Functional Role of Glycosylation in a Human IgG_4 Antibody Assessed by Surface Plasmon Resonance Technology

Rosie B. Wong[1], Ming Zeng[1], An-Horng Lee[1], T. Shantha Raju[2] and Kuang-Chuan Cheng[*,1]

[1]*Bristol-Myers Squibb, Pennington, NJ 08534, USA*

[2]*Centocor R&D Inc, 145 King of Prussia Road, Radnor, PA 19382, USA*

Abstract: Fc glycosylation of immunoglobulins is necessary for antibody effector functions. These glycans of immunoglobulins are often referred as glycoforms and they can be heterogeneous due to the variations in glycosylation machinery present in the endoplastic reticulum (ER) and in the Golgi. In the absence of a generic culturing protocol that can render a consistent glycosylation pattern, monitoring glycoforms of monoclonal antibodies from cultured cells is becoming essential. Accordingly, quantification of glycosylated and deglycosylated heavy chains of an IgG_4 monoclonal antibody was accomplished using an Agilent Bioanalyzer Lab-On-the-Chip electrophoresis system. In addition to the native antibody, completely deglycosylated antibody prepared by treating with PNGase F and a $F(ab')_2$ fraction were evaluated for their antigen binding kinetics using Biacore surface Plasmon resonance (SPR). The equilibrium binding constants KD are found to be comparable at 1.81E-09M, 1.96E-09M, for the native and deglycosylated antibody, respectively, and 5.79E-10M for the $F(ab')_2$. An *in vitro* biological activity employing a competition binding assay was also developed to demonstrate the role of the Fc glycan. The results confirm that for a neutralizing antibody therapeutic the biological activity of the native MAb-1 and deglycosylated antibody are comparable, thus indicating that the Fc glycan does not contribute to the antigen binding or the biological function. The kinetics and competitive assays performed on an SPR instrument are quick and reliable. Combined with the on-chip electrophoresis method they can be used as monitoring methods for process development and quality control.

Keywords: Surface Plasmon resonance (SPR), on-chip electrophoresis, bioanalyzer, non-glycosylated heavy chain (NGHC), equilibrium dissociation constant (KD), competitive binding assay.

INTRODUCTION

A wealth of literature on the role of Fc glycan in therapeutic monoclonal antibody has been published [1-8]. Discovery of the class of immunoglobulin superfamily receptors contributed to the knowledge of cellular immune mechanism. The current knowledge of cellular immune response involves the Fc glycan moiety of the immunoglobulin which interacts with the Fc receptors on effector cells, or the complement associated receptors. The interaction between these glycoproteins results in a cascade of signal transduction events in various cells *in vivo* stimulating phagocytosis, microbe killing, endocytosis, activation of cells, antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) [9-10]. Furthermore glycosylation may also confer stability of the antibody [11-12] by preventing proteolysis leading to increased *in vivo* half-life.

In addition to cellular immune responses, antibodies can also function to neutralize a receptor-ligand interaction or physically block a receptor *in vivo*. In these instances, antibody-antigen complex is formed at the binding epitope or the variable regions of the immunoglobulin molecule residing in the $F(ab')_2$ region. Many reports were published in which the glycan moiety of the immunoglobulins were a) removed by sequential glycosidase enzyme treatment [11], b) prevented from forming by culturing in the presence of tunicamycin [12] or c) eliminated when the N-linked carbohydrate site at Asn_{297} was replaced through site-directed mutagenesis [13]. The effect of these modifications were mostly focused on the effector functions, immunosuppression or circulating half-life. In Winkelhake and Nicolson's publication [11] purified polylonal antibody and enzyme treated partially de-glycosylated products were used to determine the average intrinsic association constants Ka to the antigen by equilibrium dialysis. The Ka values were quite similar among all the preparations, in the range of $2.6x10^6$ to $7.6x10^6$. The development of surface Plasmon resonance (SPR) technology has revolutionized molecular interaction kinetics studies, while monoclonal antibody production methods have allowed mass production of homogeneous populations of immunoglobulins. With these two technologies at hand, the question of the role of glycosylation in neutralizing antibodies was addressed again. This report demonstrates that antigen binding kinetics at $F(ab')_2$ region of an IgG_4 human monoclonal antibody MAb-1 to ligand are comparable for the glycosylated, deglycosylated and $F(ab')_2$ fragment by SPR analysis.

To further support the conclusion that efficacy of this MAb-1 (a neutralize antibody) does not require a glycan moiety, an *in vitro* competition assay with the natural receptor was developed. The results confirm that the

*Address correspondence to this author at the Bristol-Myers Squibb, Analytical Development and Testing, 311 Pennington-Rocky Hill Road, Pennington, NJ 08534, USA; Tel: 609-818-3085;
E-mail: kuang-chuan.cheng@bms.com

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biological activity of the native MAb-1 and deglycosylated antibody are comparable.

Additionally, deglycosylated IgG has been reported in monoclonal antibody products such as Tocilizumab and plantibodies of Herceptin. Quantitation was achieved by separating the glycopeptide either by CE [14] or HPLC-UV-MS [15] methods. Recently, a Caliper Life Sciences microchip method was also reported [16] where the non-glycosylated heavy-chain (NGHC) can be separated and quantified under reduced condition by SDS electrophoresis. We used the Agilent Bioanalyzer, a similar reduced SDS electrophoresis system, to separate and quantify the NGHC in the MAb. Lot to lot variations of NGHC can be easily quantified on the same run in 30 minutes. To demonstrate that the NGHC is produced during cell culture process and are not generated during storage through degradation of the glycan, the quantity of NGHC was determined for a series of samples from a stability program and found to be consistent within the experimental error of the method.

MATERIALS AND METHODS

Mab-1 was a fully human monoclonal antibody produced at Bristol-Myers Squibb following industrial production procedure (Annandale, NJ, USA). PNGase F was obtained from New England Biolabs (Ipswish, MA, USA). $F(ab')_2$ isolation kit, and NanoDrop spectrophotometer were from Thermo Scientific (Rockford, IL, USA). Recombinant antigen specific for MAb-1, recombinant receptor (R) and monoclonal anti-receptor antibody (anti-R) were from R &D Systems (Minneapolis, MN, USA). BIAcore T100 instrument, immobilization chemistry kit, CM5 chips and Biacore T100 maintenance kit were obtained from GE healthcare Bio-sciences AB (Uppsala, Sweden). Agilent 2100 BioAnalyzer and Protein 230 kit were from Agilent Technologies Inc. (Waldoromm, Germany). All other chemicals were of analytical grade and were from Sigma-Aldrich (St. Louis, MO, USA).

Deglycosylation of MAb-1

About 250µg of MAb-1 protein was centrifuged with Milli Q water using a 10K centrifugal filter to remove excipients and concentrated to about 40µL. Thus prepared protein was treated with 2500 units of PNGase F digestion at 37°C for one hour. After the digestion, the product absorption at 280 nm was determined using a NanoDrop spectrophotometer to estimate the protein concentration to prepare proper dilutions for SDS electrophoresis on the Protein 230 chip as well as for competition assays. Sample preparation and running conditions followed the manufacturer's recommendations.

Preparation of $F(ab')_2$ Fragments

Pepsin digestion of MAb-1 was performed with immobilized pepsin supplied in the $F(ab')_2$ preparation kit. Digestion time as well as product purification procedures all followed the manufacturer's recommendation. The $F(ab')_2$ product was also subjected to SDS-electrophoresis on the Bioanalyzer to determine purity.

Antigen Binding test by Surface Plasmon Resonance on Biacore T100

The recombinant antigen was immobilized onto a CM5 chip using the NHS/EDC chemistry. A target of 5000 RU was found to achieve optimal binding. MAb-1 at 10µg/mL was used at flow rate of 10µl/min and 60 seconds contact time for antigen surface binding test. The experimental extinction coefficient of the whole molecule was used for concentration estimation of the glycosylated and deglycosylated MAb-1. For $F(ab')_2$ concentration, the theoretically calculated extinction coefficient equation was utilized.

Molar extinction coefficient = (# Tryptophan)(5,500) +(#Tyrosine)(1490) + (#Cysteine)(125) [17]

Binding Kinetics Analysis

The antigen immobilized chip at low density was used for binding kinetics analysis using the single cycle mode. The single cycle mode allows five concentrations of antibody solution (25nM, 50nM, 83nM, 125nM and 250nM) to be injected sequentially without regeneration. The molecular weights of the proteins were required to allow a fitting algorithm to generate the kinetics constants using the BIA-Evaluation software version 2.0.1. The molecular weight for the whole Mab-1 was obtained experimentally by mass spectrometry, the deglycosylated protein molecular weight was calculated based on the amino acid sequence, and that of the $F(ab')_2$ was estimated from the amino acid sequence of the protein subtracting the Fc portion based on the theoretical pepsin cleavage site. The equilibrium binding constant KD is calculated based on the ratio of the binding rate (k_a) and dissociation rate (k_d).

$$KD = \frac{\text{Dissociation Rate Constant } k_d}{\text{Association Rate Constant } k_a}$$

Receptor Competition Assay (*In Vitro* Biological Assay)

The efficacy of the MAb-1 as a therapy is to prevent an antigen from binding with its natural occurring receptor (R). Thus the in vitro bioassay was designed to first allow the two molecules (MAb-1 and R) to compete for the antigen sites on the chip. For comparing the biological activities of the MAb-1 and deglycosylated MAb-1, each was mixed in varying concentrations with R at 10µg/mL in pH 7.2 HBS-EP buffer and injected onto the chip. The flow rate of the competition mixture was 5µL/min, contact time was 240S. Dissociation time of 240S was necessary to eliminate non-specific or low affinity binding molecules. Next, an anti-receptor antibody (anti-R) at 1µg/mL in HBS-EP buffer was injected for 120S at 5µL/min. Since R reacts to the anit-R antibody, the RU response found in this step quantifies specifically the amount of R bound to the chip surface. The surface was regenerated with 50mM NaOH at 100µL flow rate for 20S injected twice.

Keeping the R concentration constant while increasing the MAb-1 concentration in the competition, till arrived at a point where no anti-R was bound. This indicates that MAb-1 is truly a substitute for R to its antigen as a neutralizing therapeutic. The sensorgram in Fig. (**2**) illustrates a typical competition assay.

On Chip SDS-Electrophoresis Analysis Using Bioanalyzer

The Protein 230 kit was used throughout. All protein concentrations were estimated by absorption at 280nm using the extinction coefficients described above. The small

amount of PNGaseF remaining in the deglycosylated sample was not subtracted from the protein concentration estimation. All samples were prepared with the ß-mercaptoethanol (BME) containing reducing buffer. Running conditions were that recommended by the manufacturer. Quantitation and protein sizing were achieved by comparison to a sizing ladder and internal standards run simultaneously with the samples. Weighted peak areas by their respective migration times (1/MT) were calculated using the Bioanalyzer software.

RESULTS

Bioanalyzer SDS-electrophoresis analysis showed that the native MAb-1 contains about 5.8% of the total stained bands as the 56.7kD NGHC and 58.2% as the glycosylated HC at 61.2kD under reduced condition (Fig. **1**, panel **A**). The PNGase F treated sample contained 57.7% 57kD NGHC and no 61.2kD (HC) species under reduced condition (Fig. **1**, panel **B**). The absence of the 61.2kD glycosylated heavy chain suggests that PNGase F treatment completely removed the glycan from the antibody. The reduced profile of the

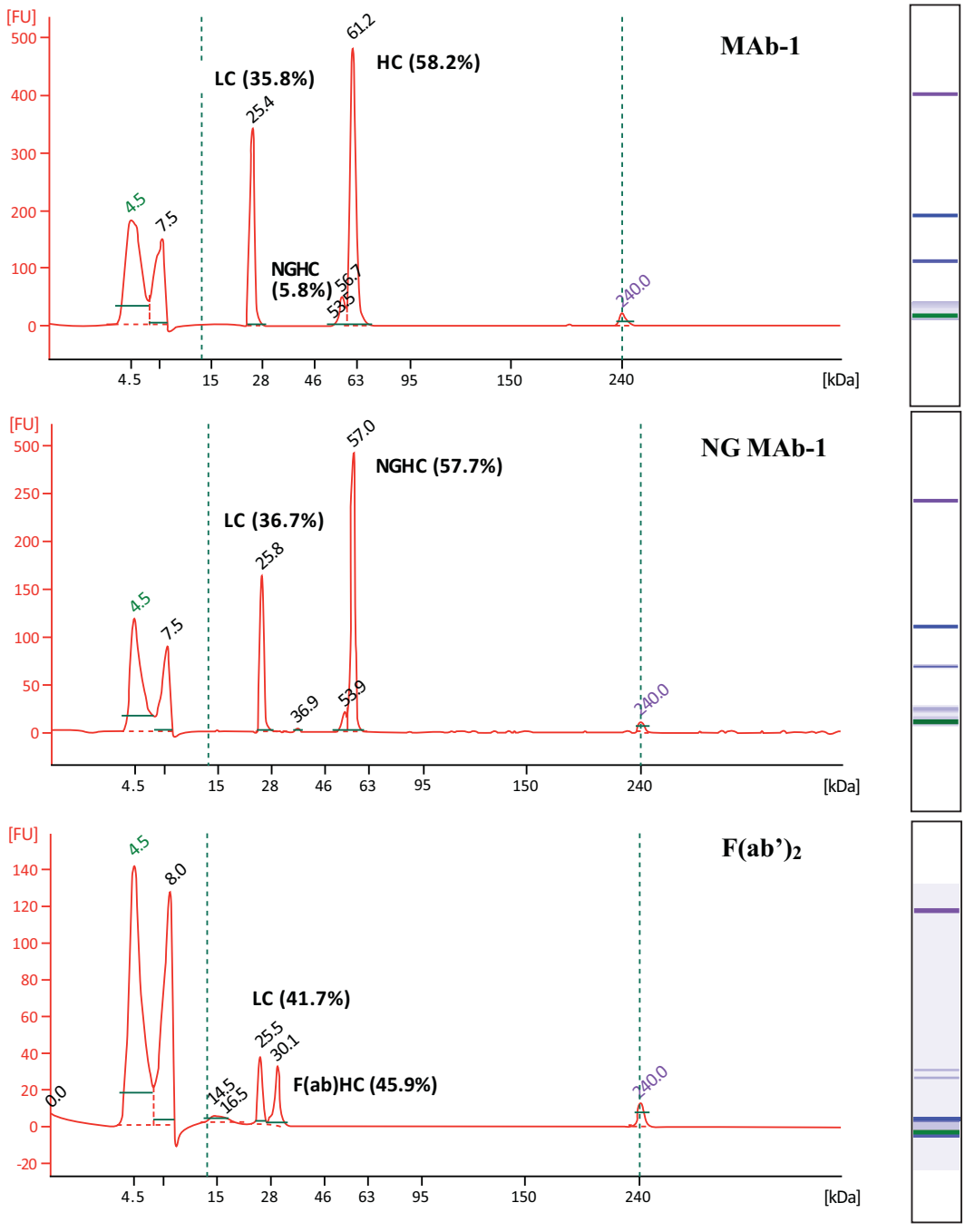


Fig. (1). Electrophoregram of MAb-1, deglycosylated MAb-1 and F(ab')$_2$ using bioanalyzer in the reducing mode. The sample labels are on the right upper corner in each electrophoregram. Numbers listed above the peaks represent the apparent molecular weights in kDs. The numbers in brackets represent the percent of the total stained bands intensity for each specific band. LC = light chain, HC = heavy chain, NGHC = non-glycosylated heavy chain and F(ab')HC = F(ab') portion of the heavy chain. The profiles in gel form are presented on the right of each electrophoregram respectively.

Table 1. Kinetic Binding Constants of MAb-1, Deglycosylated-MAb-1 and F(ab')$_2$

Sample	k_a (1/Ms)	k_d (1/s)	KD (M)	Tc	R_{max} (RU)	Chi^2
MAb-1	2.38×10^5	4.31×10^{-4}	1.81×10^{-9}	1.39E+17	45.4	1.52
Deglycosylated MAb-1	2.39×10^5	4.68×10^{-4}	1.96×10^{-9}	1.56E+17	44.2	1.27
F(ab')$_2$	8.59×10^5	4.97×10^{-4}	5.79×10^{-10}	4.99E+22	31.1	3.28

N = 3 with SD < 5%.

F(ab')$_2$ contained 45.9% 30.1kD and 41.7% 25.5kD bands confirming that the preparation has removed the Fc portion reducing the heavy chain from 61.2kD to 30.1kD (Fig. **1**, panel C). The electrophoregram of the samples as well as the percentage distribution of the visible bands are shown in Fig. (**1**).

The antigen binding kinetics (Ka, Kd and KD) of MAb-1, deglycosylated MAb-1 and the F(ab')$_2$ samples are in Table **1**. The Ka and Kd and KD values for MAb-1 and deglycosylated MAb-1 are practically the same, with Ka (on rate) at 2.38E+05 1/Ms and 2.39E+05 1/Ms and the Kd (off rate) at 4.31E-04 1/s and 4.68E-04 1/s respectively. Thus the KDs (equilibrium binding constant) are again very comparable (1.81E-09 M and 1.96E-09 M). The Rmax values are relatively low for the single cycle mode and it is reflected in the high Tc values. Thus, mass trassport issue was not a concern. The low chi^2 values indicate good fit with the 1:1 binding model. With the F(ab')$_2$, about a four fold increase in Ka was observed compared to the native MAb-1, while the Kd remained very similar. This difference may be due to the protein concentration estimation of the F(ab')$_2$. The protein concentration of F(ab')2 was estimated by using theoretically calculated extinction coefficient equation [17]. The extinction coefficient for measuring protein concentration of Mab-1 and deglycosylated Mab-1 was determined by amino acid analysis [18]. As we observed in the Bioanalyzer analysis, the major 30kD and 25kD bands constituted 87% of the total protein band areas. Thus, an over estimation of the F(ab')$_2$ concentration may be causing the higher Ka value. While the Kd value is independent of the concentration, the dissociation rate of F(ab')$_2$-antigen complex again is comparable to that of the MAb-1.

In Fig. (**2**), a typical competitive binding assay sensorgram is shown. The first injection contained a mixture of MAb-1 and the competing receptor R. The signal represented binding of either component to the antigen and thus does not discrimate MAb-1 from R. After a period of buffer wash to remove non-specific and low affinity binding species the anti-R was introduced. The RU increase during this injection indicates the presence of the R on the chip surface as a result of the competition. At constant concentration of R and increasing concentration of MAb-1, the anti-R binding (RU) decreases with increasing concentration MAb-1. Thus a competitive inverse relationship can be established with MAb-1. Fig. (**3**) shows the inverse dose relation of anti-R RU bound against MAb-1 concentration.

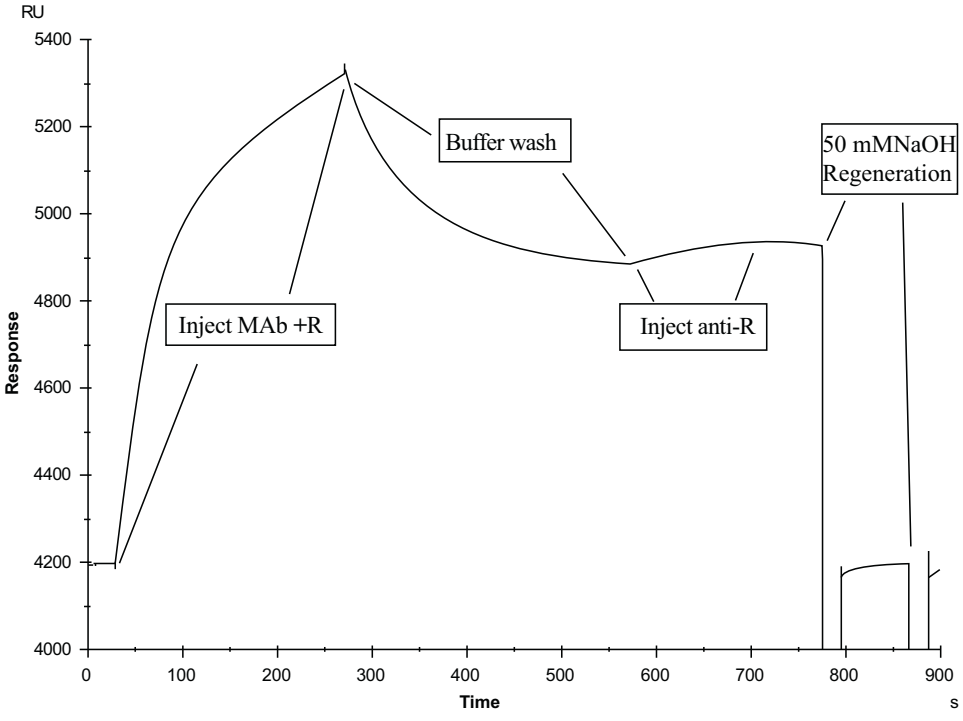
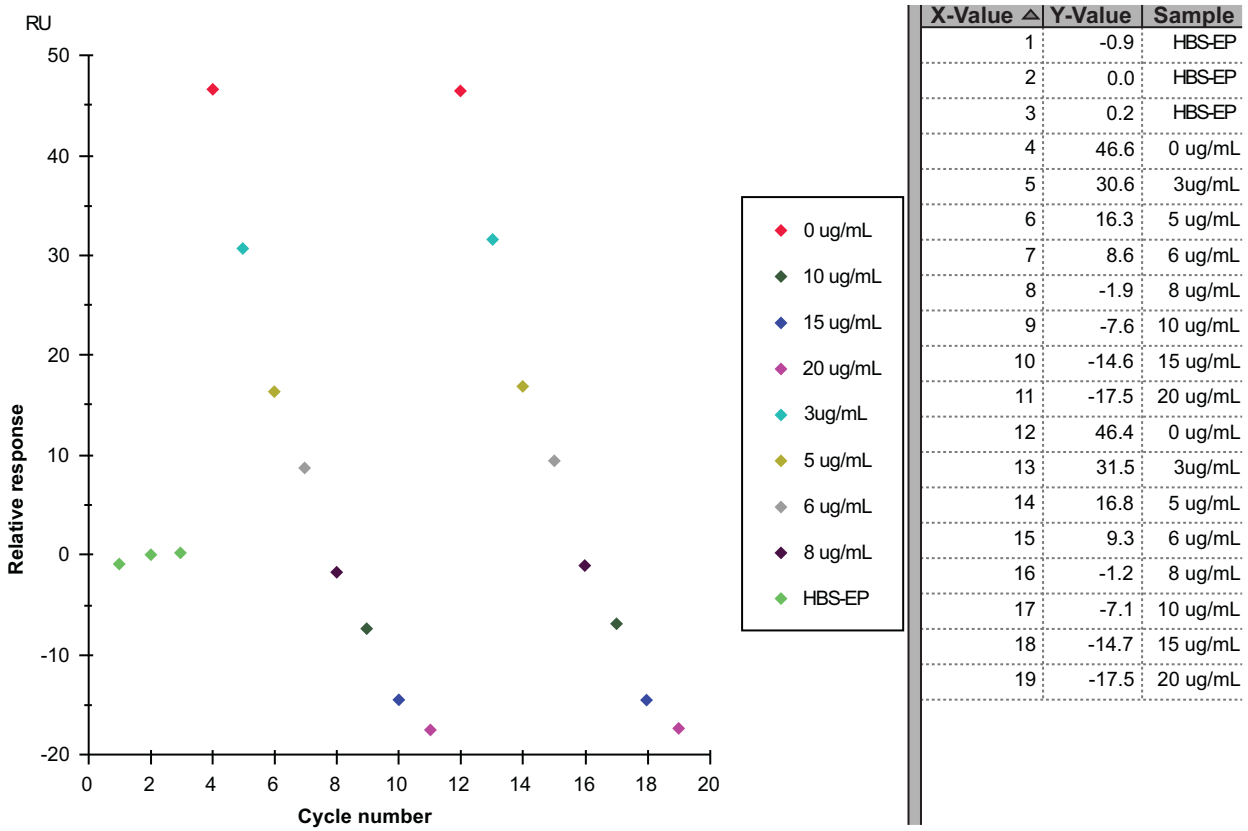


Fig. (2). Typical Sensorgram of the Competitive Assay. The steps of the assay are self explanatory. The lines directed to each step represent the duration of each step. The steps of the assay are self explanatory. The lines directed to each step represent the duration of each step.



X-Value	Y-Value	Sample
1	-0.9	HBS-EP
2	0.0	HBS-EP
3	0.2	HBS-EP
4	46.6	0 ug/mL
5	30.6	3ug/mL
6	16.3	5 ug/mL
7	8.6	6 ug/mL
8	-1.9	8 ug/mL
9	-7.6	10 ug/mL
10	-14.6	15 ug/mL
11	-17.5	20 ug/mL
12	46.4	0 ug/mL
13	31.5	3ug/mL
14	16.8	5 ug/mL
15	9.3	6 ug/mL
16	-1.2	8 ug/mL
17	-7.1	10 ug/mL
18	-14.7	15 ug/mL
19	-17.5	20 ug/mL

Fig. (3). Typical Dose Response Data of MAb-1 Concentration and Quantity (in RU) of Anti-R Bound.

Fig. (**3**) presents the data of anti-R amounts bound with changing concentration of MAb-1. Fig. (**4**) compares the competition dose response of the native and a-glyco-MAb as average of duplicate runs. The dose responses of the two samples are similar within experimental error.

In Table **2**, MAb-1 samples stored in three temperatures up to six months are analyzed using the Bioanalyzer. The percentage of the NGHC (between 57kD to 58kD) ranged from 4.5% to 6.2%. That is within the experimental limit of quantitation for the system. It was concluded that the amount

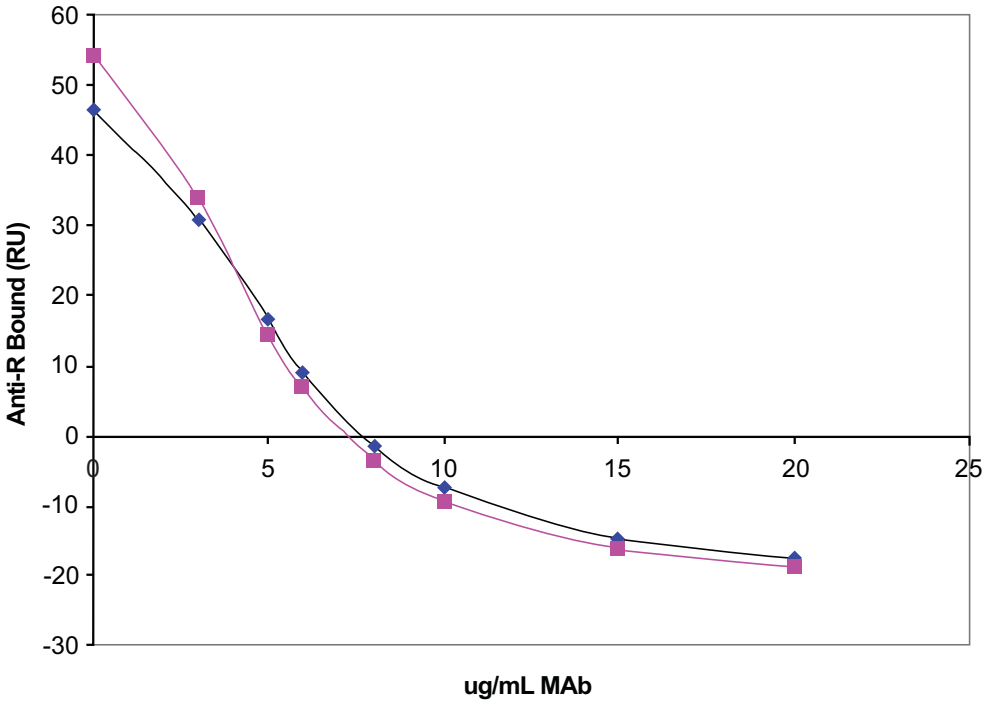


Fig. (4). Comparison of native and a-glyco MAb competitive dose response. ♦ native MAb; ■ a-glyco MAb.

Table 2. Relative Distribution of HC, NGHC and LC in the Thermo-stability Samples by On-Chip Electrophoresis in Reducing Mode

Sample Name	HC		NGHC		LC	
	Size (kD)	%Total	Size (kD)	%Total	Size (kD)	%Total
MAb-1 at 2-8 ºC T=0Mo	61.5	60.1	57.3	5.1	25.9	34.6
MAb-1 at 2-8 ºC T=3Mo	61.7	60.2	57.5	4.9	26.1	34.7
MAb-1 at 2-8 ºC T=6Mo	62.4	58.2	57.9	6.2	26.4	35.3
MAb-1 at 23-27 ºC T=3Mo	62.7	58.5	58.2	5.3	26.7	36.2
MAb-1 at 23-27 ºC T=6Mo	63.1	57.7	58.8	5.9	26.9	36.3
MAb-1 38-42 ºC T=1Mo	62.3	60.8	58.0	4.6	26.4	34.4
MAb-1 38-42 ºC T=2Mo	62.9	59.2	58.5	4.5	26.3	33.9
MAb-1 at 38-42 ºC T=3Mo	63.2	57.5	58.6	4.9	26.5	34.6
MAb-1 at 38-42 ºC T=6Mo	62.6	54.7	58.5	5.2	26.1	33.6

N = 3 with SD < 5%.

of NGHC in MAb-1 did not change upon storage. Decrease in relative percentage (%) in HC and LC is due to degradation under thermal stress (23-27^{0}C or 38-42^{0}C) in aged samples.

DISCUSSION

The presence of NGHC in a monoclonal antibody may change the efficacy of an antibody that renders cellular function such as ADCC, CDC *in vivo*. In the case of neutralizing antibodies, the antibody antigen complex formation does not involve the Fc glycan, thus theoretically a population of deglycosylated antibody in a monoclonal drug (as NGHC shown in reduced SDS-electrophoresis) should not affect the potency of the antibody drug. This report confirms that indeed the presence of some non-glycosylated antibody, in the case of MAb-1, about 5-6%, did not affect the antibody from binding to its natural antigen. This was shown by comparing the Ka, Kd and KDs of the glycosylated and deglycosylated proteins to the natural antigen. Furthermore, the kinetics constants are also similar when the entire Fc portion of the antibody was removed. The seemingly four fold higher Ka for the F(ab')$_2$ can be due to the inaccuracy in the concentration estimation by theoretical extinction coefficient of the F(ab')$_2$ preparation. The biological function of this blocking antibody was also evaluated in an *in vitro* assay using the receptor as the competing molecule for antigen binding. Again, the results showed that MAb-1 with or without glycan exert similar biological functions, that is, occupying all the antigen sites on the chip surface and prevented the receptor from binding to the antigen. As a result, no anti-receptor antibody was bound during the second injection step. The F(ab')$_2$ sample was not subjected to the competition dose response test since the preparation was not 100% pure thus the concentration estimation may cause erroneous conclusions.

A survey of NGHC quantity in various stability samples indicated that the NGHC was consistently present in the preparation and not a product of storage. This provides confidence during process monitoring and storage that the NGHC does not affect the potency of the therapeutic product.

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CONFLICT OF INTEREST

Declared none.

ABBREVIATIONS

F(ab')$_2$ = Fragment antigen-binding dimmer

F(ab') = Fragment antigen-binding

Fc = Fragment constant

PNGase F = Peptide: *N*-glycosidase F

NGHC = Non-glycosylated heavy chain

MAb-1 = Monoclonal antibody-1

KD = Equilibrium dissociation constant

Ka = Association constant

Kd = Dissociation constant

BME = ß-mercaptoethanol

HBS-EP = HEPES buffer with EDTA and P20 detergent.

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