Glycoprofiling Strategies to Support Production of Recombinant Therapeutic Proteins

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Why Monitor Glycosylation?

- Impacts bioavailability/clearance due to terminal sialic acid and galactose residues
- Effects folding (FSH, LH, CG), protease susceptibility (Fibronectin), aggregation (Fibrinogen) and stability (Thyroxine-binding globulin) (Varki,1993)
- Influences biological activity (EPO, FSH etc.)
- Ensures product batch-to-batch consistency and thus clinical efficacy

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Glycosylation in Antibodies

Lund et. al., 1996 and Jefferies et. al., 1998

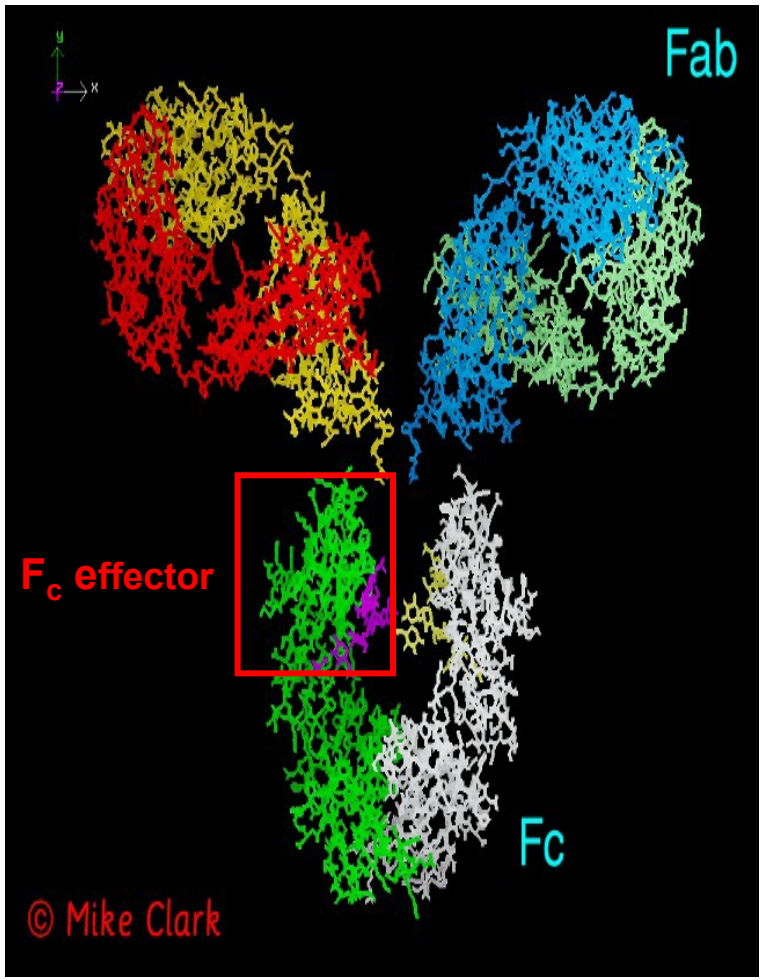
- Influences confirmation and thus affects folding
- $GlcNAc_2$-Man important in Fc receptor binding and is involved in complement-mediated lysis

Davies et. al., 2001 and Shields et. al., 2002

- Lower fucose and bisecting GlcNAc content improves FcγRIIIA receptor binding and enhances antibody-dependent cell-mediated cytotoxicity (ADCC)

Kaneko et. al., 2006

- G0 variant is pro-inflammatory and Fc sialylation is anti-inflammatory



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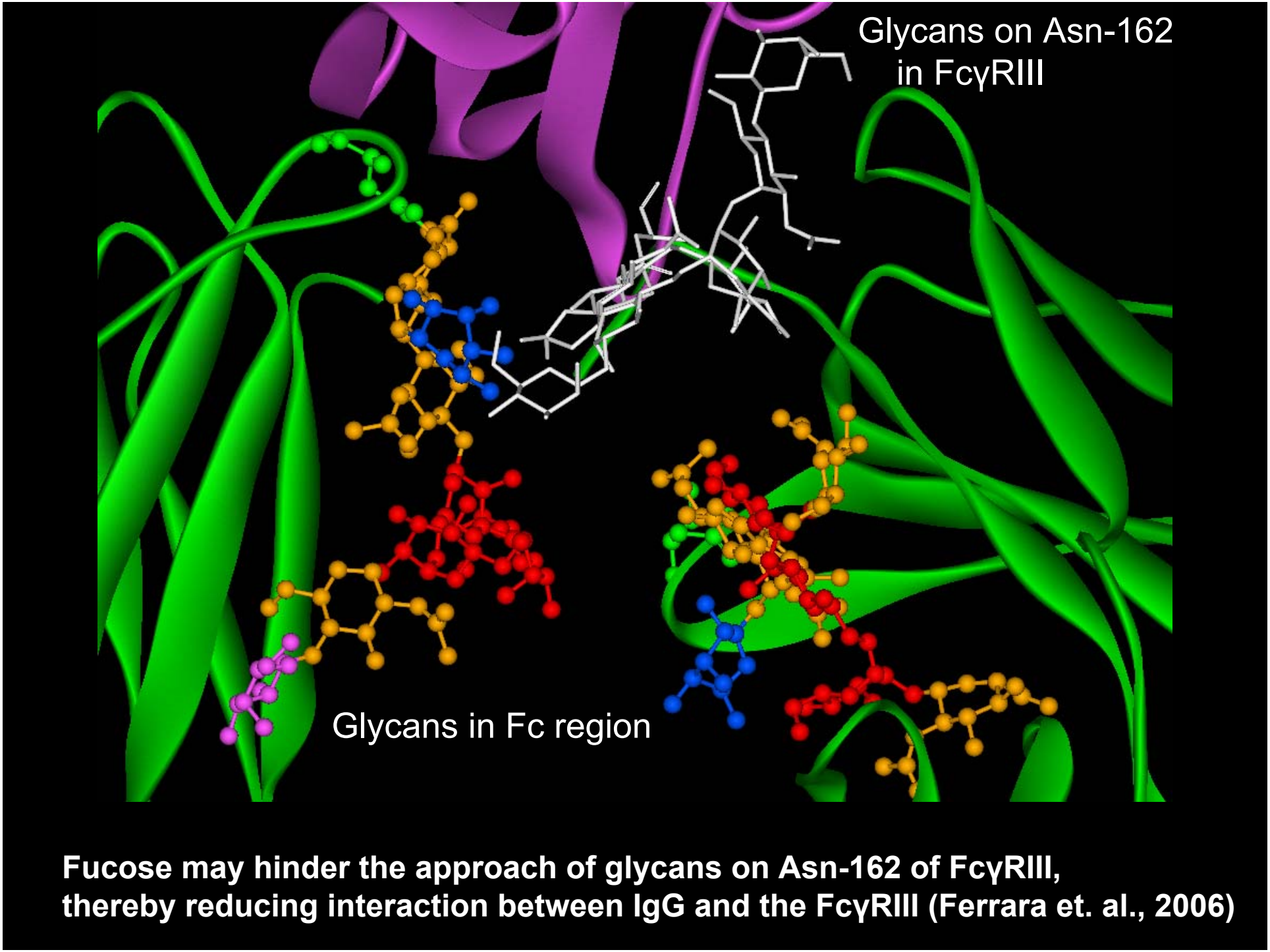
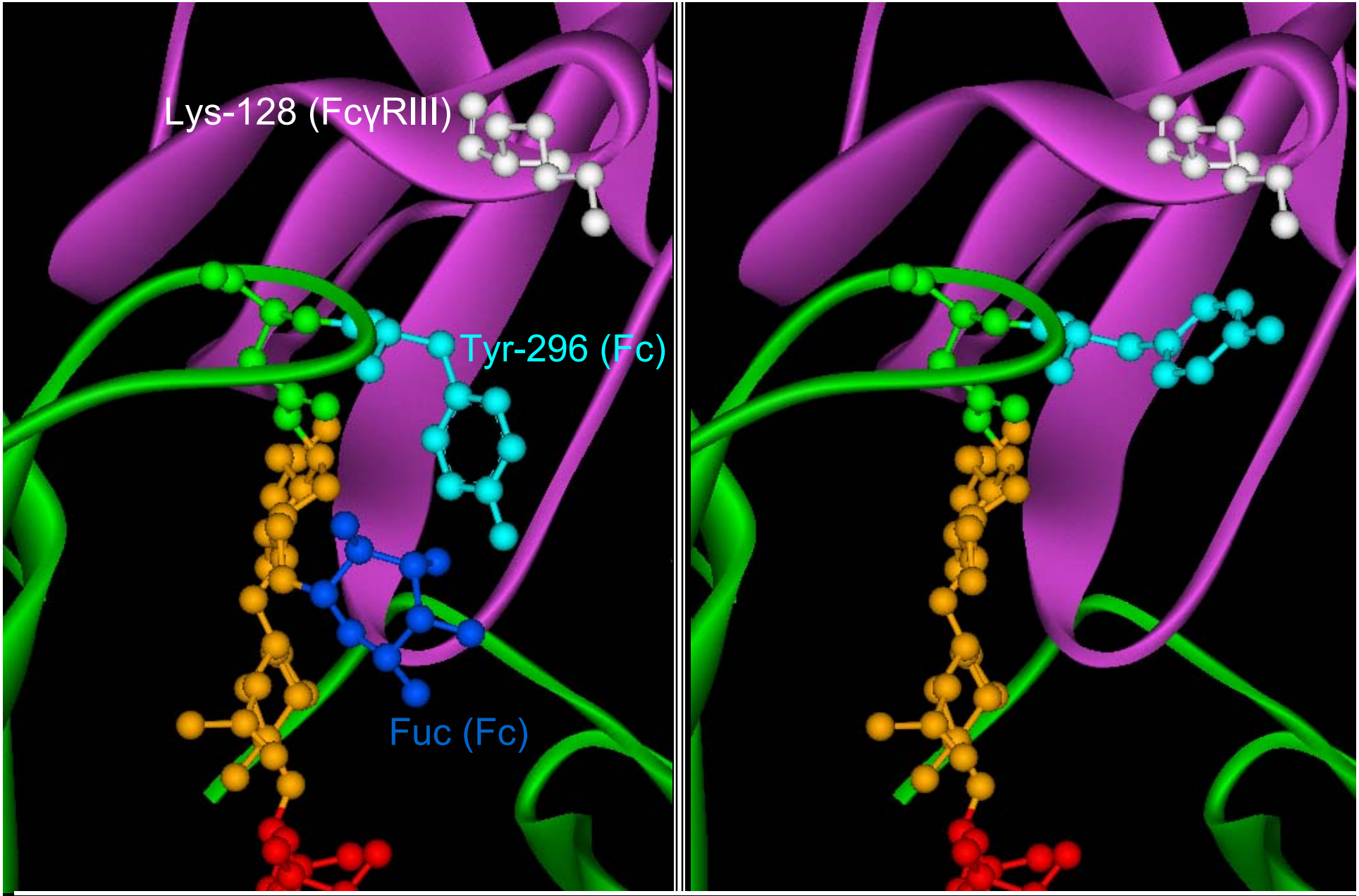


Fucose may hinder the approach of glycans on Asn-162 of FcγRIII, thereby reducing interaction between IgG and the FcγRIII (Ferrara et. al., 2006)



Depletion of Fucose from IgG Fc may allow Tyr-296 to interact with Lys-128 in FcγRIII, thereby increasing affinity (Okazaki et. al., 2004)

Factors Influencing Glycosylation

- Expression system (CHO, NS0, Pichia, Plants, Baculovirus)
- Cell age
- pH
- Cell density
- pCO_2
- Ammonium concentration and other metabolites (i.e. sodium butyrate, Jenkins et. al.,1996)
- Downstream processing

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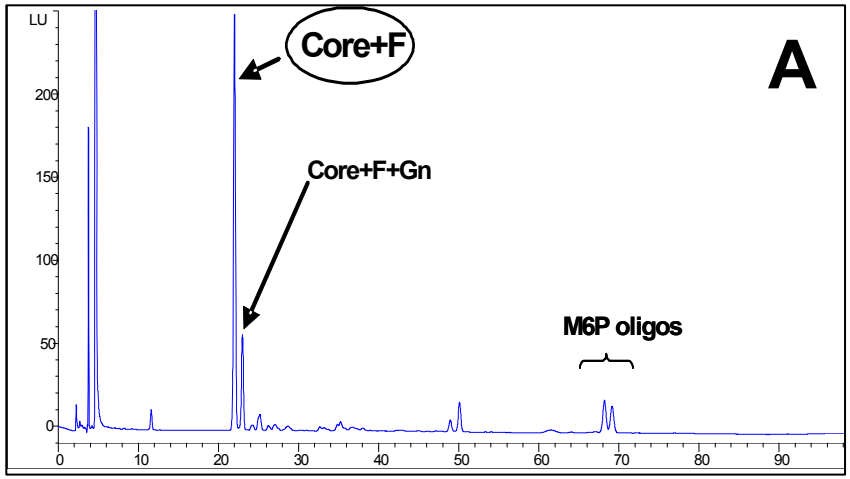
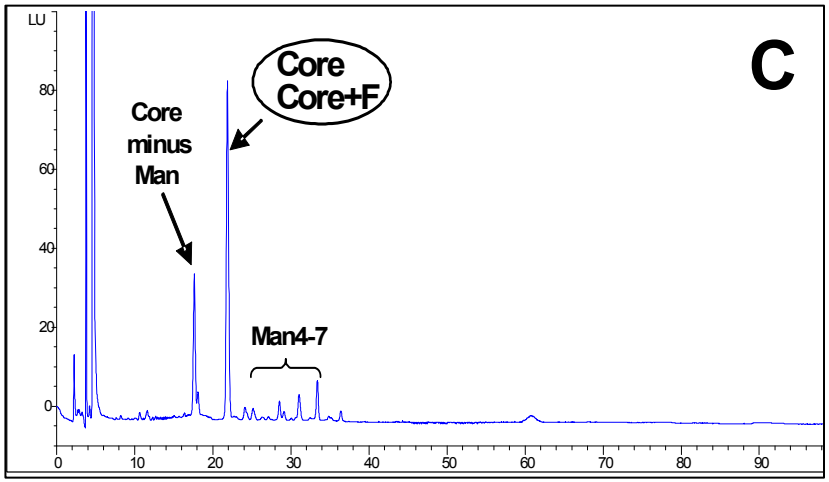
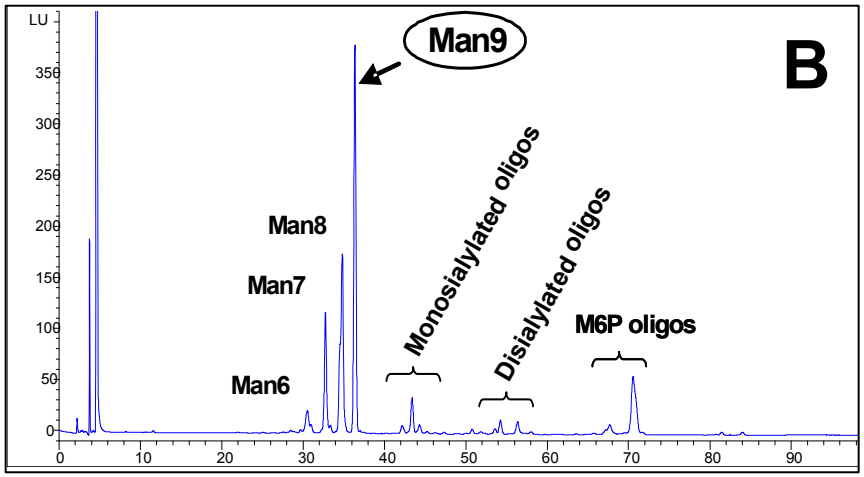
Expression System and Glycosylation



CHO

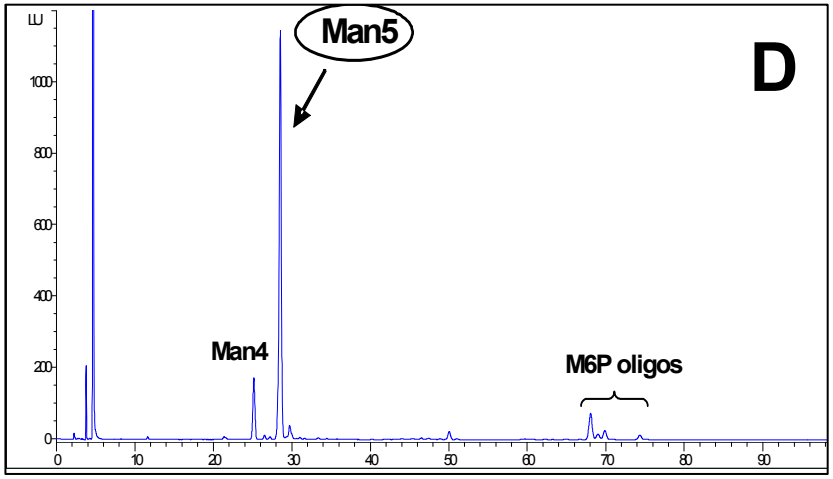


Baculovirus



CHO + Kifunensine

vanPatten et al. Glycobiology, 2007 (accepted)



CHO - Lec1 mutant

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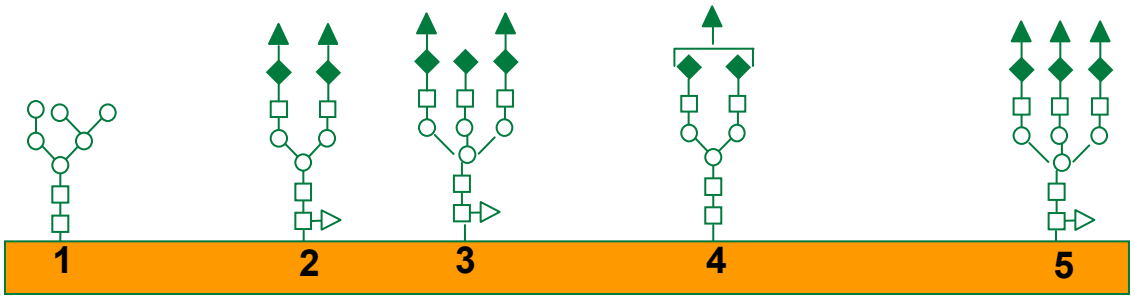
Molecular Complexity - Example



Significant glycan structures/site	**10**	**8**	**11**	**9**	**5**

~ 4.0 x 10^4 possible glycovariants

- Robust analytical platforms are critical to assess product quality and consistency

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Monitoring Glycosylation

- Individual glycan structure
- Intact protein glycoforms
- Site-specific glycosylation

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Monitoring Individual Glycan Structure

- Monosaccharide compositional analyses
  - Monosaccharides (Fuc, Gal, GlcNAc, Man)
  - Sialic acid
  - Mannose-6-phosphate
- Oligosaccharide Profiling
  - Oligosaccharide profiling monitors potential changes in oligosaccharide structures and their relative population

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Oligosaccharide Profiling Methods

- Unlabeled glycans
  - High pH Anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)
  - Mass Spectrometry (MALDI-TOF)

- Fluorescently labeled glycans
  - Anthranilic acid, AA ⟶ NP-HPLC
  - 2-aminobenzoic acid, 2-AB ⟶ NP-HPLC
  - 8-aminopyrene-1,3,6-trisulfonate, APTS ⟶ CE-LIF

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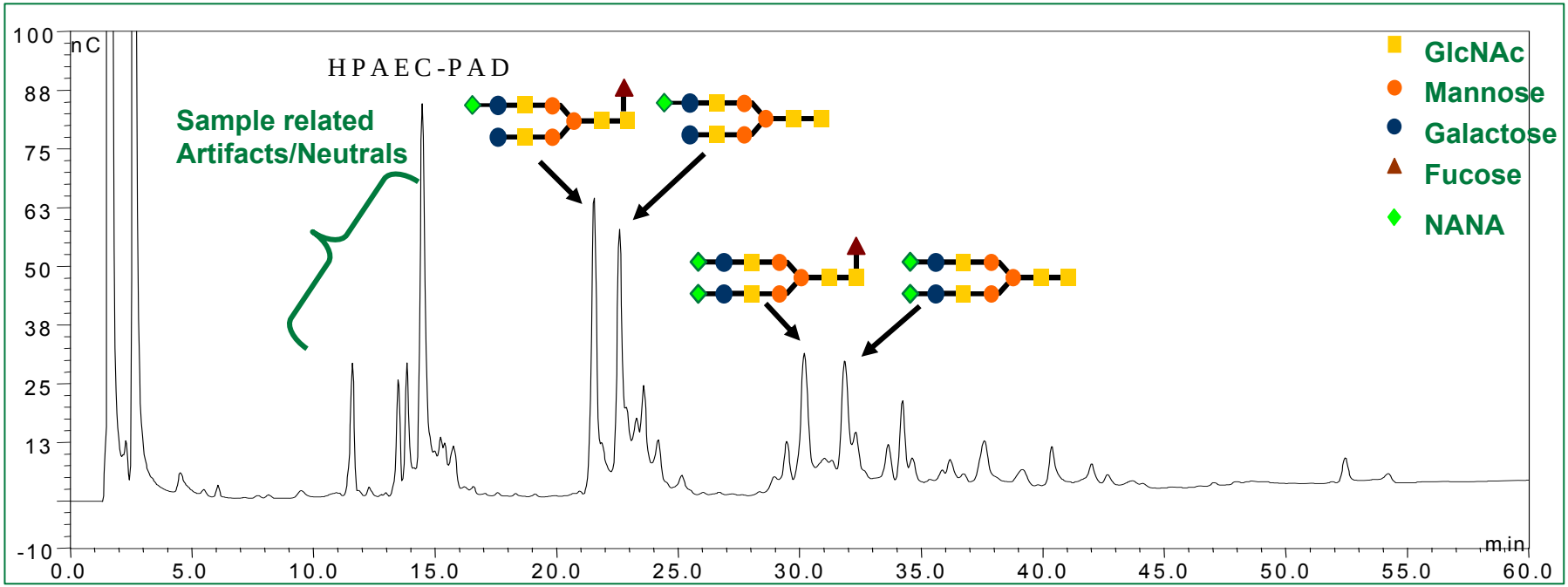
Unlabeled Glycans by HPAEC-PAD



Column : Dionex PA-100 column (250 X 4.6 mm) at 35 °C
Mobile Phase A: 100 mM Sodium Hydroxide
Mobile Phase B: 100 mM Sodium Hydroxide + 500 mM Sodium Acetate
Gradient: 0%-55%B in 85 minutes
Detection: Pulsed Amperometric Detection (4-stage waveform)

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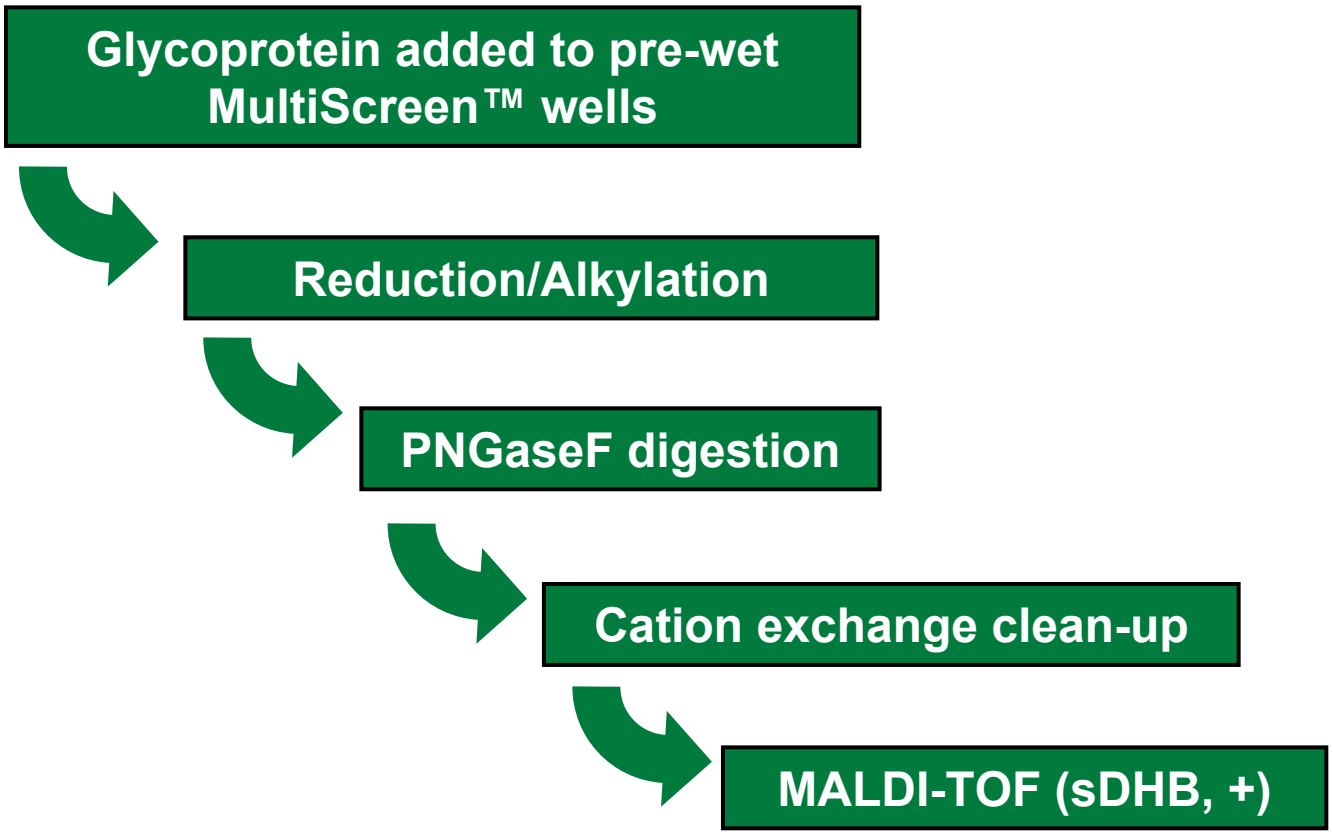
Unlabeled Glycans by MALDI-TOF



Papac et al. Glycobiology, 1998, 8, 445

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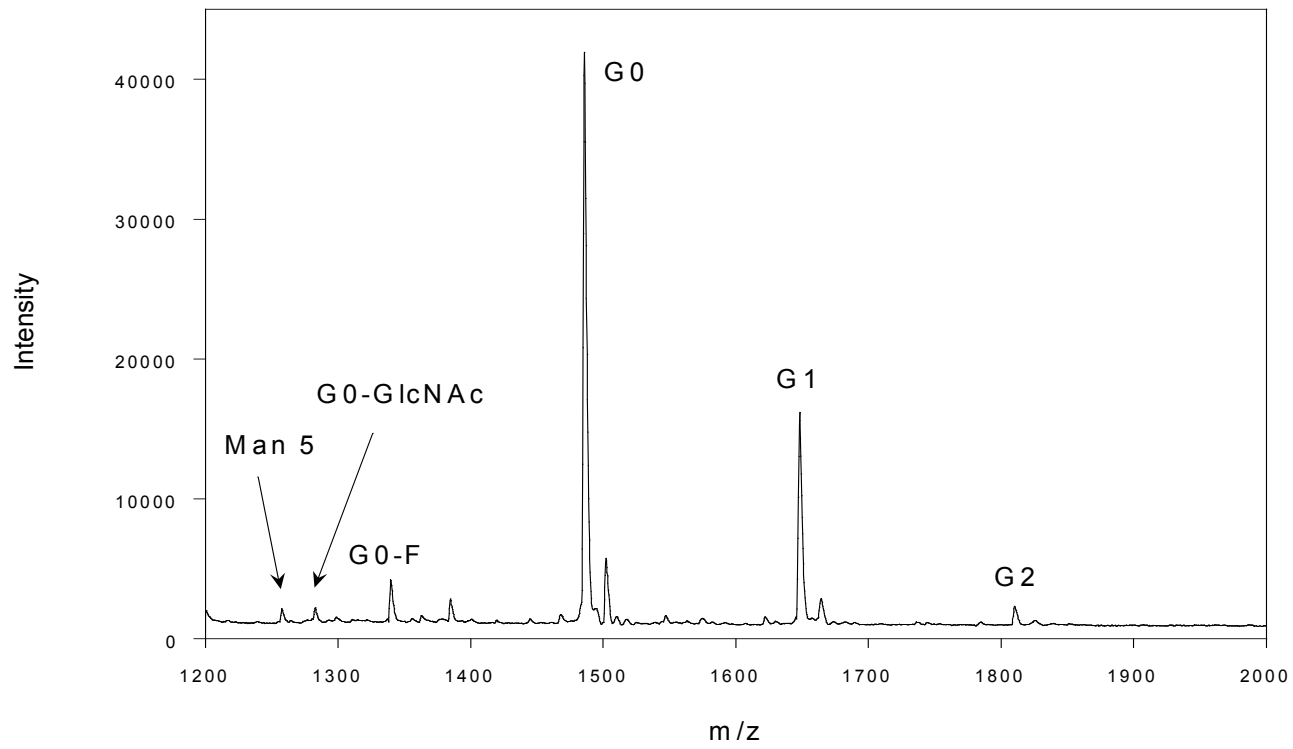
mAb: MALDI-TOF of Unlabeled Glycans



Stacey Ma, Genentech

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Fluorescence Labeling of Glycans

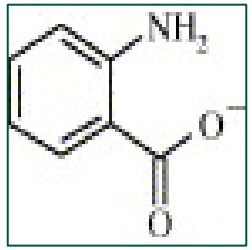
Glycoprotein

PNGaseF digestion

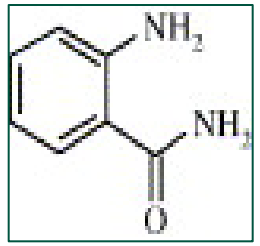
Reductive amination/labeling*

NP-HPLC/CE-LIF

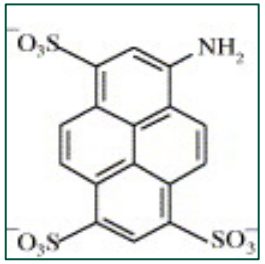
On-line Mass Spectrometry
LC-MS/MS (-)
LCQ-TOF MS (+)



AA



2-AB



APTS

*Anumula et al. *Glycobiology* 1998, *8*, 685
*S. Ma, *Anal. Chem*. 1999, 71, 5185
*Bigge et al. *Anal. Biochem*. 1995, 230, 229

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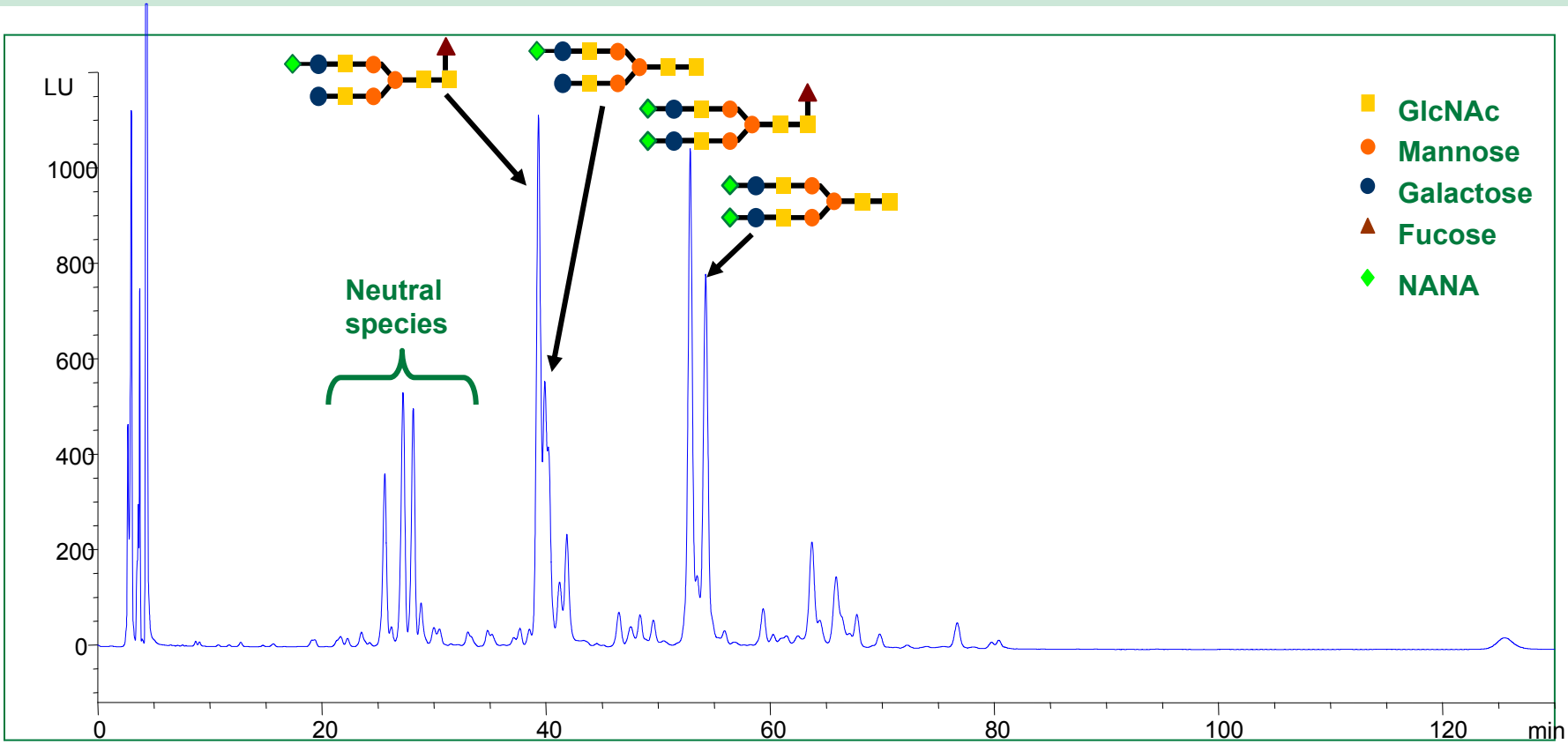
Oligosaccharide Profiling by AA-labeling/NP-HPLC



Column :	**Luna NH2, 5 µm, 100 Å (250 x 4.6 mm) at 50 °C**
Mobile Phase A:	**2% acetic acid, 1% THF in acetonitrile**
Mobile Phase B:	**5% acetic acid, 3% TEA, 1% THF in water**
Gradient:	**30%-95%B in 85 minutes**
Detection:	**ex. 230 nm and em. at 425 nm**

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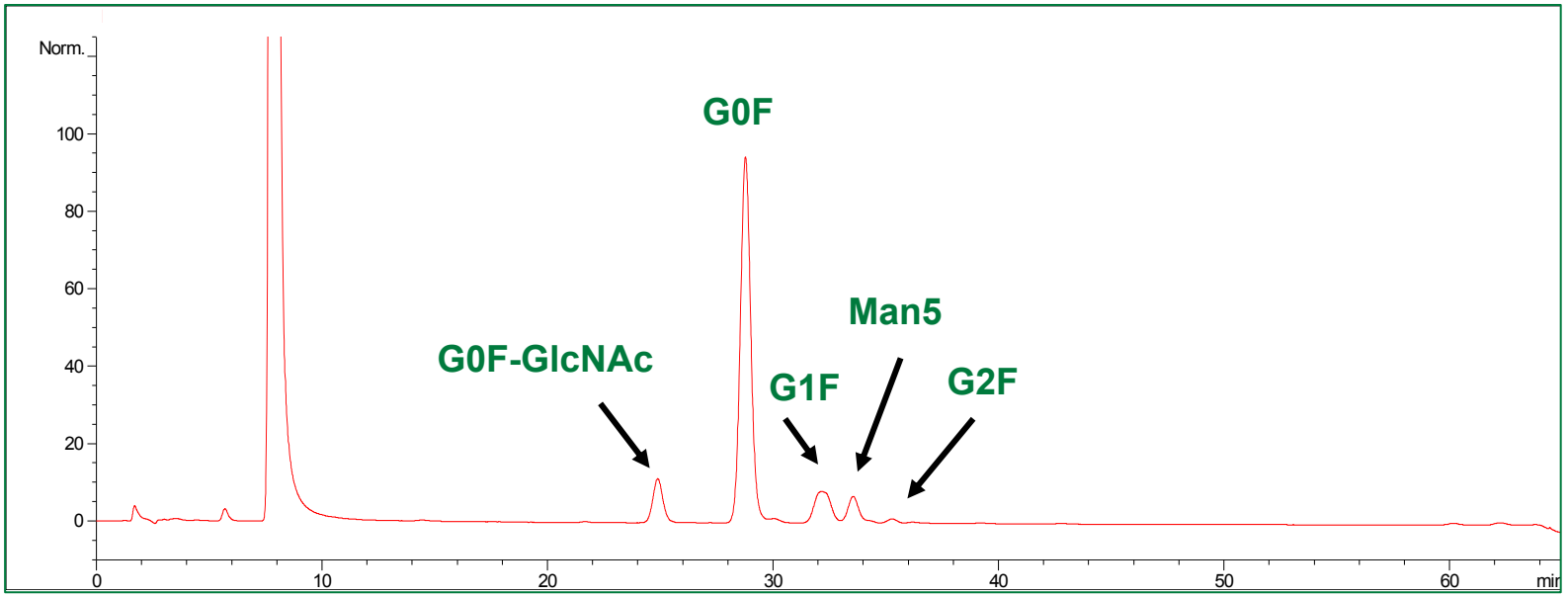
mAb: AA-labeling/NP-HPLC



Column : Asahipak NH2P-50 4D (150 x 4.6 mm) at 50 °C
Mobile Phase A: 2% acetic acid, 1% THF in acetonitrile
Mobile Phase B: 5% acetic acid, 3% TEA, 1% THF in water
Gradient: 30%-50%B in 60 minutes
Detection: ex. 230 nm and em. at 425 nm

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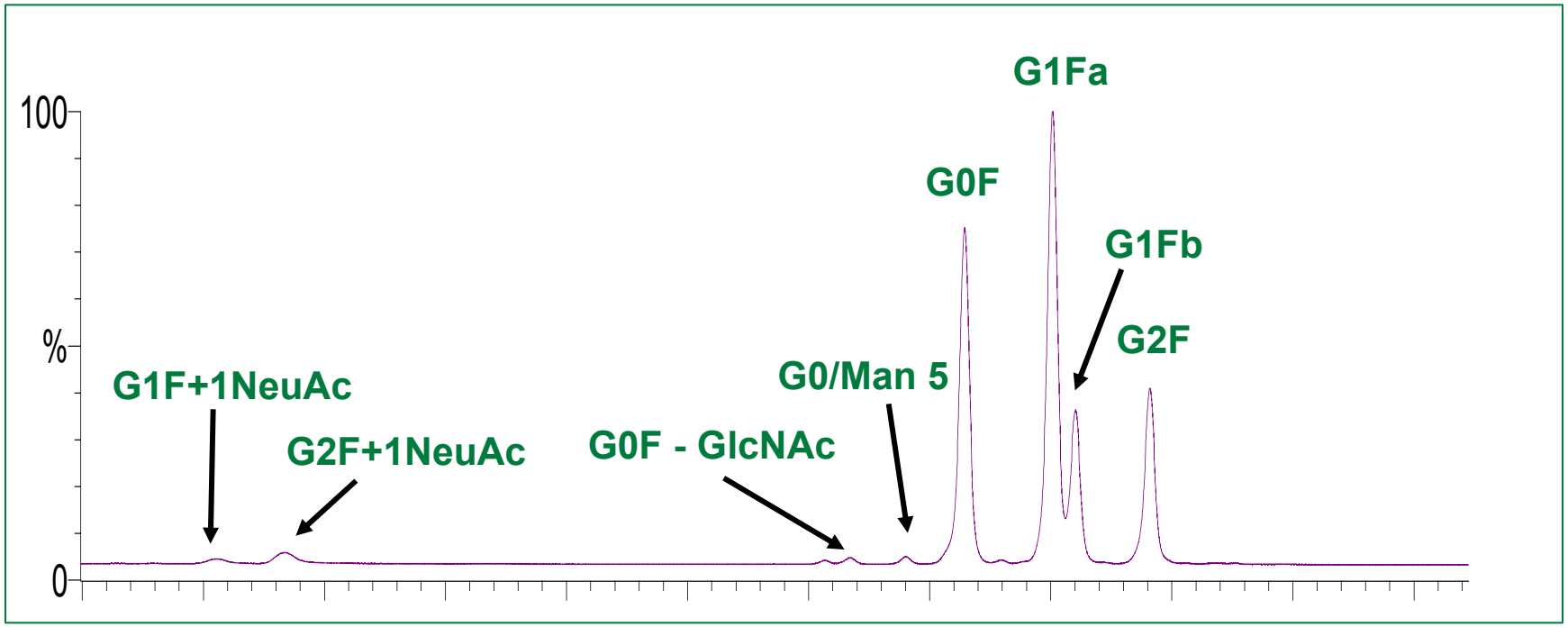
mAb: 2-AB labeling/NP-HPLC



Column :	**Polysulfoethyl A (100 x 4.6 mm) at 45 ◦C**
Mobile Phase A:	**Acetonitrile**
Mobile Phase B:	**Water**
Gradient:	**15%-41%B in 42 minutes**
Detection:	**ex. 330 nm, em. 420 nm**

Toler et al. 2006. Manuscript in preparation

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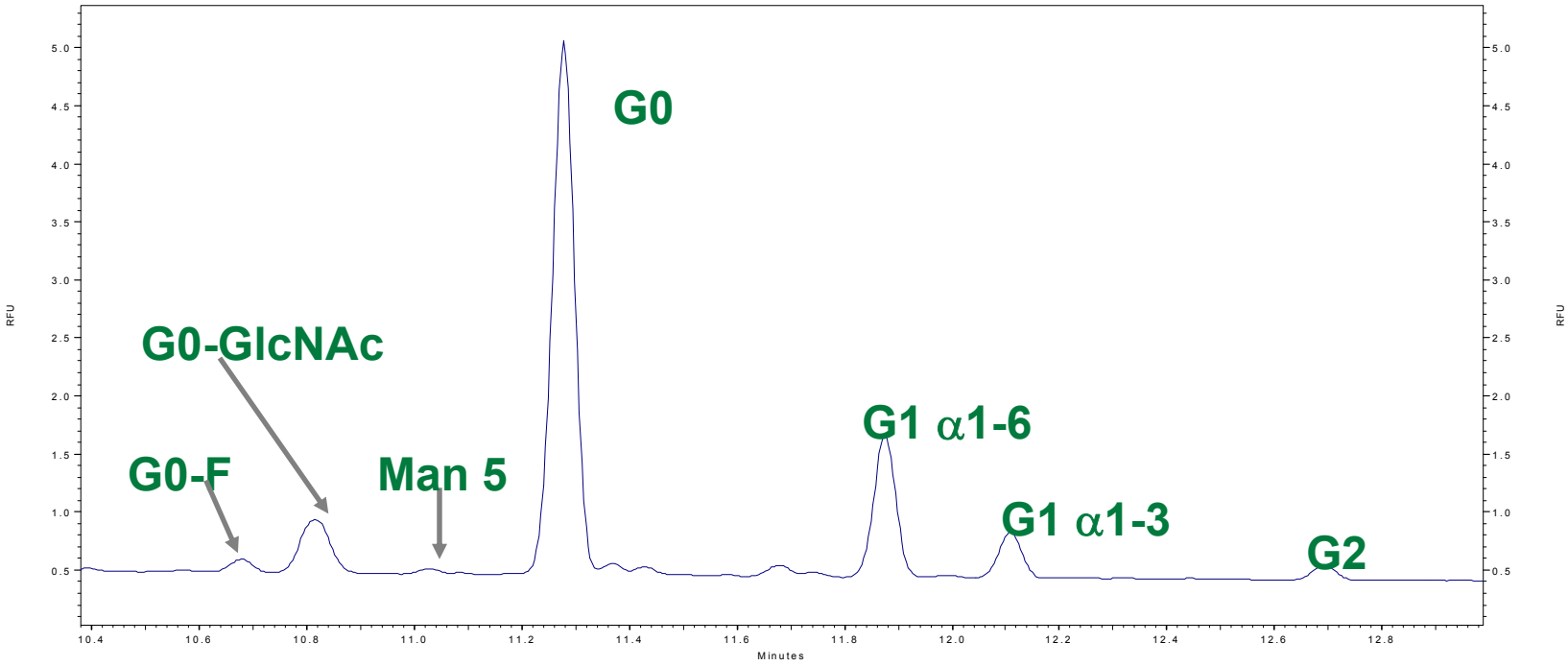
mAb: APTS-labeling/CE-LIF



Column :	**eCAP™ N-CHO capillary (50 µm x 27 cm) at 20 °C**
Cathode buffer:	**2% acetic acid, 1% THF in acetonitrile**
Anode buffer:	**5% acetic acid, 3% TEA, 1% THF in water**
Conditions:	**Reverse polarity, 20 kV, 20 µA**
Detection:	**ex. 488 nm and em. at 520 nm**

Lau et al. Poster at CE Pharm, 2006

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HPAEC-PAD versus Fluorescence

	HPAEC-PAD	Fluorescence
Charge/linkage based rugged separation	Yes	Yes
Sample derivatization and clean-up (prior to analysis)	No	Yes
Quantitative	No	Yes
Assay variability	High	Low
MS compatible	Maybe	Yes

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Monitoring Intact Protein Glycosylation

- Isoelectric focusing
  - Snapshot to examine charge heterogeneity
  - No structural detail obtained
- Mass Spectrometry (i.e. for mAbs)
  - Powerful characterization tool that provides a snap shot of glycosylation at molecular level
  - Eliminates potential selectivity associated with glycan release
  - Quantitative based on the assumption of equal ionization efficiencies for various glycoforms

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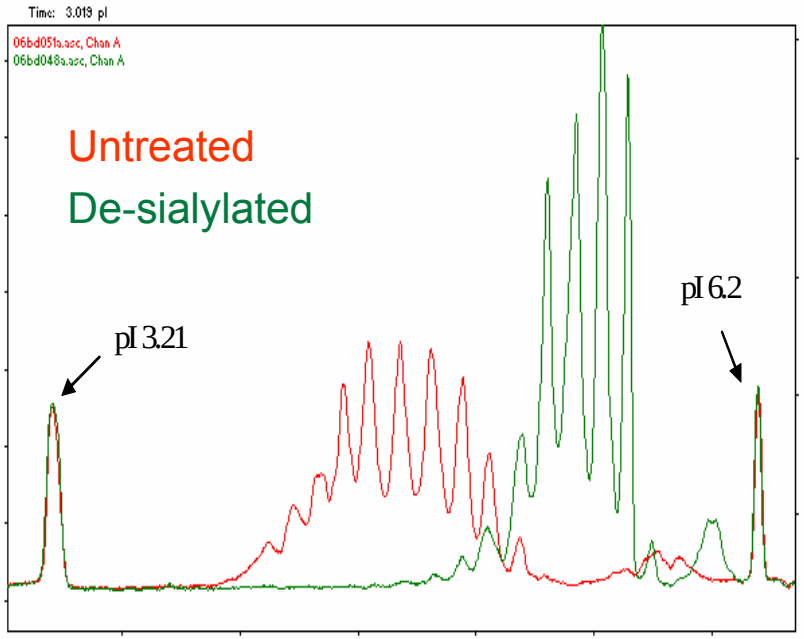
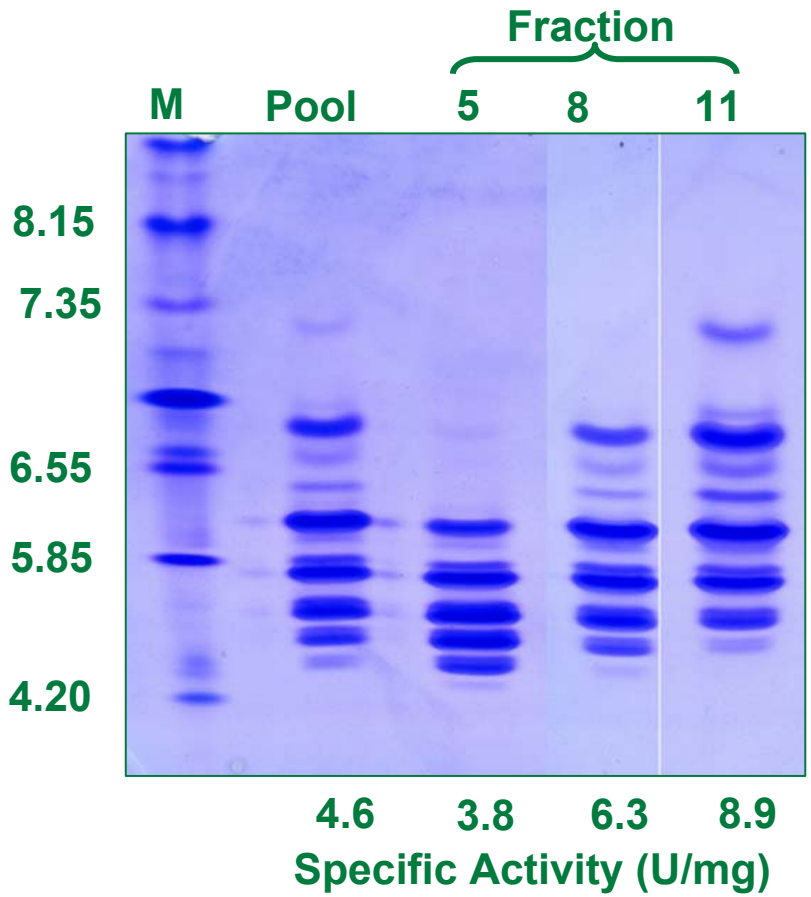
Intact Protein Glycosylation : IEF Analysis





- pI shifts with removal of sialic acid
- Sialylation affects Specific Activity of this protein

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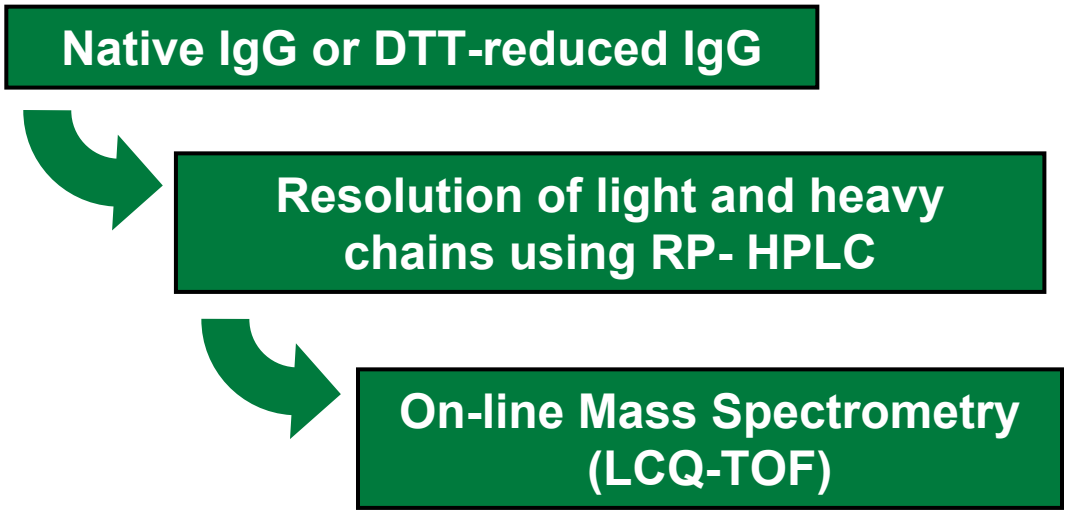
Intact Protein Glycosylation (mAb) – Mass Spectrometry



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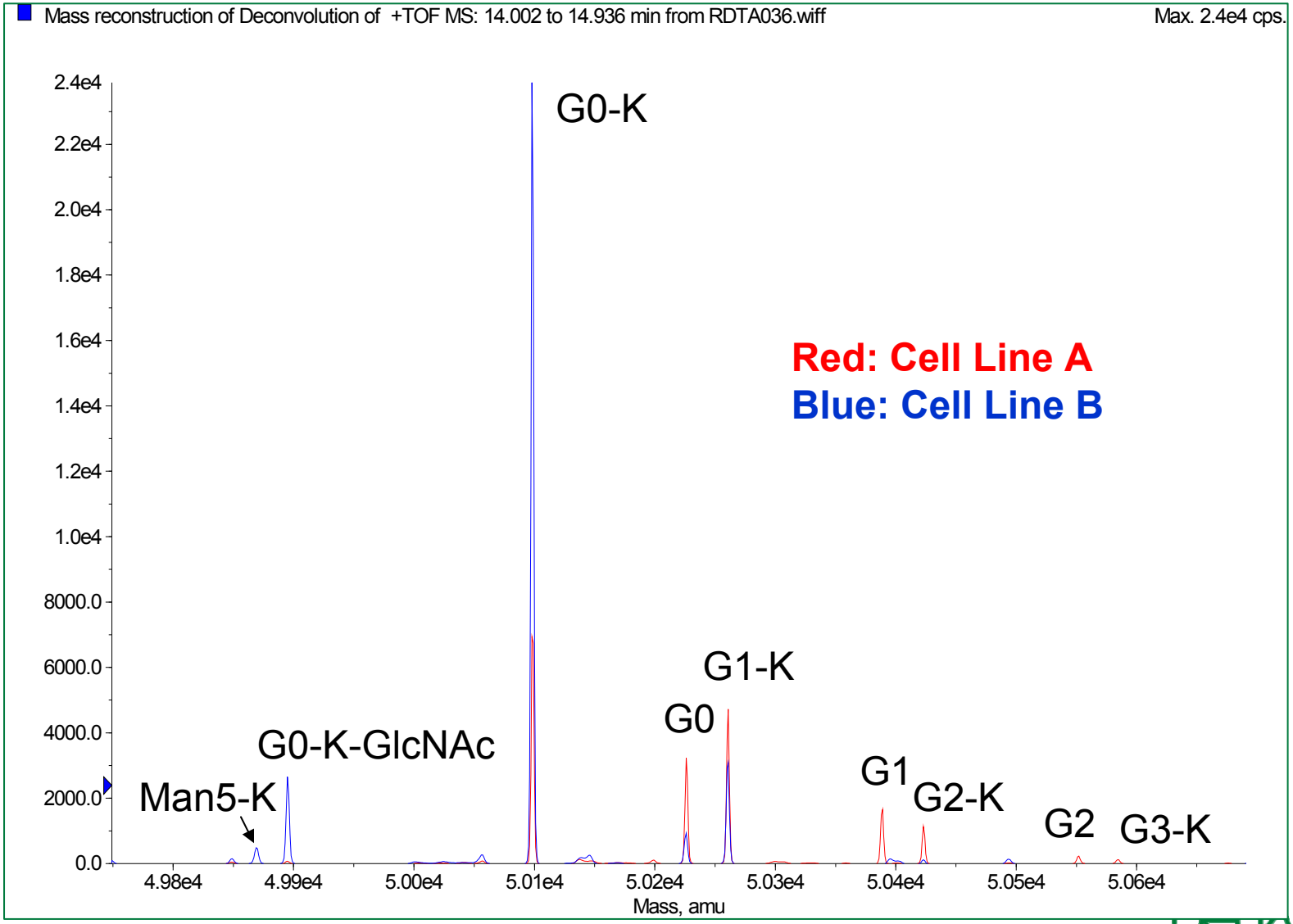
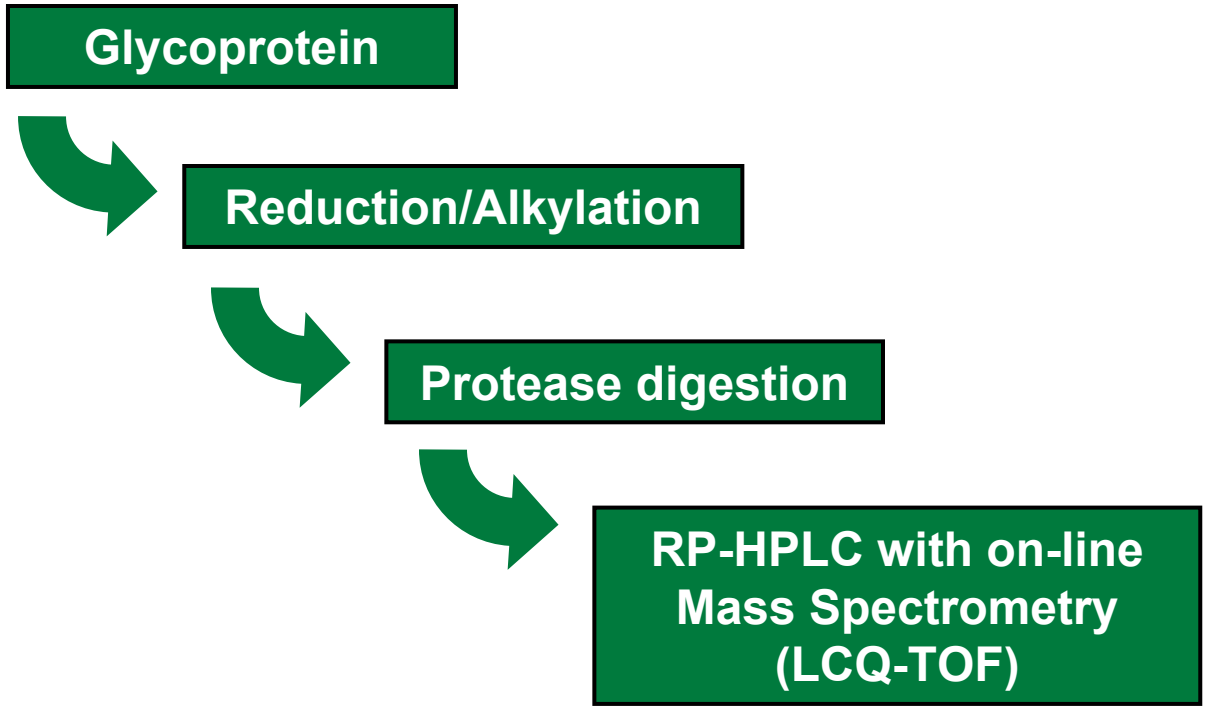
ESI-MS Spectra of mAb Heavy Chain



Monitoring Site-Specific Glycosylation



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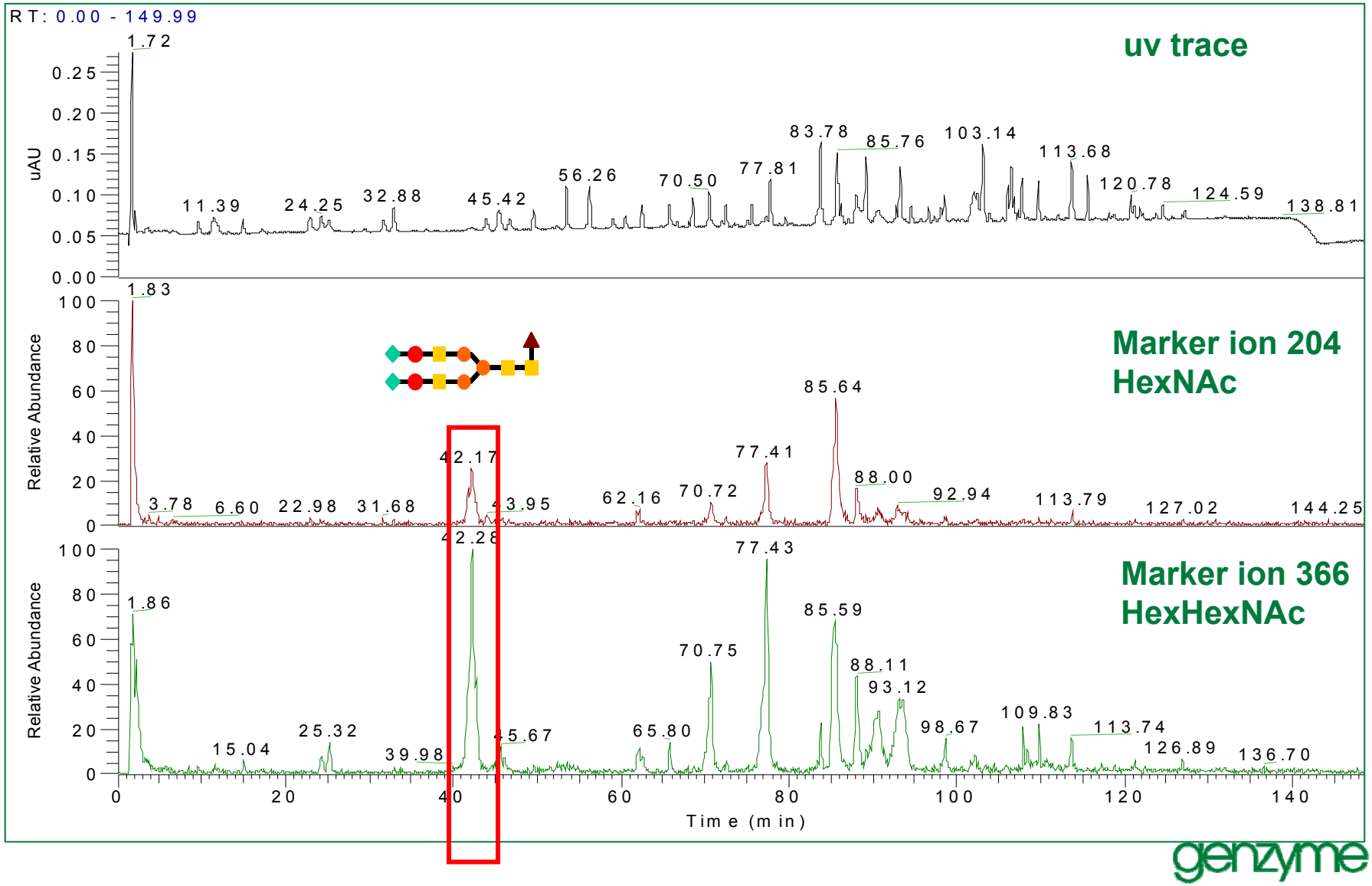
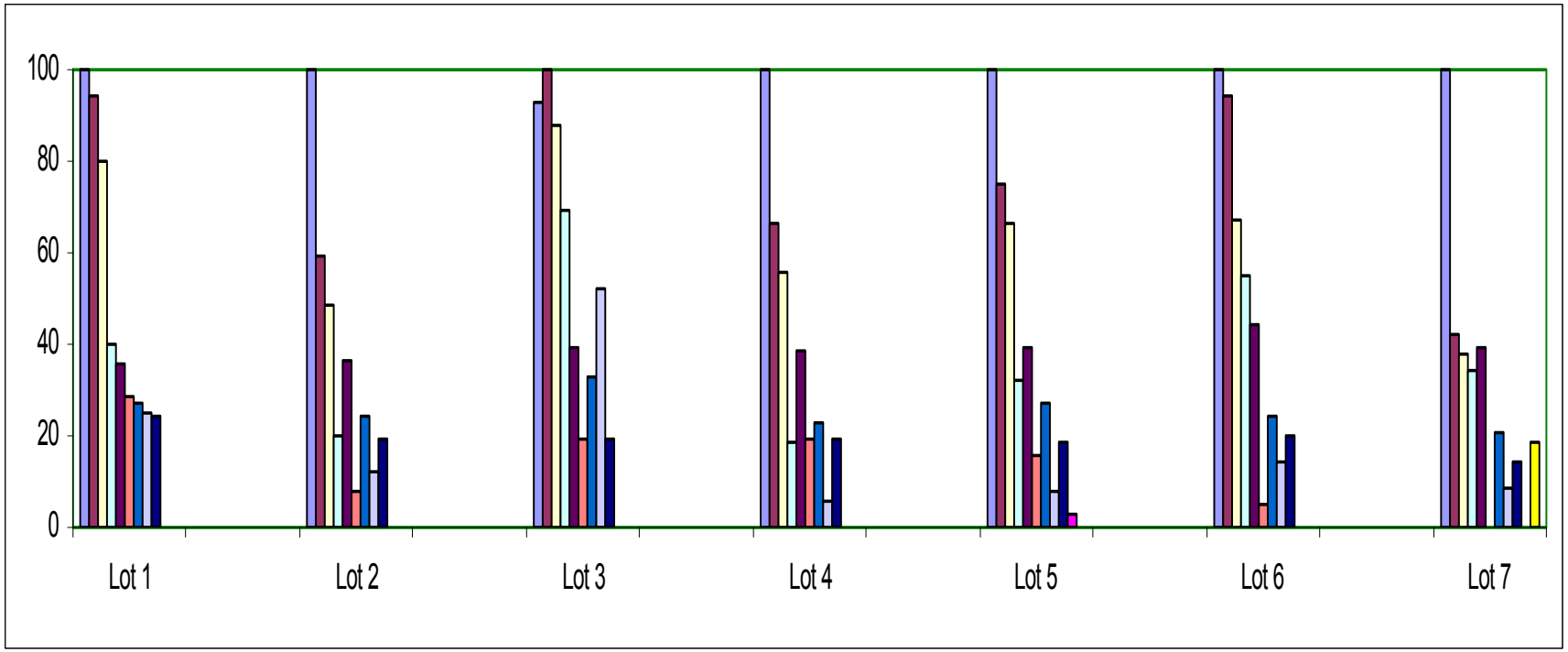
Trpytic Map of a Multiply Glycosylated Protein



High Degree of Lot to Lot Consistency

- Relative intensities of various glycans at Asn-X in 7 Drug Substance lots



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Site-Specific Glycosylation (LC/MS)

- Advantages
  - Detailed site-specific structural analysis and relative abundance of different glycan species at each site
  - Semi-quantitative and eliminates potential selectivity associated with glycan release
- Disadvantages
  - Assumes ionization efficiencies for all glycopeptides similar
  - Requires high level of technical expertise and instruments (ion trap, Q-TOF etc.)

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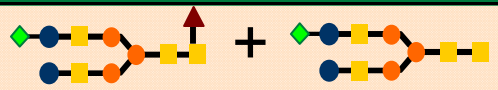
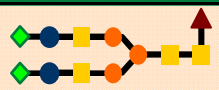
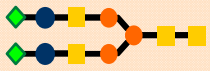
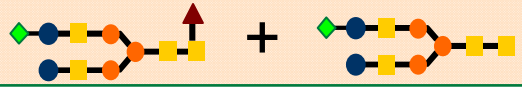
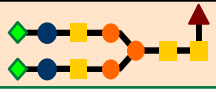
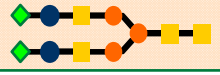
Precision : HPAEC-PAD versus AA-labeling

HPAEC-PAD	% Peak Area (N=39)		
			
Average	52.3	21.3	19.6
SD	3.3	3.2	3.0
%CV	12.6	15.0	15.3

AA/HPLC	% Peak Area (N=30)		
			
Average	50.2	24.4	20.9
SD	1.0	1.1	1.3
%CV	2.1	4.5	6.4

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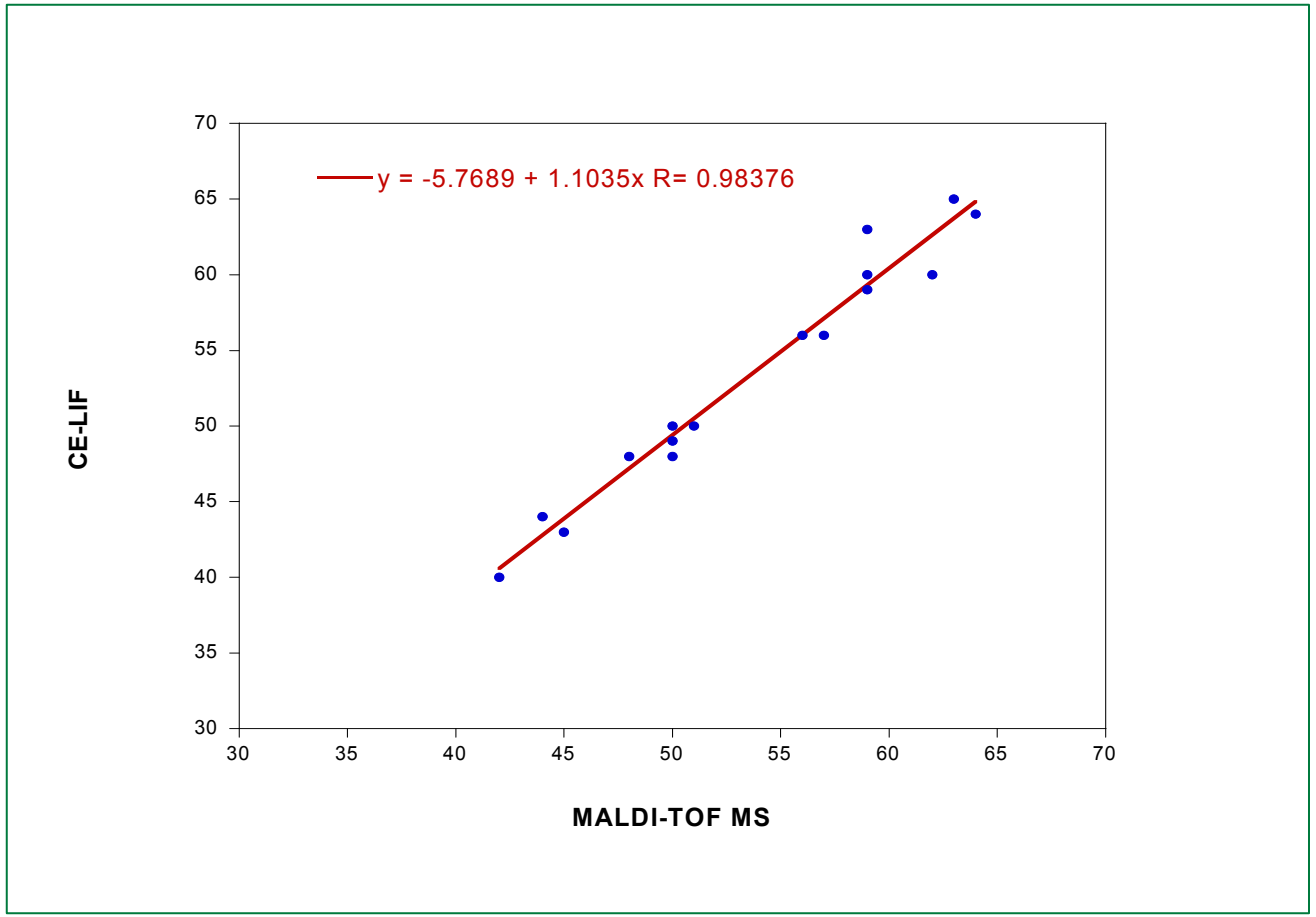
APTS-labeling/CE-LIF versus MALDI-TOF

- Relative percentage of G0 glycans



Rod Keck, Genentech

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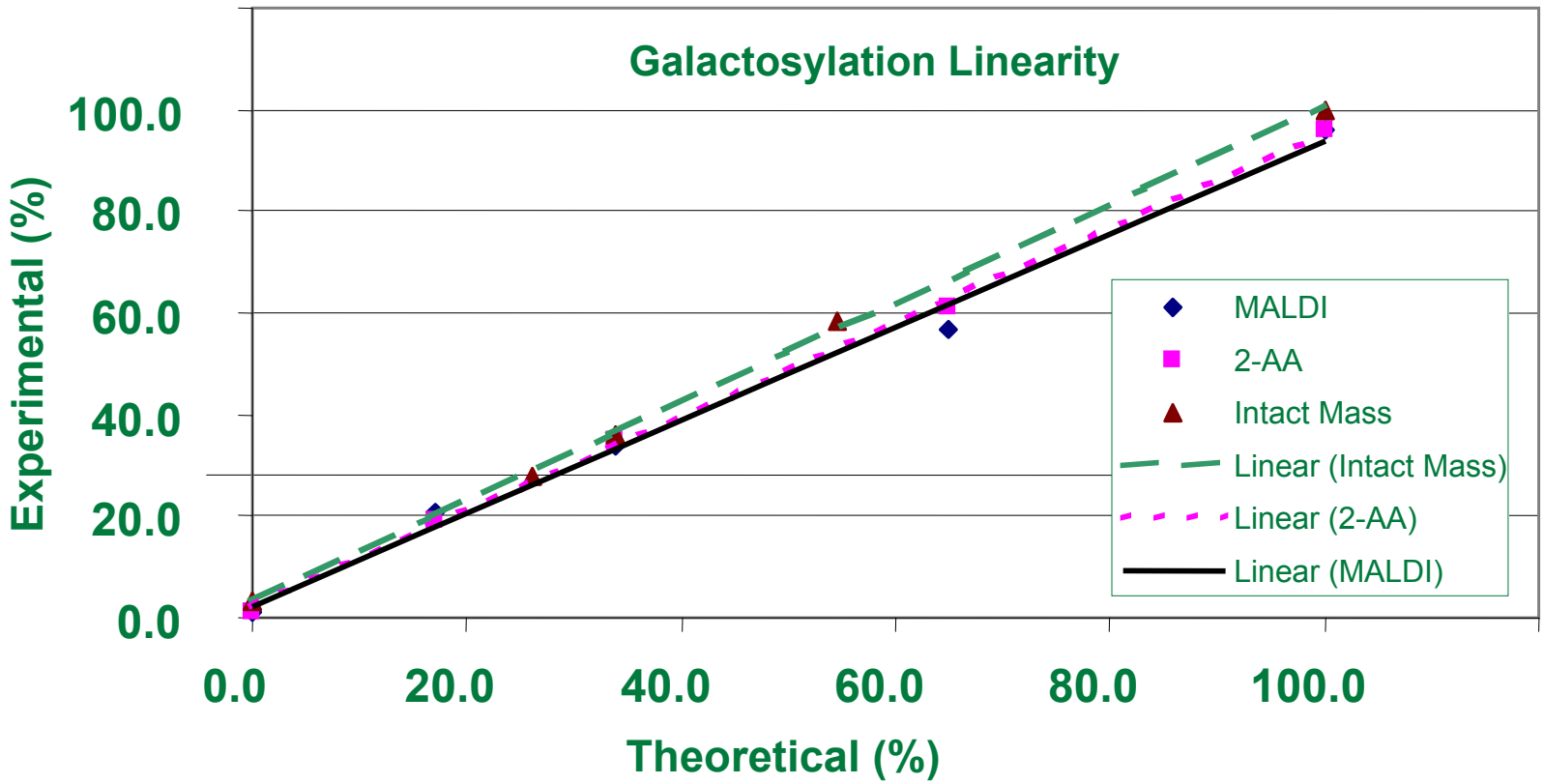
AA versus MALDI-TOF versus Intact MW



Percent Galactosylation = Total Galactose Residues / Total sites for Galactosylation

Joseph Siemiatkoski, Biogen-IDEC

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Conclusions

- Oligosaccharide profiling by HPAEC-PAD has significant limitations i.e. higher variability, non-quantitative, lack of structural information etc.
- Fluorescence-based methods offer a rugged, quantitative, alternative to HPAEC-PAD with on-line detailed structural information (LC-MS and MS/MS)
- Monosaccharides critical for initial characterization of the oligosaccharide structures must be routinely monitored
- The ultimate choice of any method depends on the glycoprotein being analyzed, analyst expertise and the successful implementation of that particular technology

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Bob Mattaliano

Joseph Siemiatkoski, Biogen-Idec
Stacey Ma, Genentech

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