An Integrated Microfluidic LC/MS Chip for Rapid On-line Deglycosylation and Characterization of N-glycans from Recombinant IgG Antibodies

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WPT 470

Introduction

Glycan post-translational modifications play an important role in therapeutic efficacy of antibodies. Characterization of the glycan profiles requires fast, sensitive and reproducible analytical methods.

We demonstrate a novel integrated microfluidic LC/MS chip for enzymatic deglycosylation with immobilized PNGase F enzyme, glycan purification, separation, identification and quantitation of N-linked glycans from IgG mAbs. With this chip, the experimental time from antibody injection to LC/MS results is reduced to 10 minutes. Current methods employ lengthy solution-phase enzyme cleavage of glycans and sample clean-up for MALDI, LC/MS or CE/MS and fluorescent labeling for CE or LC analysis.

Microfluidic Chip Designed for Speed

Total Time Duration for On-Chip Glycan Cleavage and Analysis Compared to Established Methods

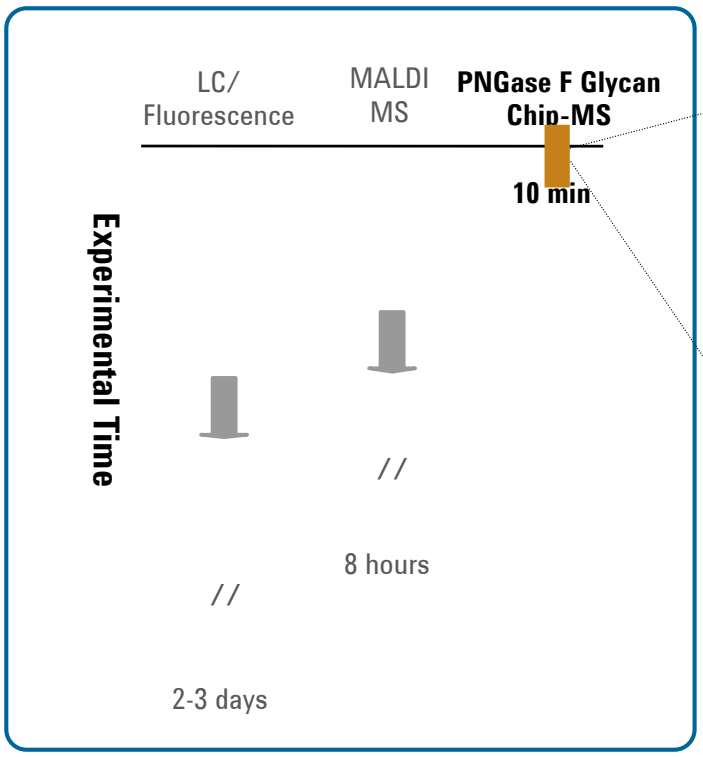


Figure 1. Time required for glycan cleavage, sample preparation and glycan analysis. This novel chip reduces the total experimental time to 10 minutes. Existing methods require at least 8 hours and include many pipetting, cleanup and labeling steps.

Integrated Microfluidic Chip Architecture

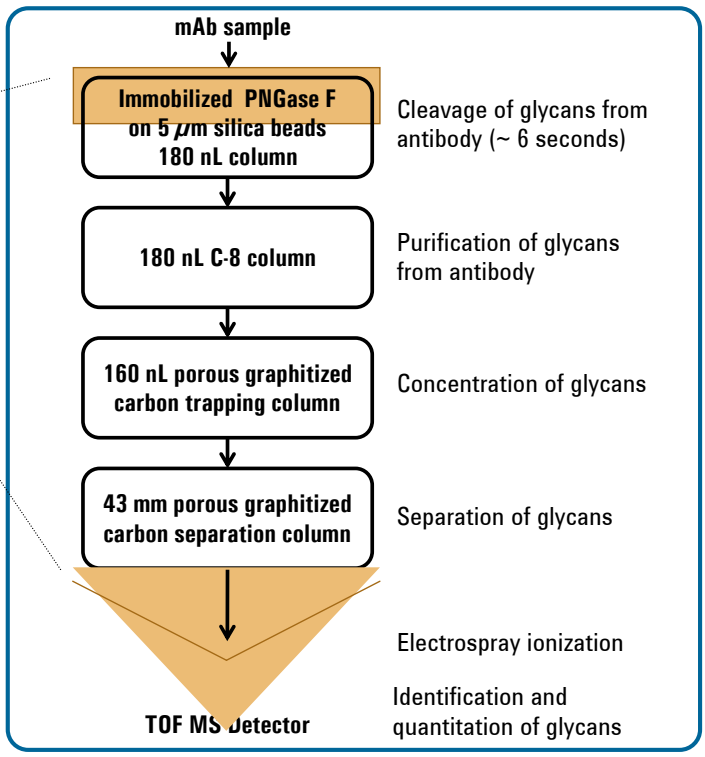


Figure 2. Novel components integrated in the microfluidic chip. Three polymer microfluidic chips were constructed using the laser ablation and lamination technologies in polyimides described previously.[1]

Experimental Details

On-Chip Deglycosylation and Analysis of Recombinant Monoclonal Antibodies

Two recombinant mAbs were obtained from Genentech, and a third from Pfizer. They are referred to as "Ab1," "Ab2" and "Ab3" respectively. The mAbs were diluted in 100 mM ammonium acetate buffer, pH 7.6, to 500 ng per µL. The total amount of mAb injected was 100 ng. 100 mM ammonium acetate was used for sample loading and deglycosylation. Typical gradient conditions for glycan separation were used.

1. Yin, H.; Killeen, K.; Brennen, R.; Sobek, D.; Werlich, M.; van de Goor, T. *Anal. Chem.* 2005, *77,* 527-533.
2. Rasmussen, J. R.; Davis, J.; Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* 1992, *114,* 1126.

Integrated Microfluidic Chip Operation

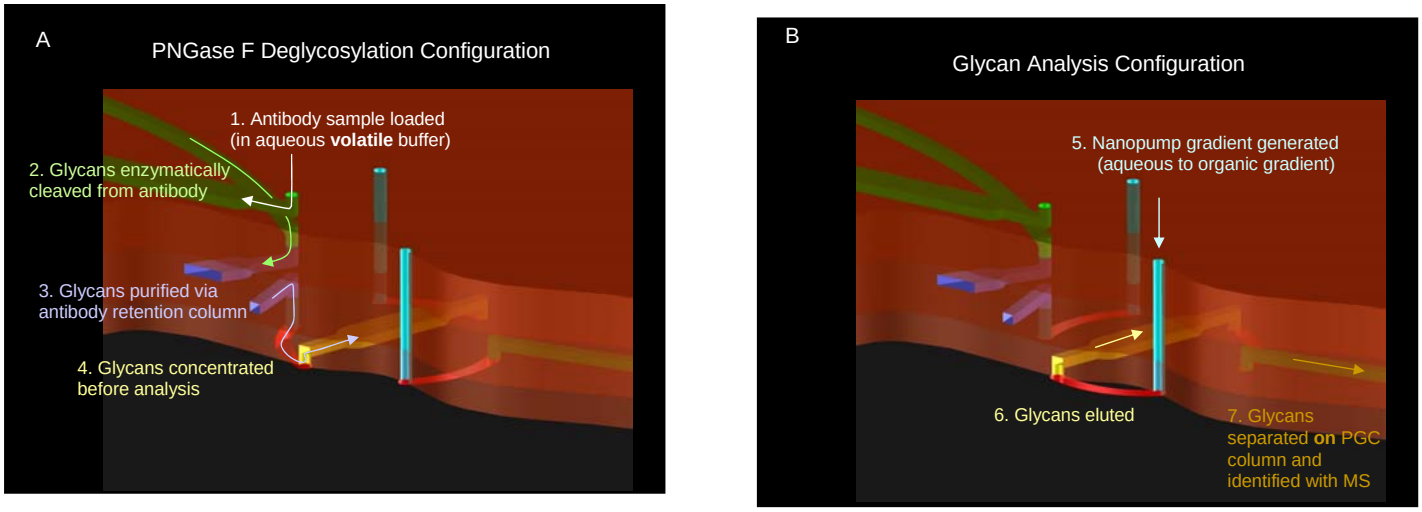
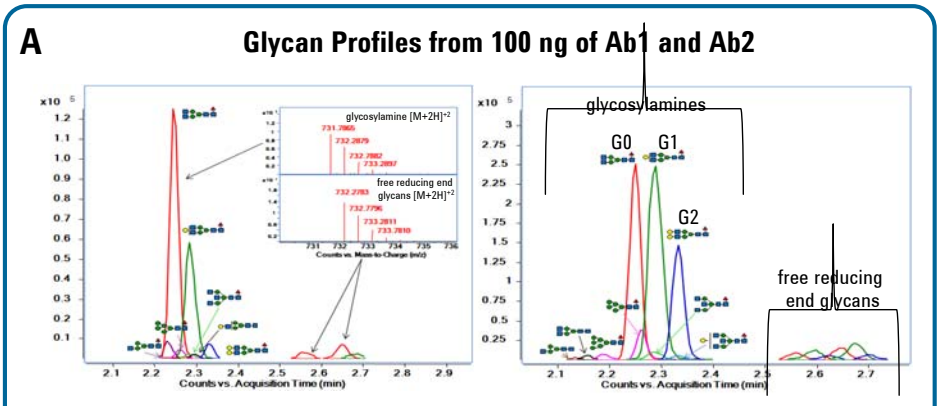


Figure 3. HPLC-Chip flowpath diagram during deglycosylation (A) and analysis mode (B). While in deglycosylation mode (A), the glycosylated mAb travels into enzyme reactor where glycans are cleaved. Both deglycosylated antibody and released glycans then travel into the C8 bead-packed channel where antibodies are retained. Free glycans travel further, via a rotor groove (shown in red), to enrichment column on HPLC-Chip where glycans are captured. Figure 3B illustrates the valve configuration showing the flow path during HPLC MS analysis. In this configuration, Agilent nanopump delivers gradient to elute glycans from the enrichment column and separate glycans on the separation column before electrospray of the glycans to the mass spectrometer

Results and Discussion: Antibody Deglycosylation and Analysis in 10 min.



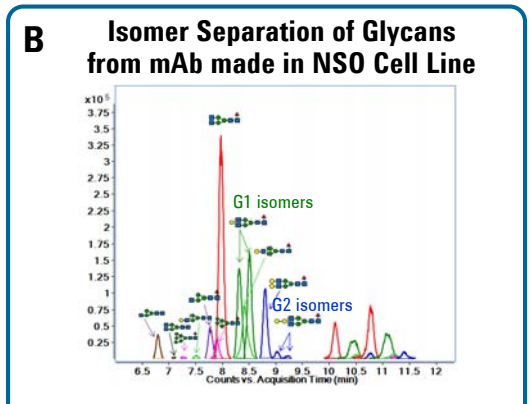


Figure 4A. Extracted Compound Chromatogram (ECC) of glycans obtained with on-chip deglycosylation, antibody removal glycan capture and glycan separation. The β-glycosylamine intermediates eluted between 2.2-2.4 minutes, and the free reducing end glycans eluted between 2.5-2.7 minutes. Color code: Red trace=G0, Green trace=G1, blue trace=G2. The inset in Figure 4A shows the mass spectra for doubly charged β-glycosylamines (top) and free reducing end glycans (bottom.) The mass difference is 0.984 Da, which is the mass difference between –OH and $–NH_2$. In addition to the fucosylated glycans seen in mAb1, mAb2 contains lower abundance nonfucosylated glycans that separate from other glycans and elute between 2.1-2.2 minutes.

Figure 4B. ECC of mAb 3 from mouse NSO cell line. β-glycosylamine G1 isomers (green trace) as well as G2 isomers (blue trace) were separated using a 10 minutes LC gradient. In addition to more commonly observed glycans G0, G1 and G2, other glycans were observed and identified, such as G0-GlcNAc-F, G0-F, Man-6, G1-GlcNAc-F, G0-GlcNAc, and G1-GlcNAc.

Results and Discussion

PNGase F Deglycosylation Mechanism[2]

1. C-N bond of glycosylated asparagine side chain cleaved
2. Asparagine residue converted to aspartic acid
3. Glycan is a β-glycosylamine initially
4. The β-glycosylamine is slowly hydrolyzed to a hydroxyl and ammonia is liberated.

Most methods detect the free-reducing end glycans. With our fast deglycosylation, the β-glycosylamine are detected directly.

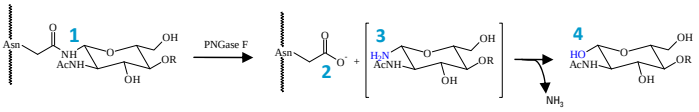


Figure 5. PNGase F deglycosylation.

Kinetics of Hydrolysis of the β-glycosylamines to Free Reducing End Glycans

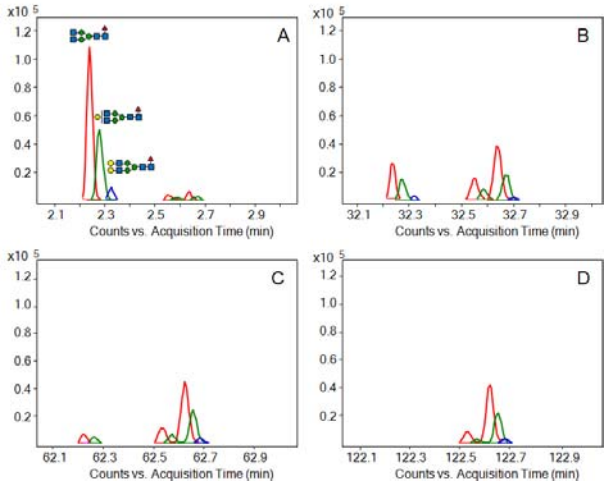


Figure 6. Time course experiments showing kinetics of hydrolysis of β-glycosylamines to free reducing end glycans. By varying the gradient delay time, the captured glycans were hydrolyzed, for varying lengths of time, to free reducing end glycans. The gradient delay times were A: 0 min, B: 30 min, C: 60 min, and D: 120 min. β-glycosylamines eluted between 2.2-2.4 minutes and decreased in intensity over time, while free reducing end glycans eluted between 2.5-2.7 minutes and increased over time. Within 120 minutes (Figure 6D) the β-glycosylamines are all hydrolyzed to free reducing end glycans. The distribution of G0, G1 and G2 is maintained.

Characterization of Intact Antibody with On-Chip Deglycosylation

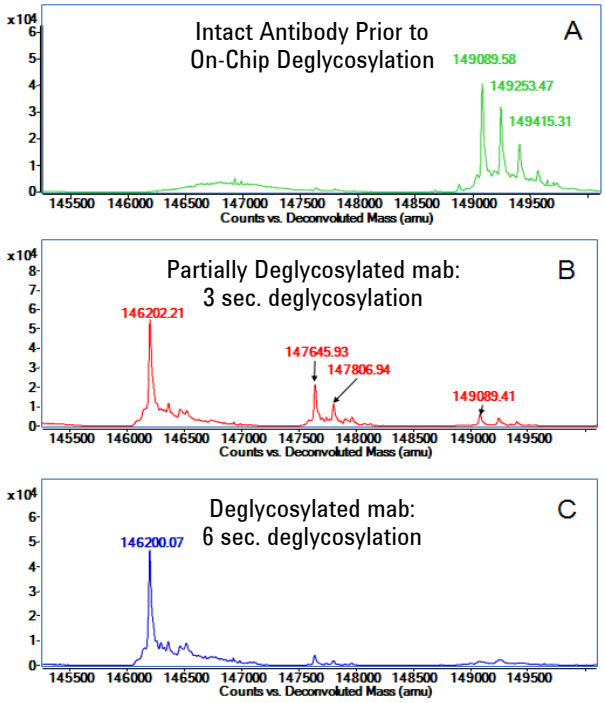


Figure 7. Analysis of deglycosylated antibody and residence time. Deconvoluted spectrum of mAb1 prior to deglycosylation is shown in Figure 7A. Three main peaks correspond to antibodies with G0/G0, G0/G1 and G1/G1 or G0/G2 glycans. Figure 7B shows partially deglycosylated antibody: 147,645.93 Da is mAb+G0 and 147806.94 Da is mAb+G1 and 146202.21 Da is deglycosylated antibody. Figure 7C shows the deglycosylated antibody.

Conclusions

- **A novel, chip-based workflow for rapid LC/MS glycan analysis of IgG mAbs demonstrated**
- **Sample-to-sample glycan profiles measured and analyzed every 10 min using < 100 ng of mAb**
- **Superior LC separation of Gx isomers over solution- phase, off-line workflows**

Acknowledgements

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