. A high binding 96-well plates were coated overnight at 4°C with 2 μg/mL
of mAb-X antigen in PBS. The plates were washed after each incubation step with PBS/0.05%
Tween 20, pH 7.4, and incubations were performed at room temperature. After coating, the
plates were blocked with 300 μL/well of assay buffer (1%BSA in PBS) for 1 hour, and incubated
for 1 hour with 0.6, 2.5, and 10.0 μg/mL mAb-X antibodies. Human C1q at 100 μg/mL was
incubated for 2 hours with the mAb-X, and the C1q binding was detected with an HRP
conjugated sheep anti-human C1q antibody (GenWay, San Diego, CA) and TMB as substrate
(Virolabs). The reaction was stopped by adding 100 μL of 1 M H₂SO₄ solution. The final signal
was measured by absorbance at 450 nm (Victor 3, PerkinElmer).
**FcRn binding assay**

A Biacore T-100 instrument (GE Healthcare Biosciences) was used to determine IgG-FcRn binding kinetics. The extracellular domain of the human FcRn protein was purified from a baculovirus system via a 6xHis tag. FcRn was immobilized onto a Biacore CM5 biosensor chip via amine coupling at densities of ~200 RU. The kinetics experiment was conducted at 25°C using PBSP (50mM sodium phosphate, 150mM NaCl and 0.05% (v/v) polysorbate 20, pH 6.0) as running buffer with a flow rate of 30 µL/min. Antibody mAb-X was allowed to bind FcRn for 3 min, followed by 2 min of dissociation. Two 30s pulses of PBSP, pH 7.5 were used to regenerate the chip.

**ADCC assay**

ADCC activity was measured using xCELLigence (RTCA-SP, ACEA Biosciences, Inc) according to the manufacturer's recommended procedure with optimized assay conditions at a ratio of effector cells to target cells of 50:1 for both PBMC and monocytes. Briefly, 15,000 target cells (SKOV3)/well were seeded into a 96 well E-plate in 100 µL of media. Cell growth was monitored with the impedance based RT-CES system until they reached log growth and formed a monolayer (about 24 hours). Effector cells were added together with different concentrations of antibodies across the plate. Target cells only, target and effector cells without antibody, and Tween 20 (2%) for 100% lysis were included as controls. The measurements were taken every 30 minutes for the first 8 hours and then every hour for the next 16 hours. Cell lysis was calculated using the cell index (CI) and the formula: (CI target only – CI sample well)/(CI target only) * 100.
Acknowledgments

We want to thank Christopher Kistler and Nihal Tugcu for producing mAb-X material and Dr. Liming Liu and Weirong Wang for C1q assay support. We are also grateful to Drs. Zhiqiang An, James Drummond, Donna Montgomery, Mary Retzlaff, Yang Wang, and Qinjian Zhao for providing critical reviews of this manuscript.
**Abbreviations:** ADCC, antibody dependent cell cytotoxicity; CDC, complement-mediated cell lysis; DSC, differential scanning calorimetry; CE, capillary electrophoresis; FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; PBMC, periphery blood mononuclear cell; SPR, surface plasmon resonance; TMB, 3,3',5,5'-tetramethylbenzidine.
Legends to figures

Fig 1. Schematic presentation of Fc N-glycan variants: Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose; Asn, asparagine

Fig. 2. Chromatograms of the CEX separation of fully- and hemi-glycosylated mAb-X mixture. A gradient of salt at pH 4.9 is used for elution. The conditions are described in the Material and Method section. The dotted blue line (---) represents the elution of the fully-glycosylated mAb-X (F), the dashed red line (---) represents the hemi-glycosylated mAb-X (H), and solid black line (—) represents the mAb-X mixture.

Fig. 3. CE-SDS analysis of the enriched hemi-glycosylated mAb-X (top, trace 1), fully-glycosylated mAb-X (middle, trace 2) and mAb-X mixture (bottom, trace 3) under reduced condition. The light chain (LC) elutes as a single peak at 15.3 min, while the heavy chain (HC) elutes as a doublet. The earlier HC peak at 18.8 min is the aglycosylated HC, and the later peak at 19.3 min is the glycosylated HC.

Fig. 4. LC-MS spectra. The x-axis represents molecular mass and the y-axis represents signal intensity. (A) Mass of the hemi-glycosylated mAb-X. (B) Mass of the fully-glycosylated mAb-X. (C) Mass of mAb-X mixture separated as hemi-(the first cluster of peaks) and fully-glycosylated IgG (the second cluster of peaks) with different glycan structures.

Fig. 5. Thermal stability of the fully-glycosylated (solid line, ——F) and hemi-glycosylated (dotted line, - - -H) mAb-X evaluated by differential scanning calorimetry (DSC).
Fig. 6. Antibody functional assays. (A) Inhibition of target receptor phosphorylation; (B) inhibition of cancer cell proliferation. The % of inhibition is calculated using the formula: (signal of mAb-X with IgG isotype control - signal of mAb-X) / signal of mAb-X with IgG isotype *100. The hemi-glycosylated mAb-X is shown as a triangle △ (dotted line) and the fully-glycosylated is shown as a solid circle ● (solid line). The y-axis error bar was calculated from n=4 separate replicates.

Fig. 7. mAb-X binding to C1q in ELISA. The box represents the 95% confidence interval, the middle line represents the mean, and the error bar shows the maximum and minimum of the data set, n=18. H, hemi-glycosylated mAb-X; F, fully-glycosylated mAb-X. The y-axis represents binding strength detected by measuring absorbance at 450nm.

Fig. 8. ADCC activities with (A) PBMC and (B) monocyte effector cells. The x-axis represents the mAb-X concentrations and the y-axis is the % of target cell lysis. Details of the method are described in the Material and Method section. The data for the hemi-glycosylated mAb-X are shown as triangle △ (dotted line) and the data for the fully-glycosylated are shown as solid circle ● (solid line).
Tables

Table 1. Binding affinities of hemi-glycosylated and fully-glycosylated mAb-X to FcγRs measured by SPR (n=3)

<table>
<thead>
<tr>
<th>Fc gamma receptors</th>
<th>Hemi-glycosylated mAb-X (K_D, nM)</th>
<th>Fully-glycosylated mAb-X (K_D, nM)</th>
<th>K_D ratio (hemi:full)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>0.45±0.02</td>
<td>0.23±0.01</td>
<td>2.0</td>
</tr>
<tr>
<td>FcγRIIA-131H</td>
<td>161±10</td>
<td>69±1</td>
<td>2.4</td>
</tr>
<tr>
<td>FcγRIIA-131R</td>
<td>234±10</td>
<td>114±4</td>
<td>2.1</td>
</tr>
<tr>
<td>FcγRIIB</td>
<td>528±10</td>
<td>260±10</td>
<td>2.0</td>
</tr>
<tr>
<td>FcγRIIIA-158F</td>
<td>38.6±0.8</td>
<td>13.1±0.1</td>
<td>2.9</td>
</tr>
<tr>
<td>FcγRIIIA-158V</td>
<td>17.4±0.5</td>
<td>7.1±0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>FcγRIIIB</td>
<td>161±9</td>
<td>84±2</td>
<td>1.9</td>
</tr>
</tbody>
</table>
References


Morgan, A., Jones, N.D., et al. (1995) The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc gamma RI and Fc gamma RIII binding. Immunology, 86, 319-324.


Shields, R.L., Namenuk, A.K., et al. (2001) High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem, 276, 6591-6604.


Figure 1.

Gal–GlcNAc–Man

Man–GlcNAc–GlcNAc–Asn$_{297}$

Gal–GlcNAc–Man

Fuc

MN2

M3N2

G0

G2
Figure 2.
Figure 3.
Figure 4.

A.

B.
Figure 5.
Figure 6.

A.

B.
Figure 7.

C1q binding, A450nm

***

Downloaded from http://glycob.oxfordjournals.org/ by guest on June 21, 2016
Figure 8.

A. PBMC

IC50

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>2 ng/mL</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>7 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>

B. Monocyte

IC50

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>10.7 ng/mL</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>36.3 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>