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

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Abstract

In order to gain a comprehensive understanding of therapeutic Monoclonal Antibody (MAb) function, it is necessary to critically characterize glycosylation associated with them. Carbohydrates 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. Because of their link to Antibody Dependent Cellular Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC), accurate analysis of oligosaccharide fucosylation, sialylation, and antennary structure is 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 8-amino pyrene 1,3,6 trisulfonic 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 methodology by which fucosylated, afucosylated, sialylated and complex antennary oligosaccharides can be differentiated from one another. Additional experimentation will focus on further development of these methods.

Introduction

Immunoglobulins or antibodies are soluble serum glycoproteins involved in passive immunity against foreign antigens. Monoclonal Antibodies (MAbs) 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 glycosylation. Glycosylation on therapeutic monoclonal antibodies is a critical post-translational modification that has been associated with their bioactivity, structure, and pharmacokinetics.

A number of different carbohydrate moieties can potentially bind to MAbs, but it is generally thought that a core group of bi-antennary and high-mannose structures make up the most commonly associated species. MAb carbohydrate heterogeneity analysis and quantitation is essential as oligosaccharides linked to their Fc region play an important role in the regulation of Complement Dependent Cytotoxicity (CDC) and Antibody Dependent Cellular Cytotoxicity (ADCC).[1]

An increase in terminal galactose (Gal) on MAb N-linked oligosaccharides has been implicated in up-regulation of CDC.[2] 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 quantitated using laser-induced fluorescence (LIF) detection technology.

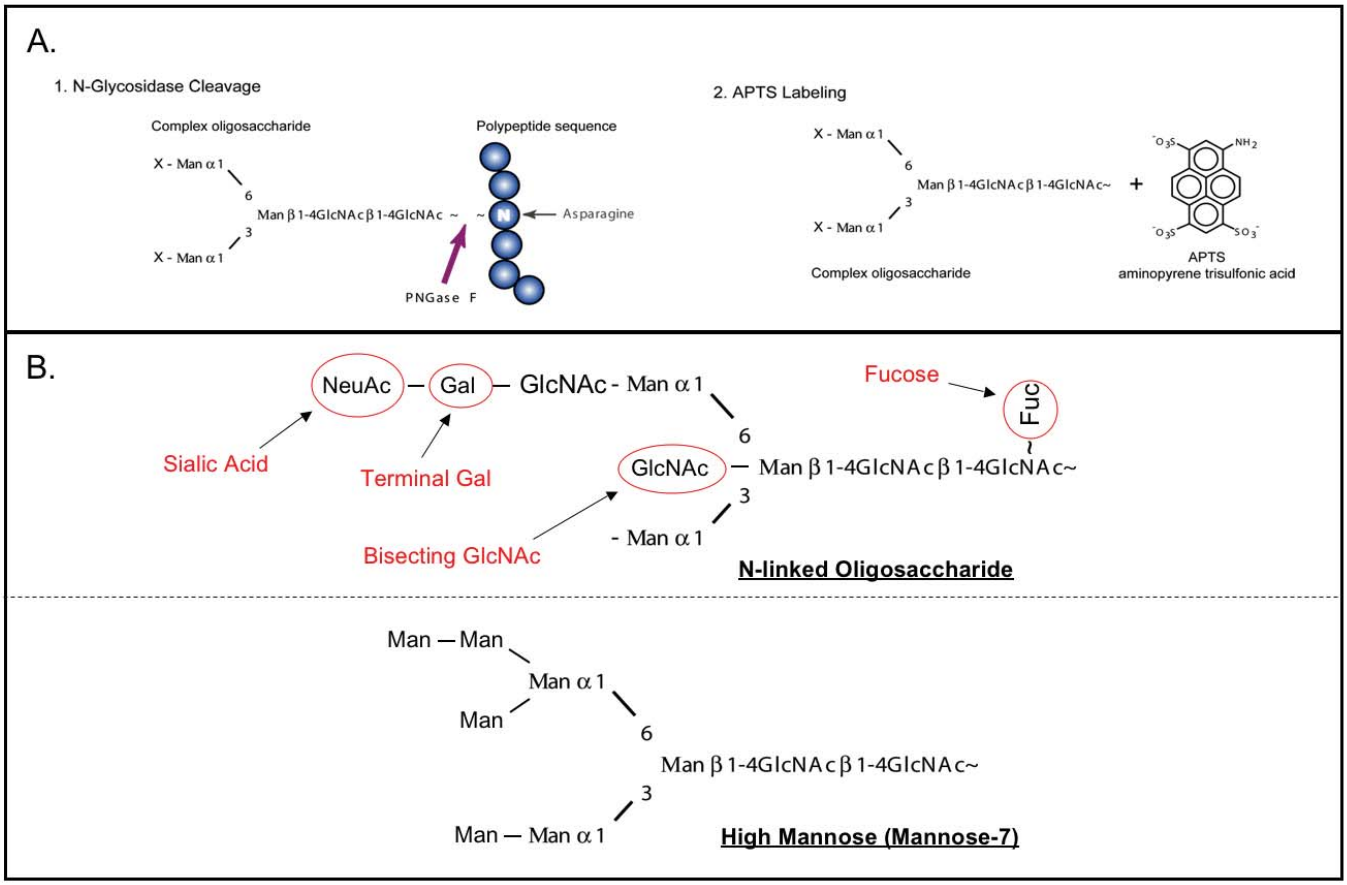

Figure 1. Schematics of glycan analysis sample preparation and various carbohydrate structures. A. Glycan cleavage and APTS derivatization strategy for analysis of N-linked oligosaccharides. B. Examples of 2 glycan species: N-linked oligosaccharide illustrating putative important modifications (left) and high mannose structure (right)

First, oligosaccharides are removed from the Asn[297] residue of the MAb backbone using the N-glycosidase F (PNGase F). This is followed by derivatization of the fluorophore 8-aminopyrene-1,3,6-tri-sulfonic acid (APTS) via reductive amination at the reducing end of the oligosaccharide (Figure 1).

Electrophoretic separation can be performed utilizing a polymeric separation matrix consisting of 0.4% polyethylene oxide (PEO). Beckman Coulter has developed and commercialized technology (Beckman Coulter p/n 477600) to automate and simplify this process. It has been shown that the principle for this gel-based CE separation of oligosaccharides is based on both 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).

Modifications on glycan structures including the presence of fucose, terminal sialic acid, or a bisecting N-Acetylglucosamine (GlcNAc) have been associated with changes in ADCC activity, thus have an impact on MAb efficacy.[4,5] High mannose structures also have been implicated in increased ADCC.[6] Because of the possibility of numerous different glycosylations, highly resolving separation of these species is necessary to accurately analyze glycan populations. Since the size difference between fucosylated and afucosylated glycans is as small as 16 daltons, and that they may have numerous positional isomers, separation has proven to be difficult for some of these species. Current methods have been incapable of resolving a large number of the major co-migrating glycan species from one another. Previously, CE conditions capable of separating fucosylated and afucosylated N-linked oligosaccharides were presented.[7] That work, in addition to the success CE technology has in resolving differences in terminal galactosylation, suggests that it should also be capable of separating glycans that are fucosylated, sialylated, or bisected from each other as well as high mannose species.

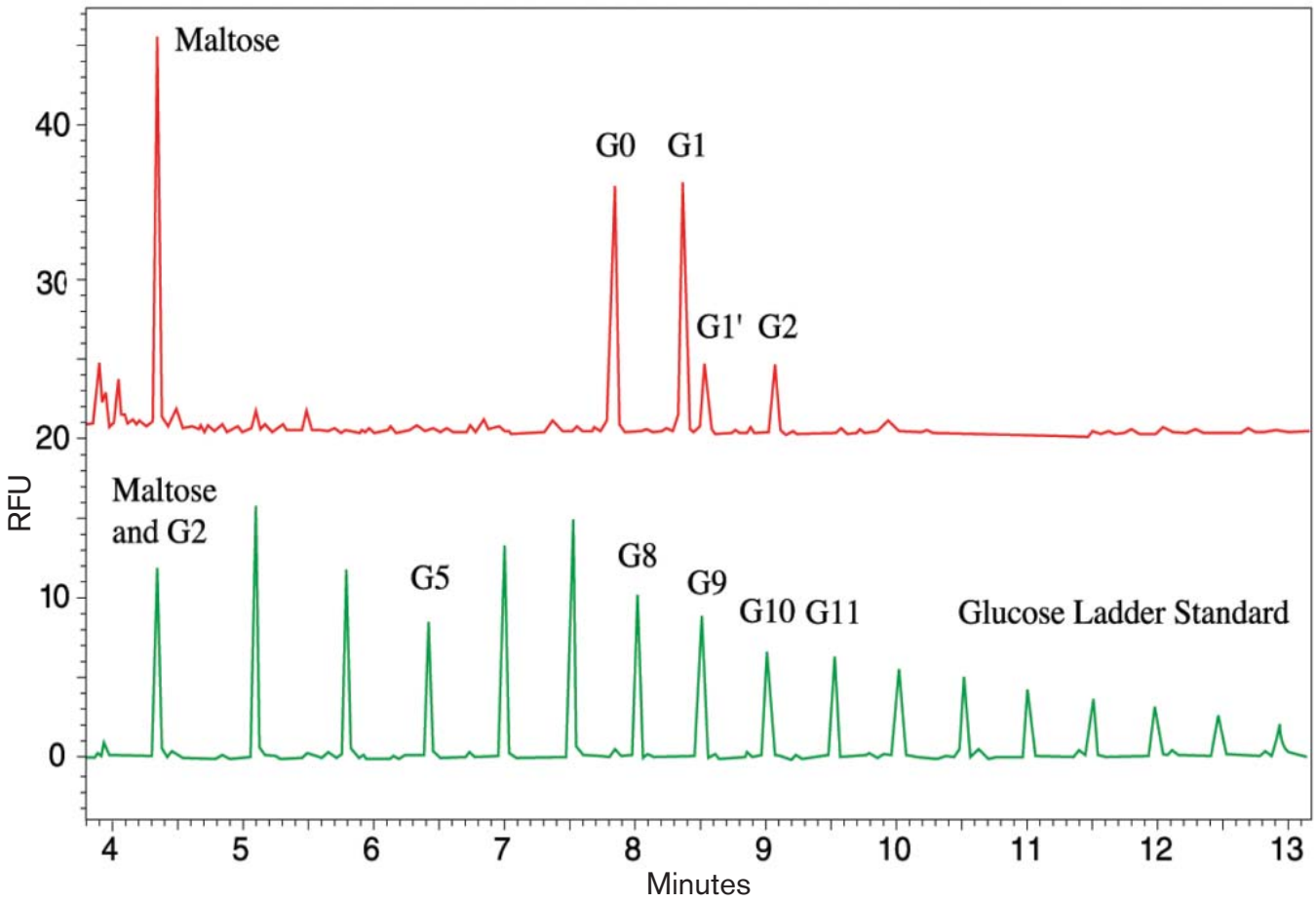

Figure 2. Separation of G0, G1, and G2 glycan species. Representative data (top trace) shows separation of N-linked oligosaccharides G0, G1, G1', and G2 using the Carbohydrate Labeling & Analysis Assay Kit (Beckman Coulter p/n 477600). G1 positional isomers are resolved from one another illustrating the mobility-based 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.

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 with 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 carbohydrate separation buffer was substituted with a new separation buffer formulation where indicated. Final concentration for oligosaccharide samples was 1.25 µM. 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 follow (unless otherwise indicated):

- 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 µm I.D.
- Injection conditions: 0.5 psi for 10 sec unless otherwise stated
- Separation Voltage: 30 kv
- Field Strength: 500 volts/cm
- Capillary cartridge temperature: 20° C
- Sample storage temperature: 10° C

Abbreviation	Description	Compound Name	MW (Da)
-G	Trimannosyl core	M3N2	911
-GF	Trimannosyl core, substituted with fucose	M3N2F	1057
Man-5	Oligomannose 5	Man-5	1235
G0	Asialo, agalacto, biantennary complex	NGA2	1317
Man-6	Oligomannose 6	Man-6	1398
G0F	Asialo, agalacto, biantennary complex, core substituted with fucose	NGA2F	1463
G1 / G1'	Asialo, monogalactosylated, biantennary complex	NA2G1	1480
Man-7	Oligomannose 7	Man-7	1560
G1F / G1'F	Asialo, mono-galactosylated, biantennary complex, core substituted with fucose	NA2G1F	1626
G2	Asialo, galactosylated, biantennary complex	NA2	1641
G0FB	Asialo-, agalacto-, biantennary, core-substituted with fucose and bisecting N-acetylglucosamine (GlcNAc)	NGA2FB	1667
Man-8	Oligomannose 8	Man-8	1722
G2F	Asialo, galactosylated, biantennary complex, core-substituted with fucose	NA2F	1787
Man-9	Oligomannose 9	Man-9	1884
G2S1	Mono-sialylate, galactosylated, biantennary complex	A1	1933
G2S1F	Mono-sialylate, galactosylated, biantennary complex, core-substituted with fucose	A1F	2079

Table 1. Glycan abbreviations and descriptions. In the course of this work, separation of standards was utilized to help identify various glycan peak positions. This table indicates the compound names, abbreviations used in the data, as well as descriptions and molecular weight of each glycan species.

Results and Discussion

The goal of this study was to achieve separation of major complex glycan species associated with monoclonal antibodies. This glycan population includes oligosaccharides with and without core fucose moieties, terminal galactose subunits, terminal sialic acids, and bisecting GlcNAc residues in addition to numerous positional isomers. We set out to characterize the separation limitations for existing separation chemistry, the Beckman Coulter Carbohydrate Labeling & Analysis Assay Kit. Employing standard protocols (included with the Carbohydrate Labeling & Analysis Assay) 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 previously demonstrated by co-migration of G0+fucose (GoF) with G1, and co-migration of G1'+fucose (G1'F) with G2[7]. 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 able to better resolve these co-migrating species (Figure 3). Additional spiking experiments illustrated the power of the CE separation developed (Figure 4).

In order to test this separation method on an antibody, we obtained a therapeutic MAb and analyzed its associated glycans (Figure 5).

Spiking with oligosaccharide standards to help identify glycan species, we showed good resolution between many of the major oligosaccharides which were previously difficult to separate by CE.

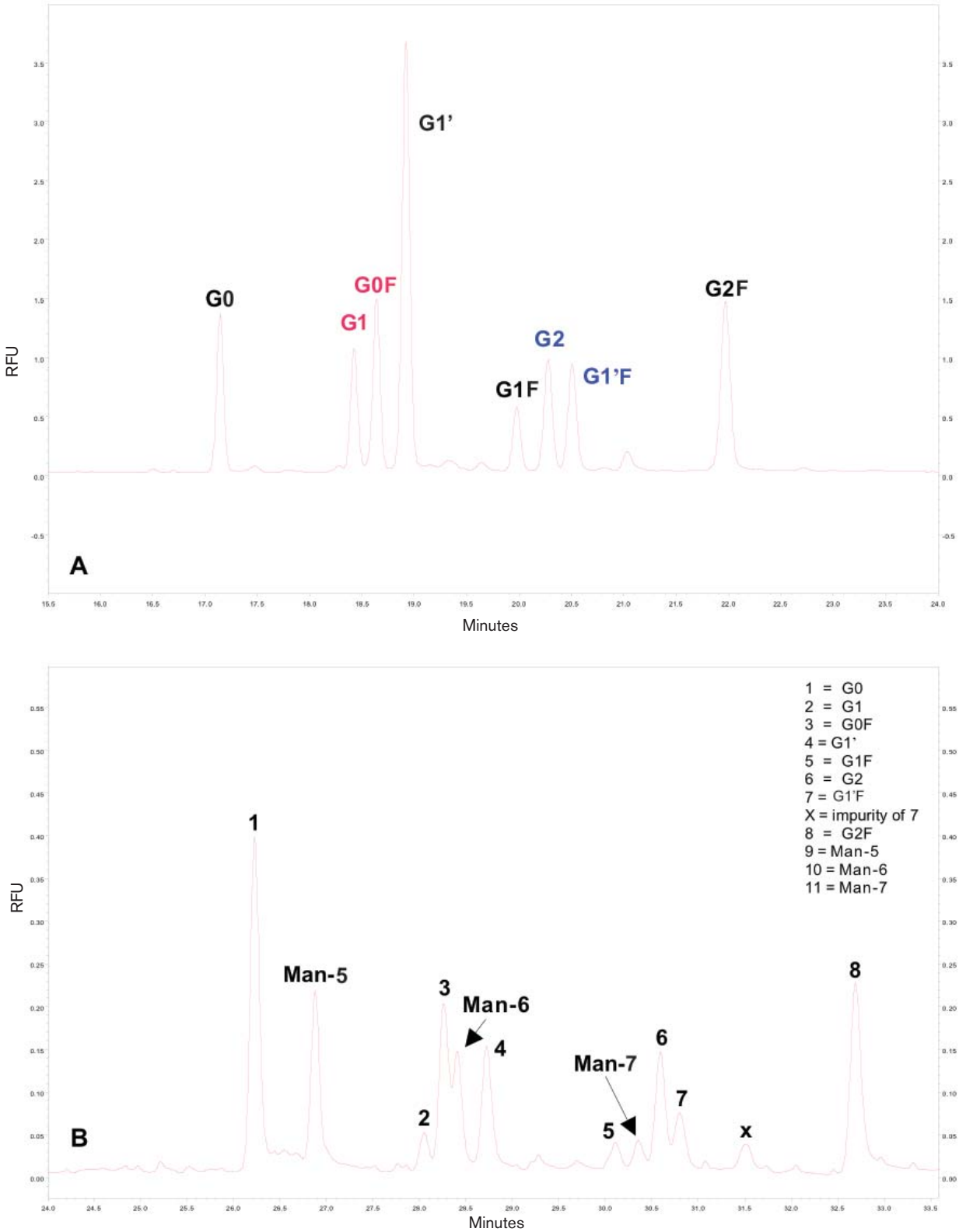

Figure 3. Optimization of the carbohydrate separation buffer allows for resolution between closely-migrating oligosaccharide pairs. Using standard sample preparation protocols, oligosaccharide standards were APTS labeled and separated by CE. Resolution of co-migrating was facilitated by combining existing 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 low percentage of linear polyacrylamide (LPA). Separations were performed on an N-CHO capillary (p/n 477600) with an effective length of 50cm. Separation conditions were 20kV following 0.5psi injection for 5 seconds. Field strength was 333V/cm. (A) Closely migrating fucosylated and afucosylated N-linked oligosaccharide standards G0F and G1 (red labels) as well as G1'F and G2 (blue labels) were separated from one another. (B) Closely migrating high mannose oligosaccharide standards were separated from one another. This separation resolved Man-5 from G0, Man-6 from G0F, and Man-7 from both G1F and G2.

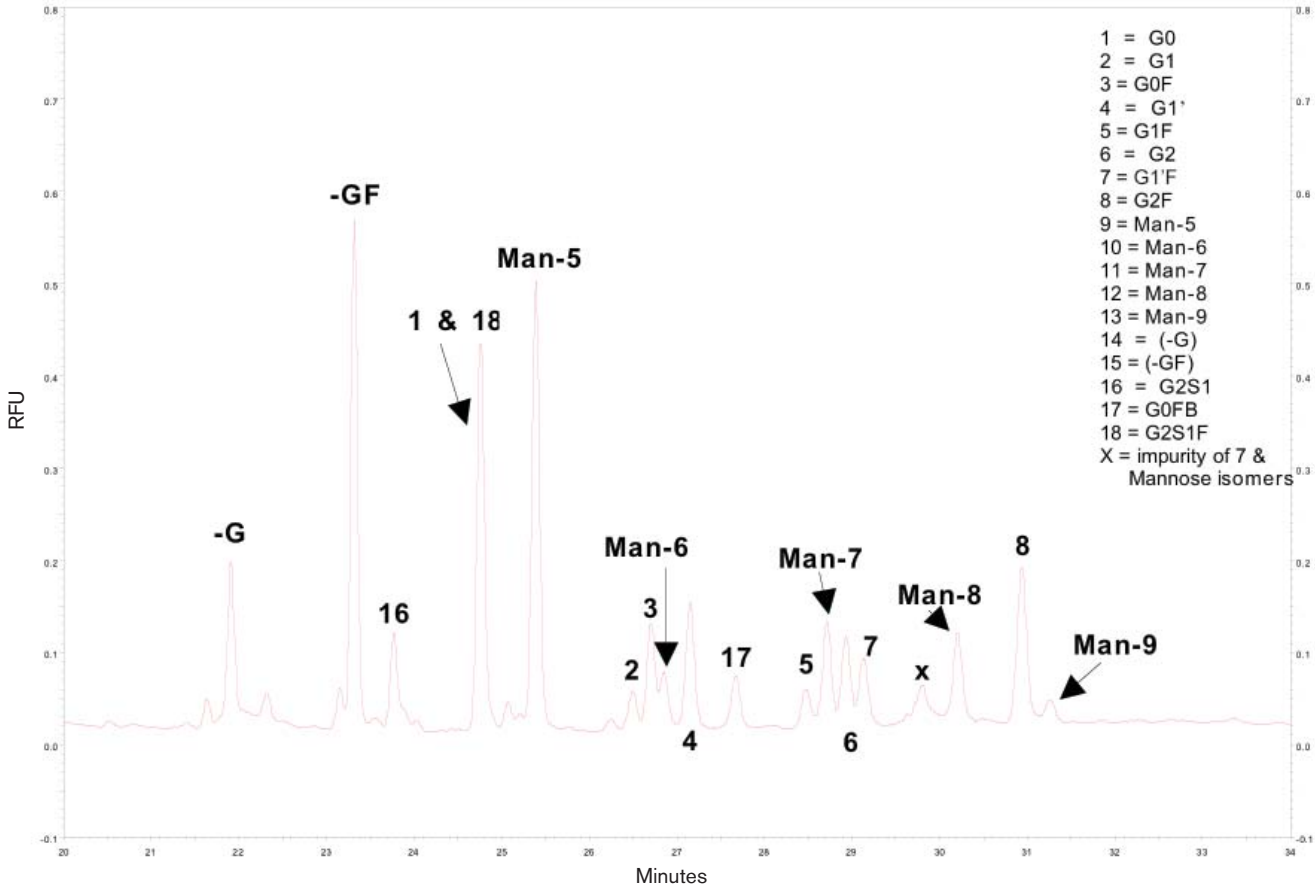

Figure 4. Optimization of the carbohydrate separation buffer allows for resolution of a number of MAb-associated oligosaccharides. Using standard sample preparation protocols, oligosaccharide standards were APTS labeled and separated by CE. Resolution of N-linked oligosaccharide standards was facilitated 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 low percentage of linear polyacrylamide (LPA). To date, we have been able to separate and identify important glycans differing in galactosylation, fucosylation, sialylation, as well as high mannose structures. Separation conditions were 20kV following 0.5psi injection for 5 seconds. Field strength was 333V/cm.

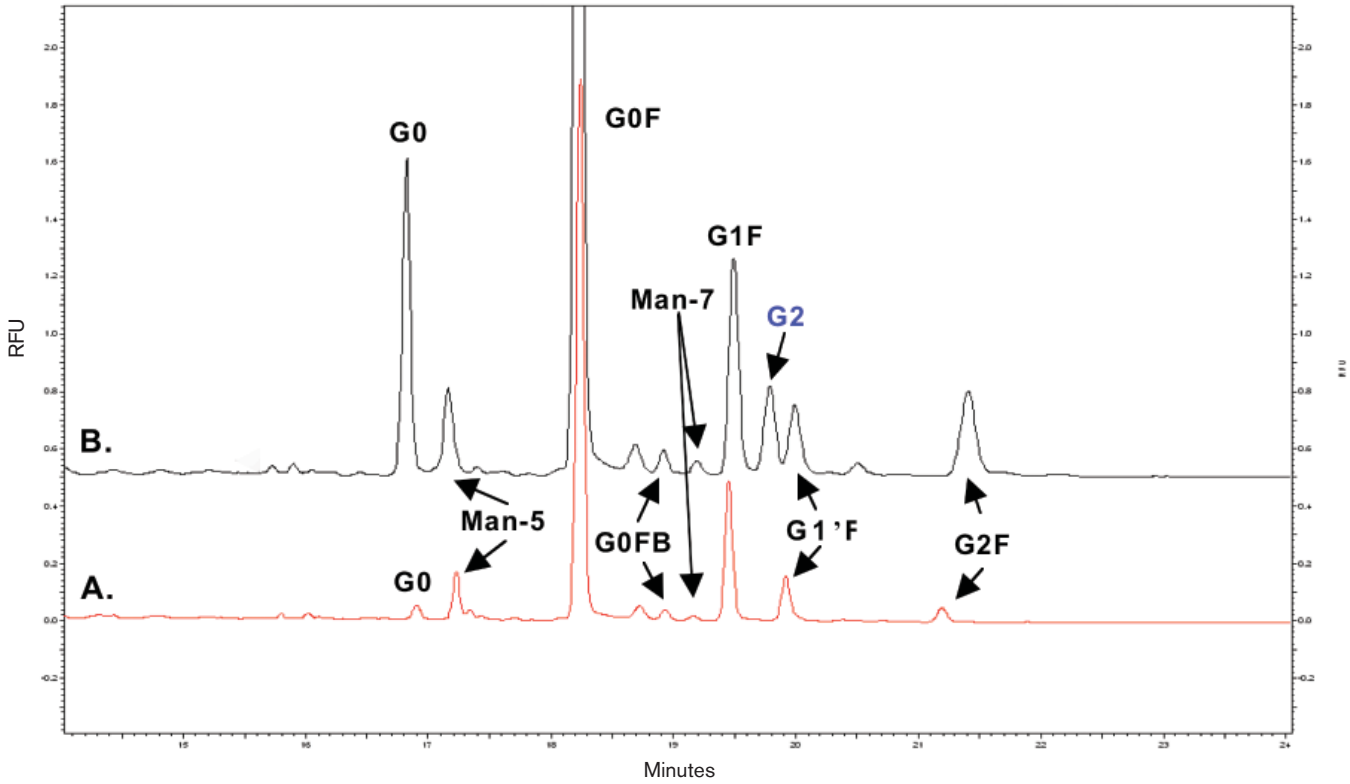

Figure 5. Separation of oligosaccharides associated with a recombinant therapeutic MAb. Oligosaccharides were cleaved from a therapeutic MAb, APTS labeled, and separated by CE using the new buffer formulation. A number of oligosaccharide species associated with this MAb were resolved from one another (A). In order to identify and help illustrate resolution between co-migrating glycan species, the MAb sample was spiked with standards (B). Relative to the oligosaccharide standards, we were able to quantifiably identify G0, Man-5, G0F, G0FB, Man-7, G1F, G1'F, and G2F. G2 standard (in blue) was also spiked into the mixture to indicate the location in the separation at which this oligosaccharide species would reside. Conditions for both separations 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.

Conclusion

High resolution CE 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 standard PEO separation gel buffer with a LPA gel buffer, we were able to separate fucosylated from afucosylated N-linked oligosaccharides, high mannose structures, and numerous other glycan moieties. This work suggests that CE can be used to successfully separate and quantify a wide array of N-linked oligosaccharides associated with MAbs. Additional experimentation will focus on further development of these methods.

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