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Comparison of orthogonal chromatographic and lectin-affinity microarray methods for glycan profiling of a therapeutic monoclonal antibody

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ABSTRACT

The N-linked glycosylation of four lots of a marketed human therapeutic monoclonal antibody (mAb) was assessed by three orthogonal chromatographic methods and a commercial lectin microarray. For chromatography, the *N*-glycans were removed enzymatically from the mAbs using PNGase F. Native glycans were determined by HPAEC-PAD using a panel of 21 *N*-glycan standards and a multi-stage linear gradient eluent profile for sequential analyses of typical neutral and sialylated glycans in one chromatographic run. The monosaccharide contents of these glycans following acid hydrolysis were confirmed by HPAEC-PAD with monosaccharide standards. Glycosylation analysis by HILIC-FD after stoichiometric labelling with two different fluorescent tags (2-AA and 2-AB) enabled direct quantitation. The 2-AA- and 2-AB-labelled versions of the same glycan standard panel yielded distinctive separation profiles suitable for orthogonal identification of mAb glycans. Glycan profiling with the lectin microarray required partial denaturation of the intact mAbs to expose the sequestered Fc *N*-glycans. Glycosylation fingerprints were obtained using a fluorescently labelled antibody directed against human IgG Fc. Fluorescence intensities from the fingerprints were deconvoluted with a proprietary algorithm to obtain semi-quantitative "glycan structural class" information. Glycosylation analyses of the four mAb lots by these four methods, which separate and detect oligosaccharides according to different principles, provided complementary and corroboratory qualitative and quantitative information. The predominant N-linked structures were core-fucosylated asialo diantennary structures with varying galactosylation. There were also trace amounts of afucosyl and bisected glycans, but no detectable sialylation by any of the four methods. The therapeutic mAb demonstrated a high degree of consistency in the types and amounts of N-linked glycans in the four lots (<6% CV), and between all four analysis methods (<6% CV). The described methods are co-supported by the excellent quantitative agreement of their results, which is particularly notable considering the orthogonality of their separation and detection mechanisms.

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1. Introduction

Therapeutic monoclonal antibodies (mAbs) constitute the largest and fastest growing class of biological drugs under development for human use [1–4]. In 2010, there were 28 mAb therapeutics marketed in the USA or EU; nine of these were considered "block-

Abbreviations: mAb, monoclonal antibody; IgG, immunoglobulin G; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; HILIC-FD, hydrophilic-interaction chromatography with fluorescence detection; 2-AA, 2-aminobenzoic acid (anthranilic acid); 2-AB, 2-aminobenzamide (anthranilamide); RODI, reverse-osmosis deionized; MWCO, molecular weight cut-off; PBS, phosphate-buffered saline; PNGase F, peptide:*N*-glycosidase F; TFA, trifluoroacetic acid; CV, coefficient of variation; DMSO, dimethyl sulfoxide.

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busters" with annual sales of more than $1 B each, and five generated over $4 B each [3,4]. Therapeutic mAbs accounted for more than $43 B in revenue, nearly half of the annual therapeutic biotechnology market [1,2,4]. Growth in sales of human therapeutic mAbs is predicted to continue at rates ~50% greater than vaccines, 3-fold more than other therapeutic proteins, and 4 to >10 times more than small-molecule drugs [1,2,4]. As of 2010, there were ~30 mAbs approved by international regulatory authorities and marketed for therapeutic use, with another 250 mAbs undergoing clinical trials, including 25–30 in late stage [1–4].

All currently approved therapeutic mAbs are of the immunoglobulin G (IgG) isotype; glycoproteins which are *N*-glycosylated at a fully conserved and completely occupied site in each of the two C_H2 domains near the hinge of the Fc region [5–11]. Unlike the complex *N*-glycans of most membrane and secreted glycoproteins, those of IgG Fc are incomplete structures with varying amounts of branch *N*-acetylglucosamine and terminal galactose, and generally minimal sialylation. The glycan structures associated with this site are derived from a library of ~35 mostly complex diantennary *N*-oligosaccharides, and few high-mannose or hybrid-type glycans [5–16]. The IgG glycoform portfolios of each mammalian species contain characteristic subsets/ratios of these structures [15,17,18]. Human IgG Fc *N*-glycans have a very high degree of core fucosylation and a low degree of bisecting *N*-acetylglucosamine [5,6,12,13,15–22].

Glycosylation is a very complex process dependent on many factors including the availability, relative activity and sequence of action of the relevant components of the glycosylation machinery. These in turn are composite functions of the species, age/gender of the organism, the type of cell/tissue, individual clonal variation, the physiological state of the organism and state of differentiation of the cell, and other biological, environmental and physicochemical factors. It is the dynamic interactions of various genetic, epigenetic and other biological and environmental factors that accounts for responsive glycosylation variability [9,10,12,23–35]. For example, the glycosylation of polyclonal IgG *in vivo* is altered during normal physiological changes such as aging [20,36–41] and pregnancy [21,22,42–45], and certain disease states such as rheumatoid arthritis [6,8,12,46–48] and systemic inflammation [11,49,50].

IgG plays important roles in both innate and adaptive immunities, in which the state of glycosylation provides the capacity to regulate the intrinsic underlying IgG functions through glycan-dependent structural effects in the receptor-binding regions of the hinge/C_H2 and C_H2/C_H3 domains [6–8,10,11,51]. These IgG glycoform conformational variants differentially interact with the FcγRs and other receptors variously expressed on immune cells and other cells throughout the body. Therefore, the absence or modification of Fc glycans can profoundly affect the therapeutic performance of mAbs by modulation or loss of intended functions, greater immunogenicity, and unfavourable pharmacokinetic profiles [6–11,14,51–54].

For these reasons, it is imperative that therapeutic glycoproteins are generated under controlled production conditions and vigilantly monitored to ensure consistent, reproducible and predictable glycosylation for the desired and expected efficacies. Many different aspects of cell culture and production have been shown to influence the glycosylation profiles and functions of mAbs and other recombinant glycoproteins, including clone selection, cell expression systems and growth conditions. Careful assessment of glycosylation profiles not only in final bulk and formulated batch products, but also during product development, scale up, process changes and production may identify factors or methods that affect glycan patterns or heterogeneity. These observations may in turn isolate potential glycosylation control points to selectively enable production of preferred glycoforms for specific functional purposes.

Glycosylation analysis by a single method is insufficient for a comprehensive assessment of the types and amounts of glycan structures in glycoproteins. The addition of orthogonal methods provides complementary and corroboratory information to clarify instances of inconclusive data. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and hydrophilic-interaction chromatography with fluorescence detection (HILIC-FD) are among the most widely used and informative methods for the determination of glycoprotein glycosylation, and are industry standards in glycan analysis [53,55–64]. However, both HPAEC-PAD and HILIC-FD require removal of glycans from the underlying protein prior to analysis, involve multiple steps are overall rather time-consuming and relatively expensive, demand significant operator experience and expertise, and involve considerable data analysis. In contrast, lectin microarrays provide medium-throughput glycan fingerprinting information using comparatively inexpensive scanner platforms already available in many laboratories [65–77]. Proficient operation is achieved with significantly less training and experience than other methods. However, the information provided from such microarrays does not detail the types and amounts of individual glycan chains, but rather is limited to grouping the data into glycan structural classes. Despite this, semi-quantitative determination of mAb glycosylation is made possible through the application of a trained algorithm.

In this study, the *N*-glycans from four lots of a marketed human therapeutic recombinant mAb product were analyzed by three orthogonal chromatographies. Native glycans were determined by HPAEC-PAD followed by confirmatory monosaccharide content analysis. For examination by HILIC-FD, the *N*-glycans were derivatized stoichiometrically at the reducing terminus (one fluorophore per N-linked glycan) with either 2-AA (2-aminobenzoic acid or anthranilic acid) label or 2-AB (2-aminobenzamide or anthranilamide) label, which resulted in distinctive elution profiles. The facility of these methods and the usefulness of their results are compared with those from a commercial semi-quantitative lectin microarray platform for the ability to accurately evaluate glycan composition of IgGs/mAbs.

2. Materials and methods

2.1. Reagents; mAb sample information and preparation

All chemical reagents were obtained from Sigma–Aldrich unless otherwise specified. All solutions, buffers and eluents were made with 18.2-MΩ cm reverse-osmosis deionized (RODI) water.

Four lots of a marketed human therapeutic recombinant IgG_1 mAb product were obtained from the manufacturer. These were stored and monitored in original packaging at 4 °C until required before indicated expiry date. To remove excipients, 400-μL samples containing 10 mg of each mAb lot were extensively dialyzed at 4 °C in pre-rinsed 20-kDa MWCO 0.5-mL dialysis cartridges (66005, Pierce Thermo Fisher Scientific) against phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4). After dialysis, samples were concentrated back to 400 μL in pre-rinsed 50-kDa MWCO centrifugal filter concentrators (42416, EMD Millipore).

2.2. Enzymatic deglycosylation of mAbs; isolation of released N-linked glycans

Monoclonal antibody samples were enzymatically deglycosylated with peptide:*N*-glycosidase F (PNGase F; E-PNG01, QA-Bio) for 24 h at 37 °C. Reaction mixtures (500 μL PBS) contained 10 mg of mAb (20 mg/mL) and 40 mU of PNGase F (80 mU/mL) to give an enzyme/substrate ratio of 4 mU enzyme/mg mAb. Reducing SDS-PAGE of the mAb lots before and after PNGase-F digestion con-

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