Separation of Fucosylated and non-Fucosylated Carbohydrates Associated with Monoclonal Antibodies using Capillary Electrophoresis

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In order to gain a comprehensive understanding of therapeutic Monoclonal Antibody (MAb) function, it is necessary to critically characterize glycosylation associated with them. Carbohydrates, and therefore glycosylation, are known to play an important role in the structure, function, and clearance of MAbs and have been shown to be responsible for invoking immune responses in humans. Changes in carbohydrate composition or concentration can significantly impact the overall efficacy of therapeutic MAbs and can also lead to side effects. Presence of fucose on monoclonal antibody associated N-linked oligosaccharides is a notable glycan modification and has been linked to a decrease in antibody dependent cellular cytotoxicity (ADCC). Accurate analysis of fucosylated and afucosylated oligosaccharides is therefore critical for a complete understanding of MAb microheterogeneity. Capillary Electrophoresis (CE) technology has been successfully used to separate major IgG N-linked oligosaccharides G0, G1, and G2 structures from one another. The basis for this separation relies on electrophoresis of oligosaccharides labeled with amino pyrene tri-sulfonic acid (APTS). The complexity of glycans associated with many molecules calls for high resolution separation in order to assess heterogeneity among carbohydrate isomers and co-migrating carbohydrate species. Since CE is already an established technology for automated and quantitative analysis of N-linked oligosaccharides, we set out to develop a methodology by which fucosylated oligosaccharides can be differentiated from afucosylated species. Optimization of chemistry and CE methods enabled separation of fucosylated and non-fucosylated carbohydrates from each another.

Introduction

Immunoglobulins or antibodies are soluble serum glycoproteins involved in passive immunity against foreign antigens. Mono-specific or monoclonal antibodies (MAb) have been developed as therapeutic reagents because of their specificity towards particular molecular targets associated with disease manifestation. There exists a high degree of structural and functional heterogeneity among antibodies, due in large part to the diversity of associated glycan populations. Glycosylation on therapeutic monoclonal antibodies is a critical post-translational modification that has been associated with bioactivity, structure, and pharmacokinetics. A number of different carbohydrate moieties can potentially associate with MAbs, but it is generally thought that a core group of bi-antennary and high-mannose structures make up the most common species.

MAb carbohydrate heterogeneity analysis and quantitation is essential as oligosaccharides linked to their Fc region play an important role in regulation of cell-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC)[1]. Increase in terminal galactose (Gal) on MAb N-linked oligosaccharides has been implicated in up-regulation of CDC[2]. Therefore, separation and quantification of Gal-containing oligosaccharides is beneficial to better understand MAb function. Glycan species varying in terminal Gal content can be readily separated and analyzed using existing CE technology. Glycan sample preparation includes addition of both charge and fluorescence properties, allowing oligosaccharides to be electrophoretically separated and then detected using laser-induced fluorescence (LIF) technology. First, oligosaccharides are removed from the Asn297 residue of the MAb backbone using the N-glycosidase PNGase F. This is followed by derivatization of the fluorophore aminopyrene tri-sulfonic acid (APTS) via reductive amination at the reducing end of the oligosaccharide (Figure 1A).

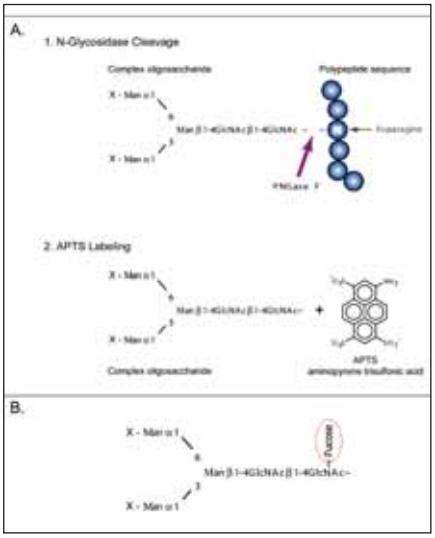

Figure 1. Schematic of glycan analysis sample preparation. A. Glycan cleavage and APTS derivatization strategy for analysis of N-linked oligosaccharides. B. Fucosylated N-linked oligosaccharide.

Electrophoretic separation can be performed utilizing a polymeric separation matrix consisting of 0.4% polyethylene oxide (PEO).

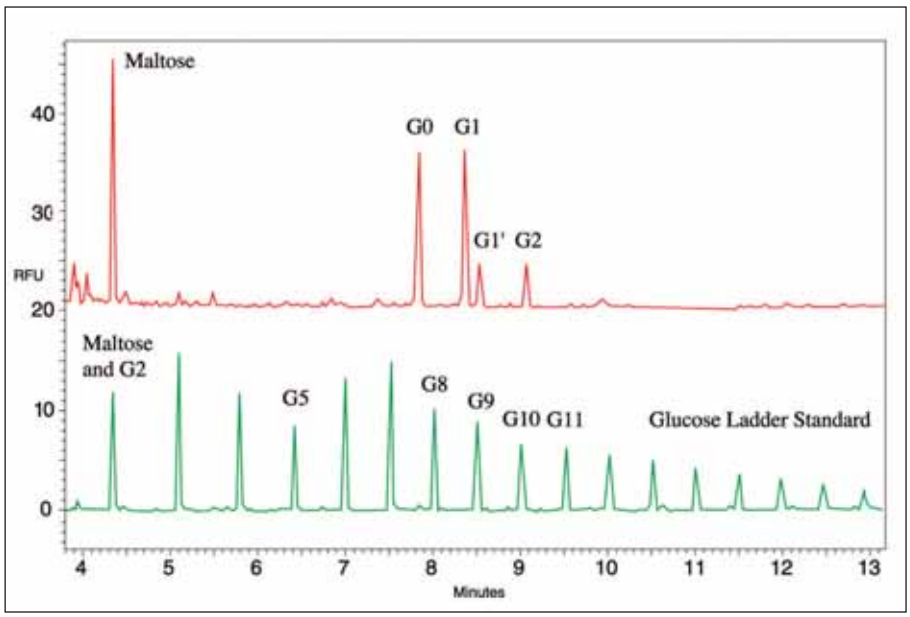

Figure 2. Separation of G0, G1, and G2 glycan species. Representative data (top trace) shows separation of N-linked oligosaccharides G0, G1, G1', and G2 using the Carbohydrate Labeling & Analysis Assay Kit (Beckman Coulter p/n 477600). G1 positional isomers are resolved from one another illustrating the mobility- and hydrodynamic volume- based separation. The bottom trace shows separation of a glucose ladder standard. The 'G' designation for the glucose ladder standards refers to the number of glucose subunits making up that standard.

Beckman Coulter has developed and commercialized technology (Beckman Coulter p/n 477600) to help automate and simplify this process. It has been shown that the principle for this CE separation of oligosaccharides is based on both their mobility and hydrodynamic volume[3]. This is illustrated in part by the fact that positional isomers, although identical in mass, can be resolved from one another (Figure 2). The presence of a core fucose moiety on MAb N-linked oligosaccharides has been associated with a decrease in ADCC activity and thus reduced efficacy[4] (Figure 1B). It is suggested that therapeutic MAbs produced in various cell lines such as the commonly used Chinese Hamster Ovary (CHO) cells contain glycans that are >90% fucosylated[5]. Because of this, separation of these species is necessary for accurate analysis. Due to a size difference as small as 16 daltons between fucosylated and afucosylated glycans, as well as the presence of numerous positional isomers, separation has proven to be difficult. Current methods have been incapable of resolving all of the major co-migrating glycan species from one another. The success for CE technology to resolve terminal Gal differences on oligosaccharide species suggests that it should also be capable of separating fucosylated from afucosylated species

Methods and Materials

All separations were performed using the PA 800 plus Pharmaceutical Analysis System configured with a 488 nm solid state laser and LIF detection using an emission band-pass filter of 520 nm ± 10 nm (Beckman Coulter Inc). N-CHO capillaries were used for separation of oligosaccharides. All other assay conditions were as described in the standard operating procedure for the Carbohydrate Labeling and Analysis Assay Kit, (Beckman Coulter p/n 477600) with the exception that the carbohydrate separation buffer was substituted with a new separation buffer formulation where indicated. Final concentration for oligosaccharide samples was 1.25 mM. Glycan standards for fucosylated and afucosylated species of G0, G1, G1', and G2 were purchased from Glyko ProZyme, Inc. (Hayward, CA). The therapeutic MAb was obtained from Genentech, Inc. (S San Francisco, CA).

Experimental details for this work were as follows:

- Carbohydrate separation gels used
- Carbohydrate assay gel (contains polyethylene oxide (PEO)) buffer or,
- New separation gel buffer was 1:1 mixture of:
- Carbohydrate separation gel buffer (PEO) – BEC p/n 477623
- dsDNA1000 separation gel buffer (LPA) – BEC p/n 477628
- Capillary length: total length = 60.2 cm, length to detector = 50 cm
- Capillary diameter: 50 mm I.D.
- Injection conditions: 0.5 psi for 10 sec
- Separation Voltage: 30 kv
- Field Strength: 500 volts/cm
- Capillary cartridge temperature: 20° C
- Sample storage temperature: 10° C

Results and Discussion

The goal of this study was to achieve separation of the major glycan species associated with monoclonal antibodies. This glycan population includes positional isomers as well as fucosylated and afucosylated oligosaccharides. We set out to characterize

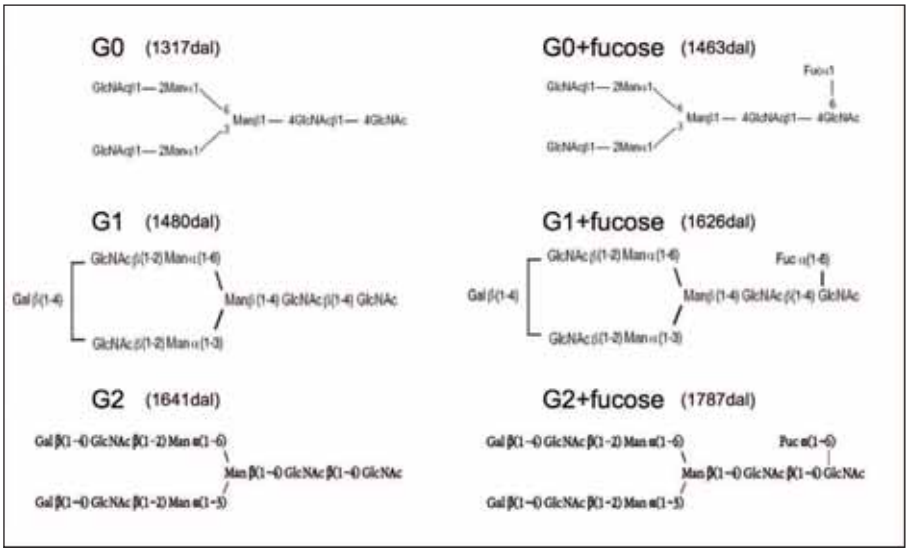

Figure 3. G0, G1, and G2 oligosaccharides. G0, G1, and G2 oligosaccharides are commonly associated with monoclonal antibodies. The 'G' designation refers to the number of galactose subunits occupying the bi-antennary termini of the oligosaccharides. Note G1 can exist as either one of two positional isomers differing in only the location of the terminal galactose.

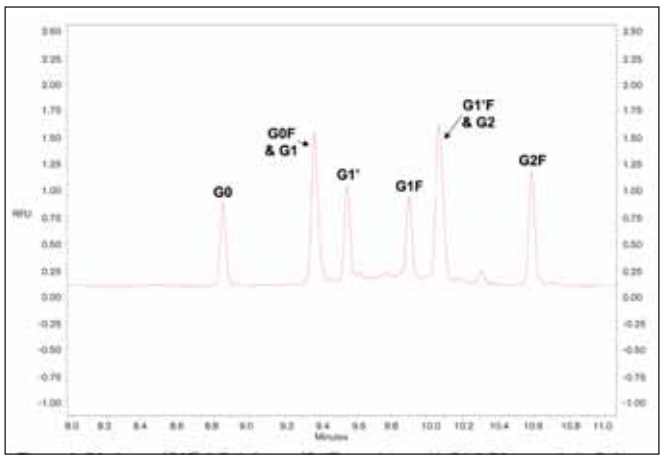

Figure 4. G0+fucose (G0F) & G1'+fucose (G1'F) co-migrate with G1 & G2 respectively. Spiking experiments were performed and illustrated co-migration of G0F with G1 oligosaccharide species as well as co-migration of G1'+fucose (G1'F) with G2. This separation was performed using the standard separation buffer and conditions described in the Carbohydrate Labeling & Analysis Assay (Beckman Coulter p/n 477600).

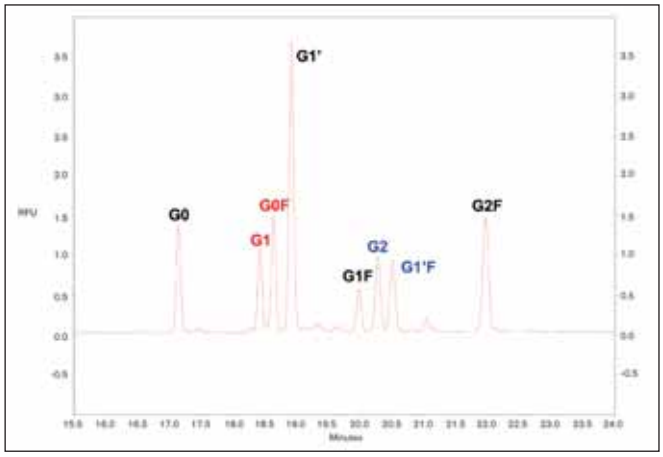

Figure 5. Optimization of the carbohydrate separation buffer allows for resolution between co-migrating oligosaccharide pairs. Using standard sample preparation protocols, oligosaccharide standards were APTS labeled and separated by CE. Resolution of co-migrating fucosylated and afucosylated N-linked oligosaccharide standards was facilitated by combining existing Beckman Coulter separation buffers. Separation buffer consisted of a 1:1 mixture of Carbohydrate Separation Buffer (Beckman Coulter p/n 477623) containing 0.4% PEO and dsDNA 1000 Gel Buffer (Beckman Coulter p/n 477628) containing a final concentration of 6% linear polyacrylamide (LPA). Co-migrating species G0F and G1 (red labels) as well as G1'F and G2 (blue labels) were separated from one another.

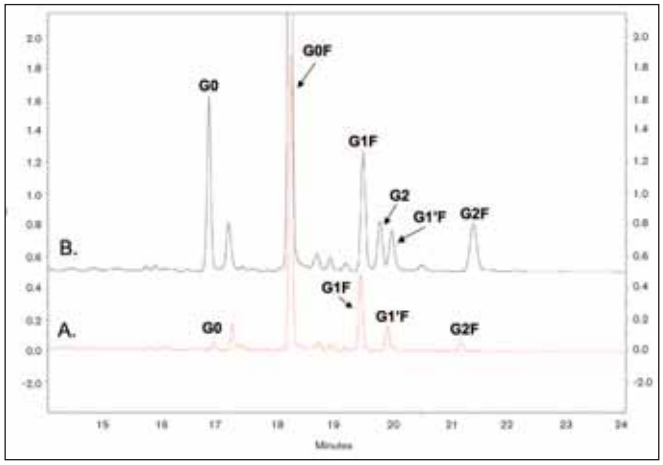

Figure 6. Separation of oligosaccharides associated with a recombinant therapeutic MAb. Oligosaccharides were cleaved from a therapeutic MAb, APTS labeled, and separated by CE using the new buffer formulation. A number of oligosaccharide species were resolved from one another (A). In order to identify and help illustrate resolution between co-migrating glycan species, we spiked the MAb sample with standards. Relative to the oligosaccharide standards, we were able to quantifiably identify G0, G0F, G1F, G1'F, and G2F. G2 standard was also spiked into the mixture to indicate the location in the separation at which this oligosaccharide species would reside. Further experimentation is being performed to identify the additional species present in the electropherogram. Separation conditions were the same except that injection for the MAb alone was 0.5 psi and that for the MAb + glycan standards was 1.5 psi.

the separation limitations for an existing separation chemistry, the Beckman Coulter Carbohydrate Labeling and Analysis Assay Kit. Employing standard kit protocols for instrument configuration, sample preparation, and separation conditions, we easily attained baseline resolution between G0, G1 positional isomers (G1 and G1'), and G2 oligosaccharide species (Figure 2). A systematic approach was devised in which standards were spiked into samples to help identify additional peaks in this separation and also to better define co-migration of glycans that may be occurring. The G0, G1 and G2 species are shown in Figure 3. Spiking experiments using oligosaccharide standards illustrated that individual separated peaks may contain multiple glycan species. This was demonstrated by co-migration of G0+fucose (G0+F) with G1, and co-migration of G1'+fucose (G1'+F) with G2 (Figure 4). Modification of separation parameters such as capillary length, separation voltage, and temperature did not offer improved resolution (data not shown). By developing a new separation buffer formulation, we were better able to resolve these co-migrating species (Figure 5). In order to test this separation method on a real molecule, we obtained a therapeutic MAb and analyzed glycans associated with it (Figure 6). Spiking with oligosaccharide standards to help identify glycan species, we showed good resolution between many of the major oligosaccharides including G1'+F and G2, which were previously difficult to separate by CE.

Conclusions

High resolution capillary electrophoresis separations based on mobility and hydrodynamic volume have been developed for quantitative analysis of glycans. Using published protocols and commercially available reagents, we have shown this technology to be capable of separating oligosaccharides differing in terminal galactose. We also showed that by combining the standard PEO separation gel buffer with a LPA gel buffer, we were able to separate fucosylated from afucosylated N-linked oligosaccharides. This work suggests that CE can be used to successfully separate and quantify N-linked oligosaccharide populations associated with MAbs. Additional experimentation will focus on development of these methods.

References

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