

Fast Glycan Sequencing Using a Fully Automated Carbohydrate Sequencer

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Overview

Who Should Read This: Senior Scientists, Lab Directors in the biopharmaceutical and biomedical fields.

Focus: A new automated glycan sequencing approach for comprehensive structural elucidation of complex carbohydrates.

Goal: To demonstrate an automated carbohydrate sequencing solution that significantly decreases manual steps and reduces the analysis time from 1–5 days to 1–2 hours.

Problem: Previously, carbohydrate sequencing was a tedious and time-consuming manual process. It required either consecutive addition of the sequencing exoglycosidase enzymes to a single reaction vial, which could take up to a week depending on the number of enzymes used, or arrays (combinations) of enzymes in multiple vials processing multiple sample aliquots in parallel, which could still take a full day.

Results: Using automated sample and enzyme handling, as well as optimized reaction times and temperatures for the individual exoglycosidase reactions, the SCIEX PA 800 Plus Pharmaceutical Analysis System dramatically reduces hands-on steps and provides carbohydrate sequencing in just 1–2 hours.

Key Challenges:

Current carbohydrate sequencing methods are based on manual processing that can take from one day to a full week.

Key Features:

- The precise temperature control of the sample storage compartment in the PA 800 Plus System provides proper incubation conditions for the carbohydrate sequencing reactions
- The separation capillary serves a dual role, functioning both as the separation column and as a reagent (enzyme) delivery device



The PA 800 Plus Pharmaceutical Analysis System

- Intelligent instrument control optimizes and automates the sequencing process
- The Fast Glycan Labeling and Analysis Kit vastly simplifies sample preparation and speeds glycan characterization

Introduction

Monoclonal antibodies (mAbs) are one of the fastest emerging groups within the rapidly growing protein therapeutics market. In most instances, mAbs possess a conserved N-linked glycosylation site in each of the CH₂ domains of the Fc portion of the heavy chain, but may also have additional sugar structures attached to the Fab domains.¹ Increasing evidence shows that these carbohydrate moieties play important roles in biological activity, physicochemical properties, and effector functions.² Even minor changes in the carbohydrate structures (linkage, position, and site occupancy) can influence bioactivity. For example, IgG molecules with identical amino acid sequences can gain 50-fold efficacy in terms of antibody-dependent cellular cytotoxicity (ADCC) when the core fucose residue is absent in the conserved Fc glycan.³ Terminal sialic acids, on the other hand, transform monoclonal antibodies into anti-inflammatory mediators capable of suppressing autoantibody-mediated inflammation. Due to this sensitivity to changes in carbohydrate structures, fast, efficient,

and reliable bioanalytical techniques are needed along the entire pharmaceutical processing pipeline, from clone selection to lot release.⁴

The extremely high diversity of protein glycosylation makes structural elucidation challenging. In most instances, only a combination of analytical methods can provide the desired information.⁵ The most frequently used methods for structural analysis of complex carbohydrates include capillary electrophoresis (CE), nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), and high-performance liquid chromatography (HPLC), often combined with exoglycosidase digestion techniques.

Capillary electrophoresis with laser-induced fluorescent detection (CE-LIF) is a high-resolution bioanalytical tool with excellent

sensitivity. It is capable of rapid, comprehensive profiling of complex carbohydrates, even discriminating between closely related positional and linkage isomers.⁵ Individual glycan structures, corresponding to peaks in the electropherogram, can be proposed based on their glucose unit (GU) values.⁶ However, due to the high diversity of possible glycan structures, existing GU databases might not contain the glycan structures at hand. Carbohydrate sequencing can alleviate this handicap by providing required structural identification.⁷

N-linked carbohydrate sequencing starts with the release of the sugar moieties from the polypeptide backbone of the glycoprotein, in most cases using peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase (PNGase F). For capillary electrophoresis-based N-linked carbohydrate profiling, the liberated oligosaccharides are labeled with a charged

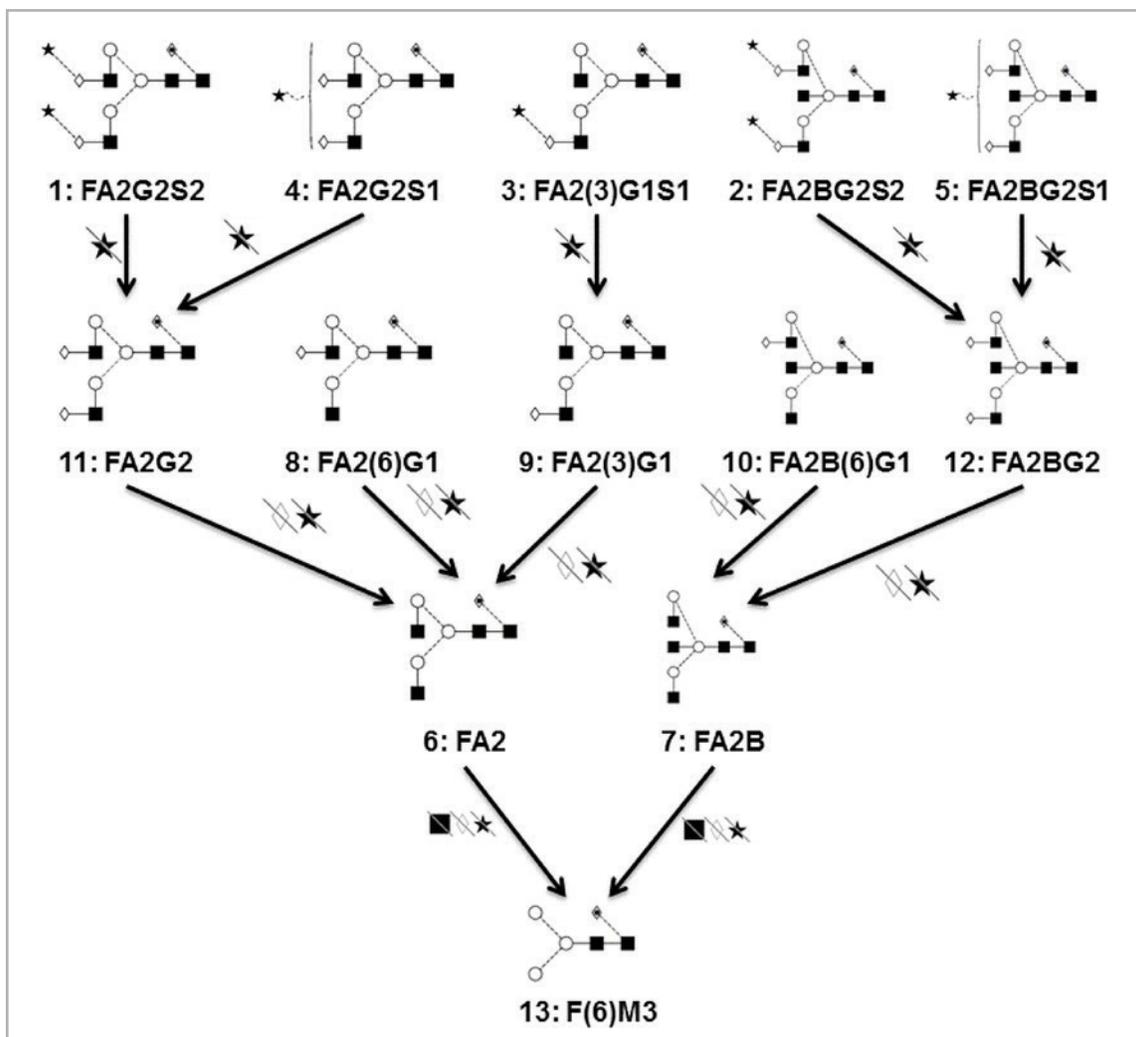


Figure 1. Exoglycosidase digestion reaction pathways of the major sugar residues of interest in monoclonal antibody N-glycans. Structure numbers correspond to the peak numbers in Figure 2 in the Results section of this note. N-glycan nomenclature and symbolic representations use the notation previously suggested by Harvey et al.⁹ ★: sialidase; /: galactosidase; ■: hexosaminidase.

fluorophore, 8-aminopyrene-1,3,6-trisulfonic acid (APTS), and separated by CE-LIF. In cases when the glycan structures in the sample are not found in the GU database, carbohydrate sequencing is necessary to identify the components.

Current carbohydrate sequencing strategies involve use of specific exoglycosidase enzymes to remove the sugar residues from the non-reductive end of the glycan structures. The enzymes can be used serially, or arrays (combinations) of enzymes can be applied in a parallel fashion.⁷ In both instances, following the reaction steps the resulting glycan pools are analyzed by CE-LIF and the migration time shifts of all the peaks are recorded. Glycan sequencing starts with cutting the out-most sugars from the non-reducing end (in most instances sialic acids), followed by removal of all galactose and N-acetyl-hexosamine residues (including antennary and bisecting) until the trimannosyl-chitobiose core structure of all N-linked sugars is reached (fucosylated and/or non-fucosylated). This enzyme reaction sequence is represented in Figure 1. If antennary specification is needed, linkage specific mannosidases (1-3 or 1-6) can be applied.⁸

For serial exoglycosidase-based sequencing, enzymes are added to the same reaction vial individually in series. Each reaction step takes approximately 16 hours at 37° C. Using the parallel approach, on the other hand, specific arrays (combinations) of enzymes are prepared in an equal number of sample vials.¹⁰ In the case of monoclonal antibody N-glycans, the first vial would contain sialidase, the second vial sialidase and galactosidase, and the third vial sialidase, galactosidase, and hexosaminidase. Sample aliquots are processed concurrently in each vial. For the same set of exoglycosidases, this parallel approach requires only 16 hours for the reactions to reach completion.

This note presents semi-automated (use of exoglycosidase arrays) and fully automated (serial exoglycosidase addition) carbohydrate sequencing strategies that take advantage of the unique hardware features of the PA 800 Plus Pharmaceutical Analysis System to accomplish the sequencing task in only 1–2 hours. The method works without the need to isolate any of the individual structures from the initial glycan pool. GU values are used to calculate the mobility shifts for full structural elucidation.

Experimental

Chemicals

Human immunoglobulin G (IgG), acetonitrile, and sodium cyanoborohydride (1 M in THF) were obtained from Sigma Aldrich (St. Louis, MO). PNGase F, sialidase A ($\alpha 2 \rightarrow 3,6,8,9$), jack bean β -galactosidase ($\beta 1 \rightarrow 3,4,6$) and jack bean β -N-acetyl-hexosaminidase ($\beta 1 \rightarrow 2,3,4,6$) were obtained from New England Biolabs (Ipswich, MA).

Glycan Release and Labeling

Following the protocol of the Fast Glycan Sample Preparation and Analysis Kit (Part Number B94499PTO, SCIEX, Brea, CA), 100 μ g of IgG1 was:

1. Denatured
2. Digested by PNGase F (2.5 mU)
3. Labeled with fluorophore (8-aminopyrene-1,3,6-trisulfonate, APTS)
4. Purified using magnetic beads
5. Eluted in deionized water

Sequencing Digestion

The exoglycosidase digestion-mediated sequencing reactions were accomplished in the temperature-controlled sample storage compartment of the PA 800 Plus System. The compartment temperature was set between 40° C and 60° C, according to requirements of the enzyme reactions. Temperature drop during the tray movement (sample in and out) was less than 4° C and re-equilibration took only a few minutes.

Exoglycosidase digestions were performed by the addition of:

- 5.0 mU sialidase (1.0 μ L)
- 25 mU β -galactosidase (1.0 μ L)
- 25 mU β -N-acetyl-hexosaminidase (1.0 μ L)

to 50 μ L of glycan sample-containing reaction mixture in a premixed array form (semi-automated carbohydrate sequencing) or in a step-by-step fashion (fully automated carbohydrate sequencing). Since therapeutic monoclonal antibodies usually do not possess any antennary fucosylation, only sialidase, β -galactosidase and β -N-acetyl-hexosaminidase were used in the reactions.

Semi-automated carbohydrate sequencing

The semi-automated carbohydrate sequencing workflow is depicted in Table 1. The workflow utilized four reaction vials, all containing the APTS-labeled IgG1 glycans, and three corresponding enzyme arrays. The first vial contained only IgG1 glycans and served as the control. Arrays of exoglycosidases were added to the three other vials:

Vial 1: IgG1 glycans only (control)

Vial 2: IgG1 glycans + sialidase

Vial 3: IgG1 glycans + sialidase and β -galactosidase

Vial 4: IgG1 glycans + sialidase, β -galactosidase, and β -N-acetyl-hexosaminidase

The four vials were placed into the sample tray and incubated in the storage compartment at 60° C.

Sequencing / Separation Steps	Elapsed Time				
	12	24	36	48	60
CE-LIF analysis of IgG1 pool	30 kV				
Sialidase digestion	60° C				
CE-LIF analysis after sialidase digestion		30 kV			
Galactosidase digestion		60° C			
CE-LIF analysis after galactosidase digestion				30 kV	
Hexosaminidase digestion		60° C			
CE-LIF analysis after hexosaminidase digestion					30 kV

Table 1: Timing of the semi-automated carbohydrate sequencing workflow.

Fully automated carbohydrate sequencing

The fully automated carbohydrate sequencing workflow is depicted in Table 2. This workflow required only one reaction vial. The analytical capillary was used to deliver the appropriate exoglycosidases to the vial. Nanovials (SCIEX), each containing 5.0 μ L of one exoglycosidase, were placed in the outlet buffer tray of the PA 800 Plus System.

Similar to the semi-automated approach, first the APTS-labeled IgG1 pool sample was analyzed by CE-LIF. After this separation step, the capillary was rinsed with water and, by careful adjustment of the inlet pressure, used to deliver 1 μ L of sialidase enzyme to the sample vial. The temperature of the sample storage compartment was ramped from 40°C to 60° C during the 16 minute sialidase incubation step.

Upon finishing the sialidase digestion, the reaction mixture was injected into the capillary, which was filled with separation gel-buffer while digestion was taking place, and analyzed by CE-LIF.

Next, the capillary was rinsed again with water followed by the capillary delivery of 1 μ L of β -galactosidase into the sample vial. This reaction mixture was incubated for 32 minutes at 60° C, and then injected for analysis into the capillary.

This process was repeated for the β -N-acetyl-hexosaminidase (1 μ L), and after 32 min incubation at 60° C the reaction mixture were injected and analyzed by CE-LIF.

Capillary Electrophoresis

All capillary electrophoresis separations were performed on a PA800 Plus Pharmaceutical Analysis System with laser-induced fluorescence detection (SCIEX).

Column: 20 cm effective length (30 cm total, 50 μ m ID) bare fused silica capillary (EZ-CE cartridge)

Separation gel buffer: HR-NCHO

Separation temperature: 25° C

Separation voltage: 30 kV (0.17 min ramp time), E=1000 V/cm

Sequencing / Separation Steps	Elapsed Time						
	12	28	40	72	84	116	128
CE-LIF analysis of IgG pool	30 kV						
Sialidase digestion		40→60° C					
CE-LIF analysis after sialidase digestion			30 kV				
Galactosidase digestion				60° C			
CE-LIF analysis after galactosidase digestion					30 kV		
Hexosaminidase digestion						60° C	
CE-LIF analysis after hexosaminidase digestion							30 kV

Table 2: Timing of the fully automated carbohydrate sequencing workflow.

Polarity: Reversed (cathode at injection side, anode at detection side).

Injection steps:

1. Water at 3.0 psi for 5.0 sec
2. Sample at 1.0 kV for 1.0 sec
3. Bracketing standard (DP2 + DP15) at 1.0 kV for 1.0 sec

Detection: laser-induced fluorescence
(488 nm excitation / 520 nm emission)

Data acquisition and analysis were done using the 32 Karat software (version 10.1, SCIEX). GU values were assigned for all peaks after each sequencing step.

Results

IgG1 samples were processed and analyzed using both the semi-automated and fully automated carbohydrate sequencing workflows. After completion of each digestion step, the resulting reaction products were analyzed by CE-LIF and identified by the GU value and GU value shifts of the affected peaks (Table 3). The type and linkage information of the sugar residue losses were determined by the exoglycosidase enzyme used. The number of the residues was determined by the extent of the GU shifts. Thus, positional and linkage information were also determined by the actual exoglycosidases in the reaction mixture. Based on the GU shifts, the glycan structures were built up for all sample components after each enzyme was applied and the reaction products were analyzed.⁵

Sialidase Digestion GU Shifts						Galactosidase Digestion GU Shifts					Hexosaminidase Digestion GU Shift			
Glycan ID		9	11	12		Glycan ID		6	7		Glycan ID		13	
Structure		FA2(3)G1	FA2G2	FA2BG2		Structure		FA2	FA2B		Structure		F(6)M3	
GU Value		9.11	10.16	10.55		GU Value		7.69	8.23		GU Value		6.75	
1	FA2G2S2	5.00	—	5.16	—	8	FA2(6)G1	8.76	1.07	—	6	FA2	7.69	0.94
2	FA2BG2S2	5.14	—	—	5.41	9	FA2(3)G1	9.11	1.42	—	7	FA2B	8.23	1.47
3	FA2(3)G1S1	6.04	3.07	—	—	10	FA2B(6)G1	9.18	—	0.95				
4	FA2G2S1	6.80	—	3.37	—	11	FA2G2	10.16	2.47	—				
5	FA2BG2S1	6.98	—	—	3.56	12	FA2BG2	10.55	—	2.32				

Table 3: Identified GU value shifts of the major IgG1 glycans.

Semi-Automated Sequencing Results

At zero elapsed time, the undigested control sample was analyzed (Figure 2, lower panel), followed by the CE-LIF analysis of the exoglycosidase reaction mixtures after the relevant elapsed times:

Sialidase at 12 min (Figure 2A, NANase trace)

Sialidase and β -galactosidase at 36 min (Figure 2A, NANase+GALase trace)

Sialidase + β -galactosidase + β -N-acetyl-hexosaminidase at 48 min (Figure 2A, NANase+GALase+HEXase trace)

This semi-automated exoglycosidase array-based sequencing required only 1 hour, including the reaction times, for all enzyme digestions and the CE-LIF separations. However, manual premixing of the exoglycosidase enzyme arrays was required, and four times as much IgG1 sample was required compared to the fully automated workflow.

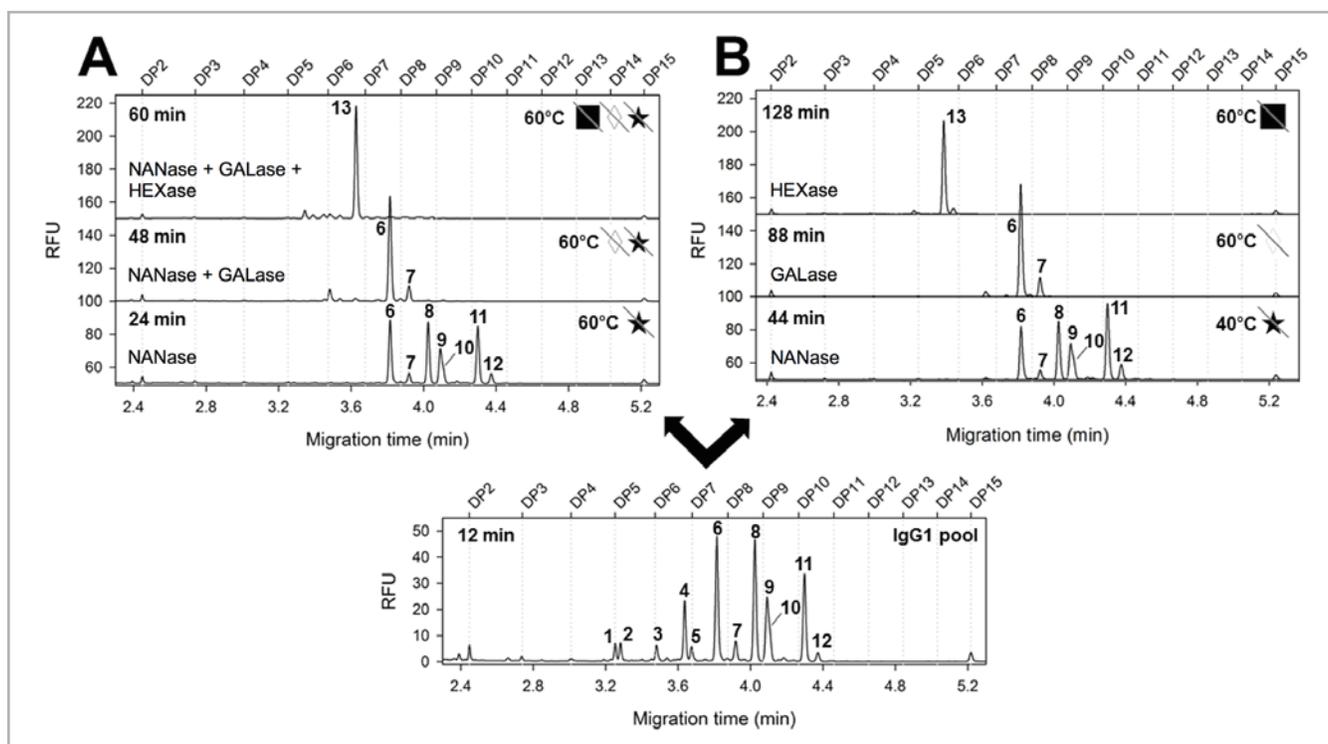


Figure 2. Automated carbohydrate sequencing of monoclonal antibody N-glycans. Lower panel: CE-LIF trace of the APTS-labeled IgG N-glycan pool. A: exoglycosidase array digestion-based semi-automated glycan sequencing. B: consecutive exoglycosidase digestion-based fully automated glycan sequencing.

Fully automated carbohydrate sequencing

Similar to the semi-automated approach, in the fully automated workflow the APTS-labeled IgG1 pool sample was analyzed first by CE-LIF (Figure 2, lower panel). The capillary was then rinsed with water and, by careful adjustment of the inlet pressure, used to deliver 1 μ L of sialidase enzyme to the sample vial. The sample was incubated according to the automated sequencing workflow outlined in the Experimental section of this note. The digestions products were sampled and analyzed (Figure 2B,

NANase trace). The process was repeated with β -galactosidase (Figure 2B, GALase trace) and β -N-acetyl-hexosaminidase (Figure 2B, HEXase trace).

This fully automated workflow did not require any pre-analysis preparation of reaction arrays, but due to the serial nature of the reactions the total sequencing time was a little more than 2 hours.

Conclusion

This work introduced the use of the PA 800 Plus Pharmaceutical Analysis System for automated glycan sequencing by CE-LIF. Both semi- and fully automated exoglycosidase digestion-based carbohydrate sequencing were accomplished by using the temperature-controlled sample storage compartment of the CE instrument as a reaction incubation chamber and using the separation capillary both for analysis and as an enzyme delivery device. Through careful optimization of reaction temperatures, enzyme concentrations, and incubation times, sequencing of the monoclonal antibody N-glycans was accomplished in 1 hour using the semi-automated workflow and in a little over 2 hours using the fully automated workflow. Conventional manual carbohydrate sequencing methods require between one day (array-based) and one week (sequential) to accomplish the same task.

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