Rapid and High Throughput CE Assays of Enzyme Activity: Liberty Ballroom B&C 13:00-14:30

Lisa Holland,
Lloyd Bwanali, Laura Casto-Boggess, Srikanth Gattu, Jessica Guerrette, John Lucas

NIH R01GM140560, R01GM114330

Greetings from West Virginia!

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Capillary Nanogel Electrophoresis Applied to Glycosylation

Enzyme inhibitor global market $178B in 2023

Glycosylated substrates are non templated therapeutics requiring sophisticated biotechnology. Complex analytical tools are costly and difficult to use

Self assembled nanogels are multifunctional and improve biomolecule separations at a cost of $0.76 for the on-board fluid.

Innovation: smart materials enhance functionality at for automated, rapid, and microscale analyses

Findings: Phospholipid nanogels provide **sieving/electrophoresis**, **enzymatic modification**, **sequencing,** and **patterning.**

Goals for Integrated Enzyme Analyses

1. Enzyme Assay: Capillary Electrophoresis is used to quantify enzyme activity and to screen and even quantify the ability of molecules to inhibit the enzyme

Innovation: nanogels pattern nanoliter reactions for automated, rapid, and cost-effective screening

2. Microscale Sequencing: Enzymes are integrated into a capillary to determine glycan (a PTM), sequence and linkage in capillary electrophoresis run

Innovation: enzyme sequencing provides cost-effective identification with no standards or MS

Unique Topics to be Addressed

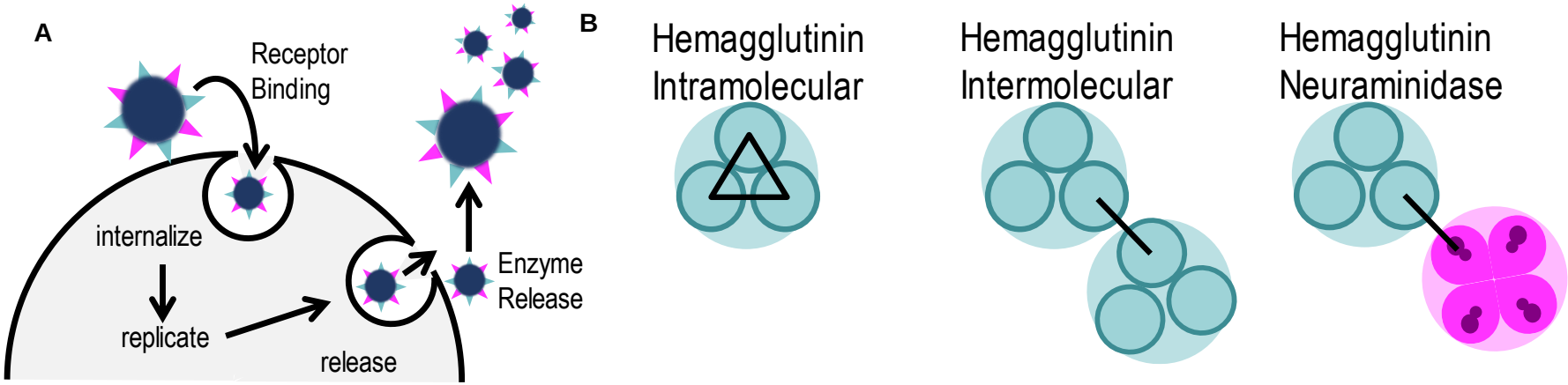
What makes oligosaccharides difficult to analyze

What is a Nanogel and how does it work?

What makes capillary electrophoresis ideal for integrated enzyme analyses

Why does it matter?

Post-COVID *Significance* of Sialylation (a form of glycosylation) in Scientific Research



Neuraminidase and hemagglutinin function and balance are implicated in virulence. The complexity of these interactions makes it difficult to develop strategies to eradicate influenza A.

Post-COVID *Significance* of Therapeutics and Glycosylation in Scientific Research

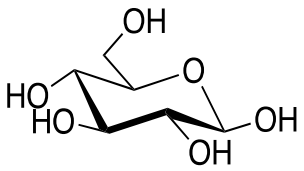
Enzyme inhibitors important therapeutics
including COVID-19

Glycosylation involved in viral infections
Implicated in binding domain positioning, immune system evasion, cell entry

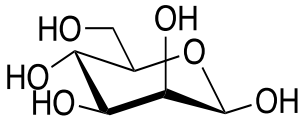
What makes oligosaccharides difficult to assay or analyze?

What makes oligosaccharides difficult to analyze

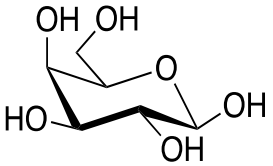
monomers



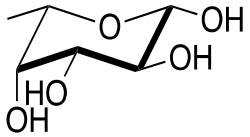
D-Glucose (●)



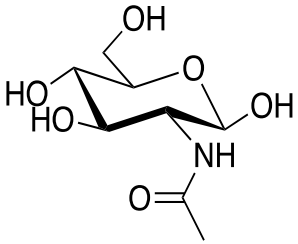
D-Mannose (●)



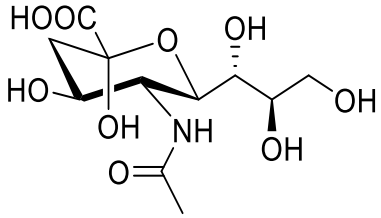
D-Galactose (●)



L-Fucose (▼)

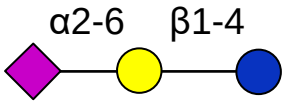


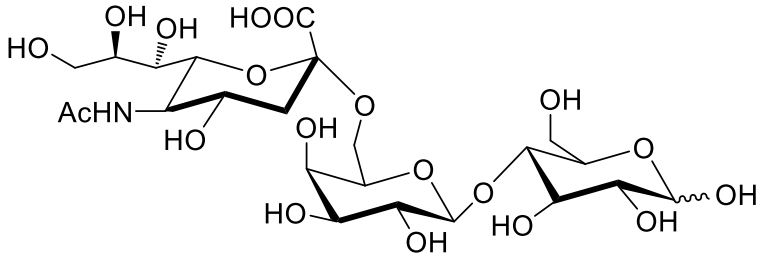
N-Acetyl-D-glucosamine (■)

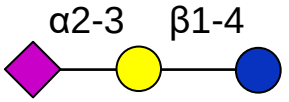


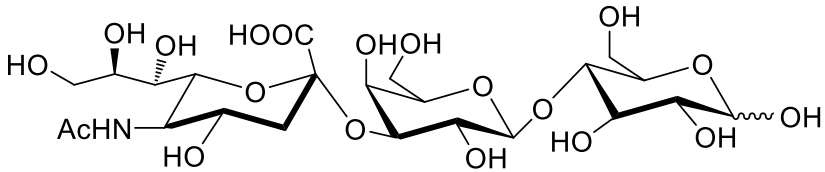
N-Acetyl-D-neuraminic acid (◆)

linkages



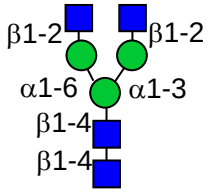






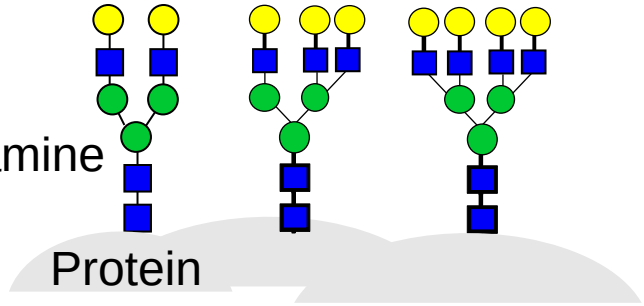
Lu, G., C.L. Crihfield, S. Gattu, L.M. Veltri, and L.A. Holland, Capillary Electrophoresis Separations of Glycans. *Chemical Reviews*, **2018**. 118(17): p. 7867-7885.

The Anatomy of a Glycan

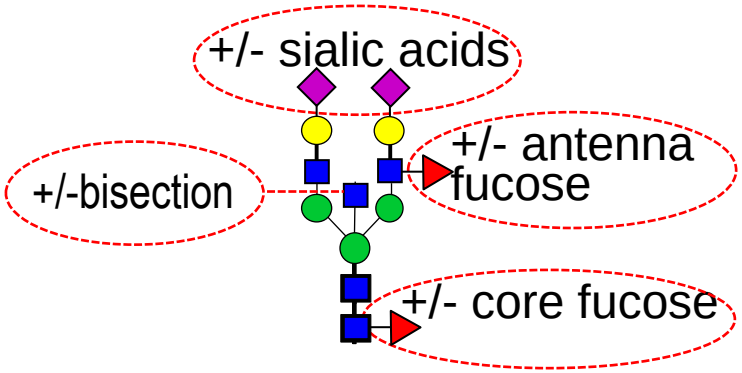


N-glycans (unmodified)

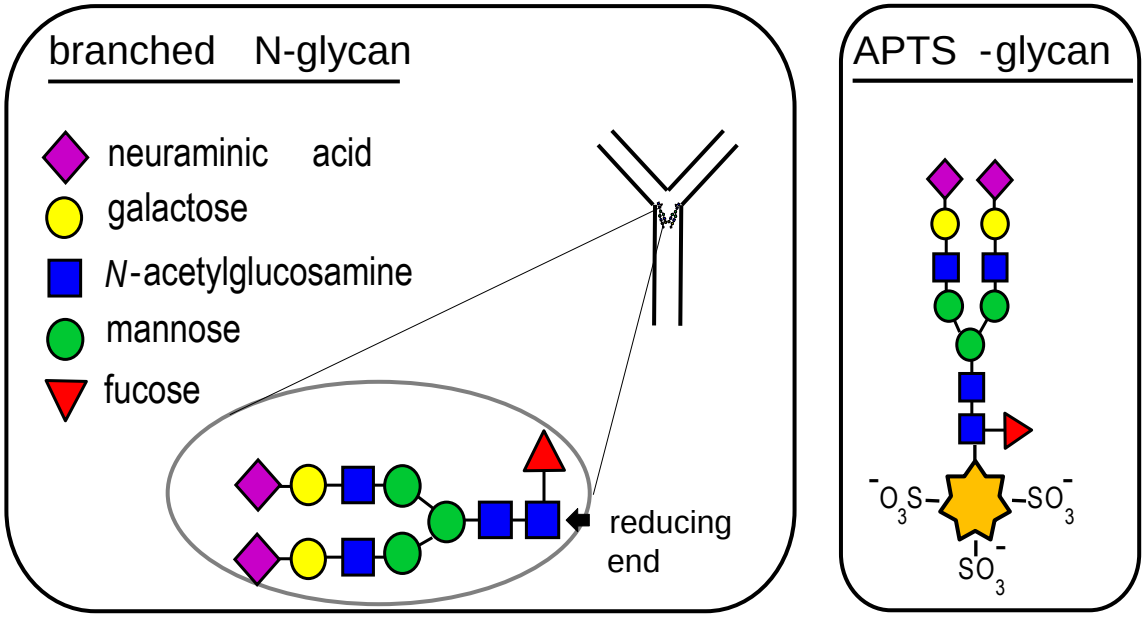
- sialic acid
- galactose
- *N*-acetylglucosamine
- mannose
- fucose



Features Monitored



Analysis of Antibody Glycosylation

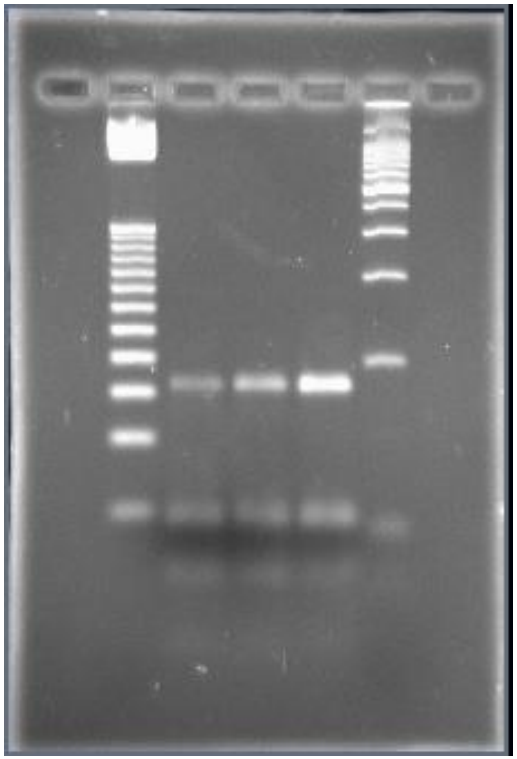


Unique Topics to be Addressed

What is a Nanogel and how does it work?

What makes capillary electrophoresis ideal for reaction-based analyses

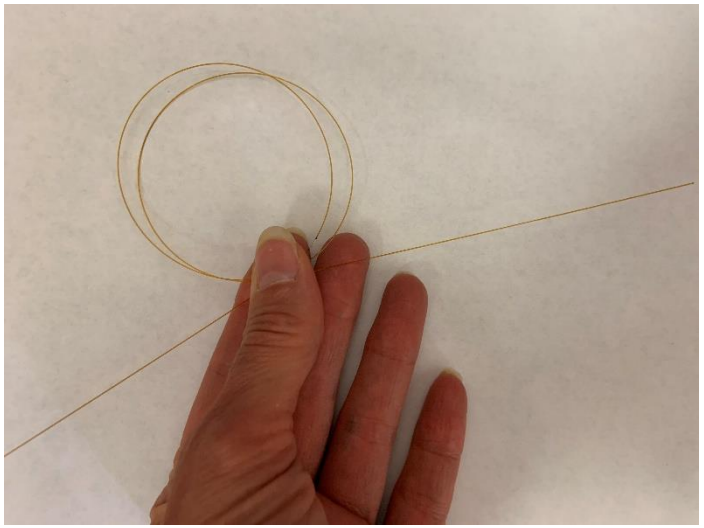
Electrophoretic separations transitioned from slab gel (1959) to capillary gel electrophoresis (1984).

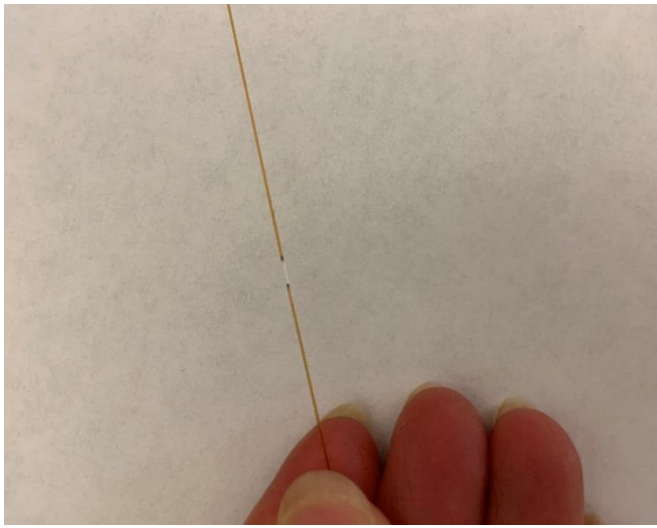


Cross-linked gels in a slab replaced with aqueous solutions or even linear gels in a capillary

+higher voltages

+improved heat dissipation





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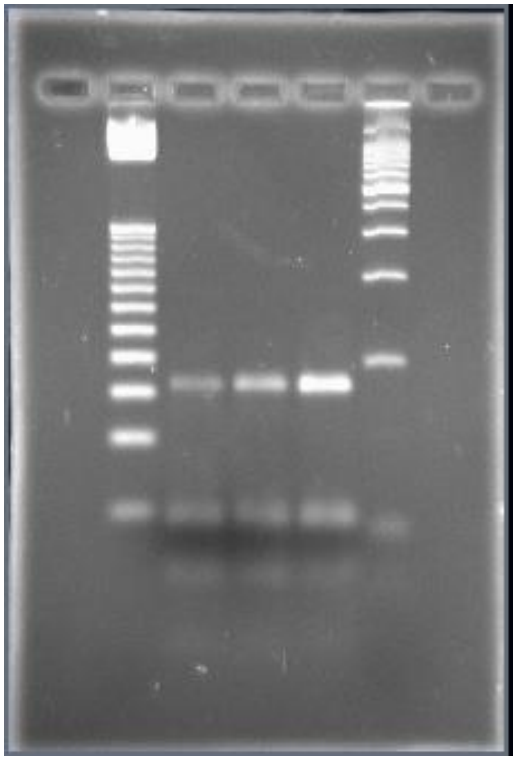

Cross-linked gels in a slab replaced with aqueous solutions or even linear gels in a capillary

+higher voltages

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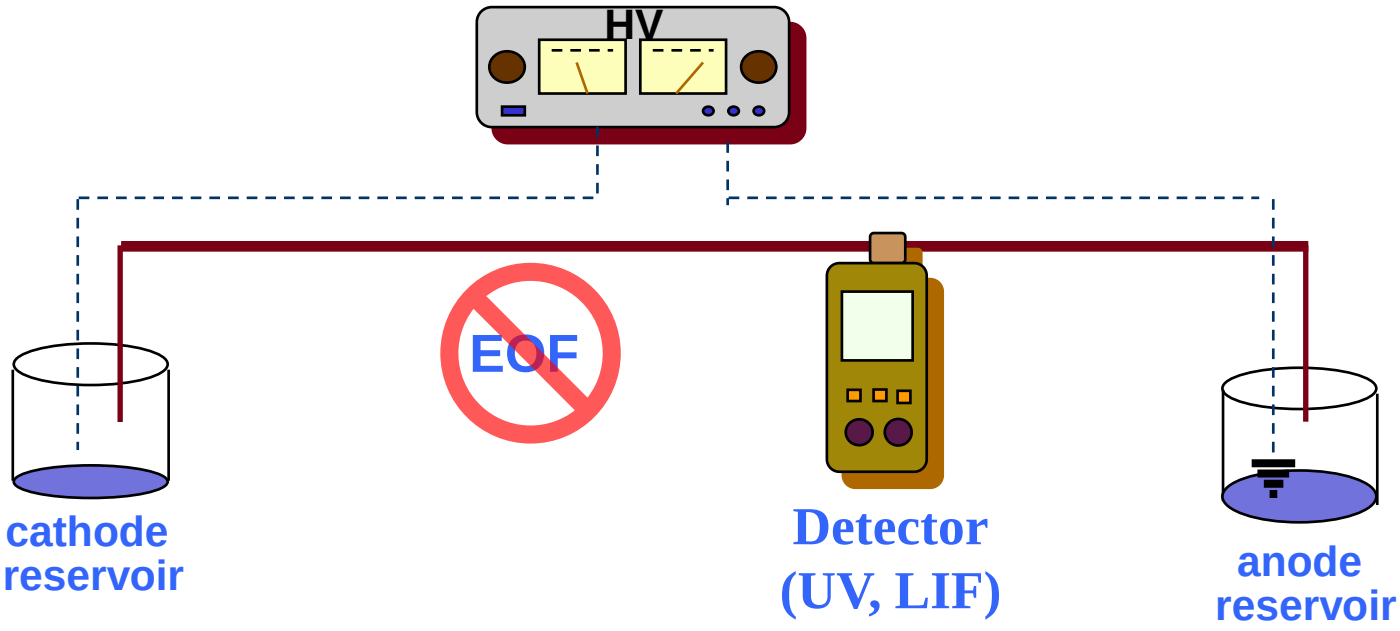
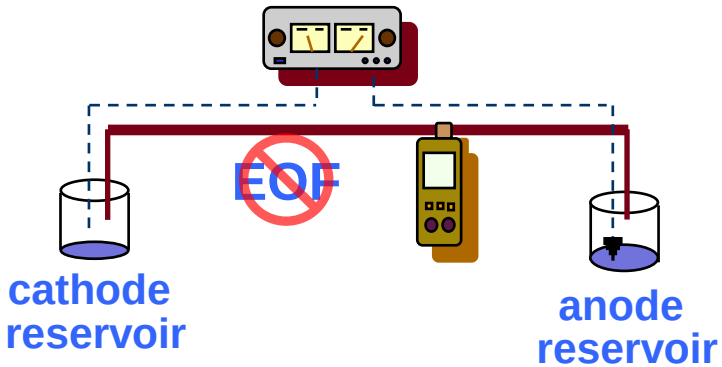
Nanogel Electrophoresis (no EOF)

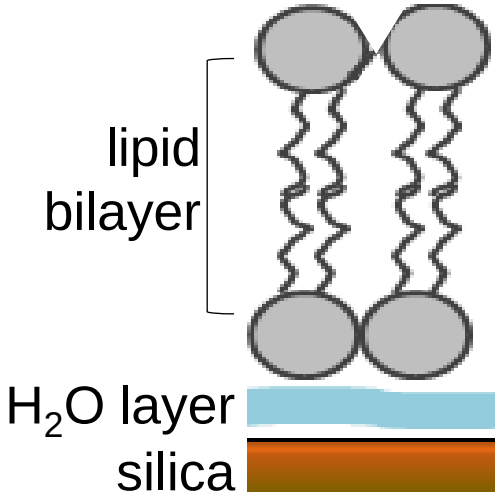


Cunliffe, J.M., N.E. Baryla, and C.A. Lucy, Phospholipid Bilayer Coatings for the Separation of Proteins in Capillary Electrophoresis. *Anal. Chem*., **2002**. 74: p. 776-783.

White, C.M., R. Luo, S.A. Archer-Hartmann, and L.A. Holland, Electrophoretic Screening of Ligands under Suppressed EOF with an Inert Phospholipid Coating. Electrophoresis, 2007. 28(17): p. 3049-55.

Nanogel Electrophoresis (no EOF)

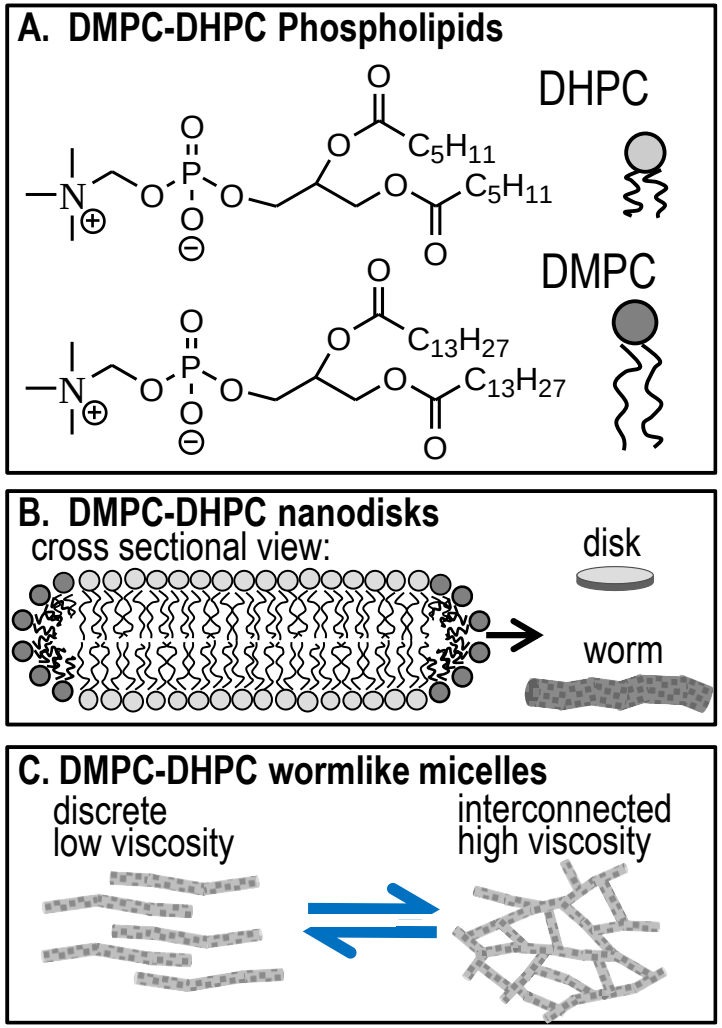




- composed of self assembled lipids
- semi-permanent
- zwitterionic
- can be further modified
- surface is compatible with nanogel
- surface is biocompatible

Multifunctional Nanogels

Fluid Steering, Sequencing and Separations



Phospholipids

5 nm nanodisks

5 nm nanoribbons

Morphology⇨Viscosity

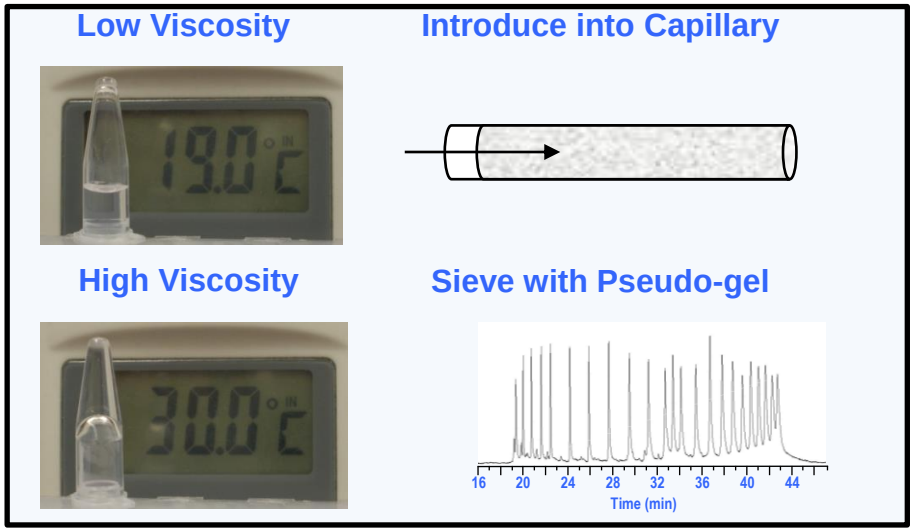
Thermally Responsive

Temperature Affects viscosity

Liquid at 24 °C

Gel at 26-31°C

Thermo-responsive Nanogels are Easily Introduced and Expelled



Temperature Affects Viscosity

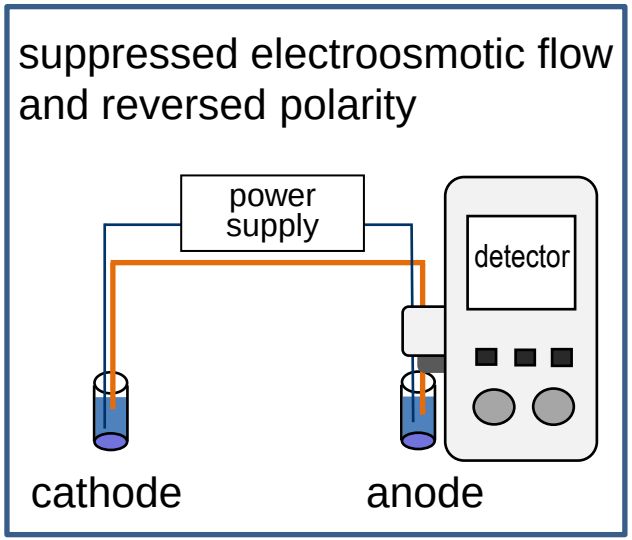
Liquid at 24 °C
Gel at 26-31°C

Fill/expel “cold”

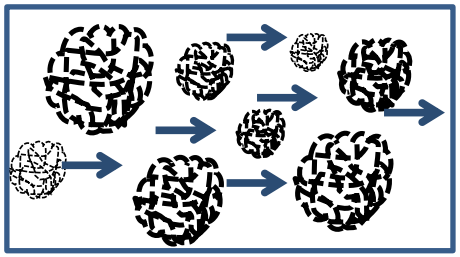
Lock “warm”

B.C. Durney, J.A. Lounsbury, B.L. Poe, J.P. Landers, L.A. Holland, **Thermally Responsive Phospholipid Pseudo-gel: Tunable DNA Sieving with Capillary Electrophoresis** *Analytical Chemistry*

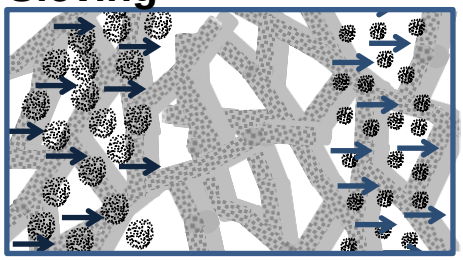
Capillary Electrophoresis Separations of Proteins



Electrophoretic mobility



Sieving

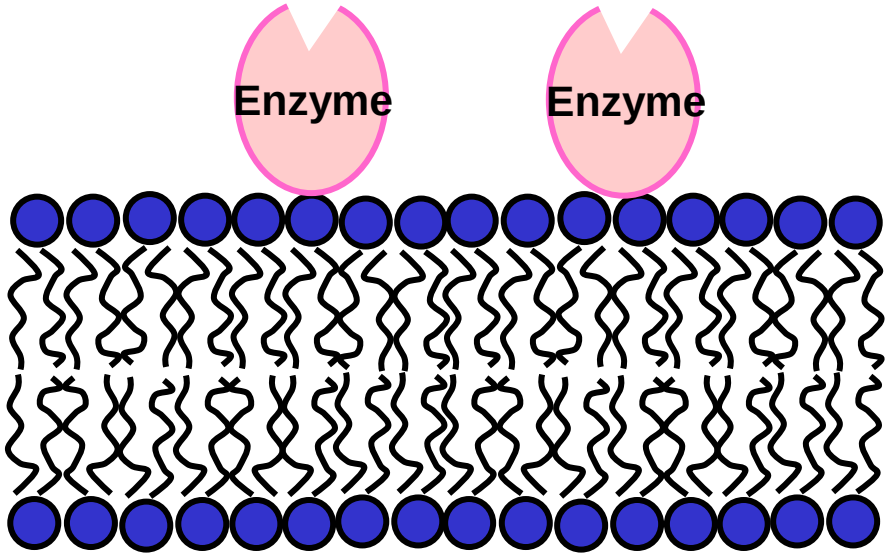


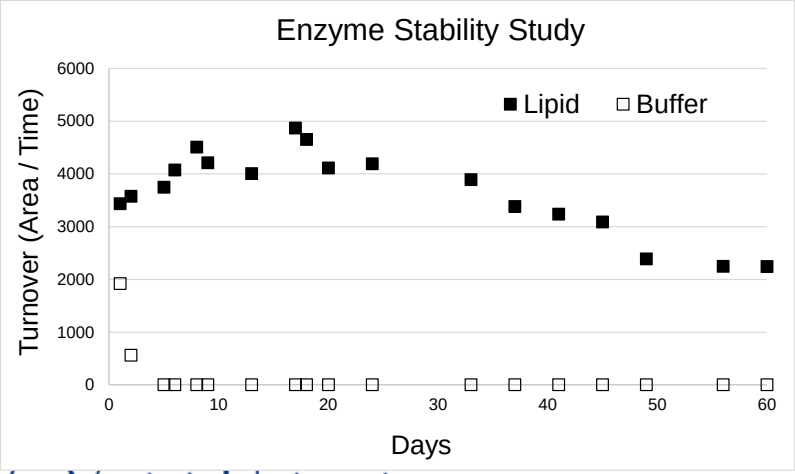
	0% nanogel	vs	**25% nanogel**
Peptide: REDV (0.57 kDa), pI 4.4,	0.000150 $cm^2V^{-1}s^{-1}$		0.000150 $cm^2V^{-1}s^{-1}$
Alpha-1-antitrypsin (52 kDa), pI 4.6	0.000110 $cm^2V^{-1}s^{-1}$		0.000071 $cm^2V^{-1}s^{-1}$

Crihfield, C. L.; Holland, L. A. **Protein Sieving with Capillary Nanogel Electrophoresis**. submitted.

B.C. Durney, C.L. Crihfield, L.A. Holland, **Capillary electrophoresis applied to DNA: Determining and harnessing sequence and structure to advance bioanalyses (2009–2014),** Anal. Bioanal. Chem., 407 (**2015**) 6923-6938.

Neuraminidase Activity/Stability in Lipid





- **Pseudo-immobilization**
- **Non-covalent**
- **Turnover double**
- **Activity > 20x**
- **Lifetime extended months**

Gattu, S., C.L. Crihfield, and L.A. Holland, **Microscale Measurements of Michaelis–Menten Constants of Neuraminidase with Nanogel Capillary Electrophoresis for the Determination of the Sialic Acid Linkage**. *Analytical Chemistry*, 2017. 89(1): p. 929-936.

Two Goals for Integrated Enzyme Analyses

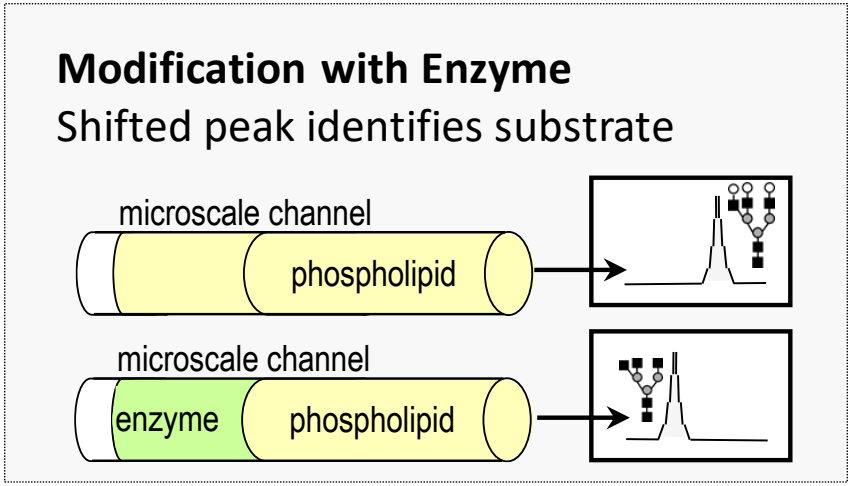
Enzyme Assay: Capillary Electrophoresis is used to quantify enzyme activity and to screen and even quantify the ability of molecules to inhibit the enzyme

Innovation: nanogels pattern nanoliter reactions for automated, rapid, and cost-effective screening

Microscale Sequencing: Enzymes are integrated into a capillary to determine glycan (a PTM), sequence and linkage in capillary electrophoresis run

Innovation: enzyme sequencing provides cost-effective identification with no standards or MS

Enzyme Shift Assays are Compatible with Capillary Nanogel Electrophoresis



- Nanoliter volumes of enzyme/lectin (↓cost)
- Pattern in capillary (enables processing)
- Retains enzyme/lectin activity (↓cost/repeatability)
- Replace each run (eliminate carryover)

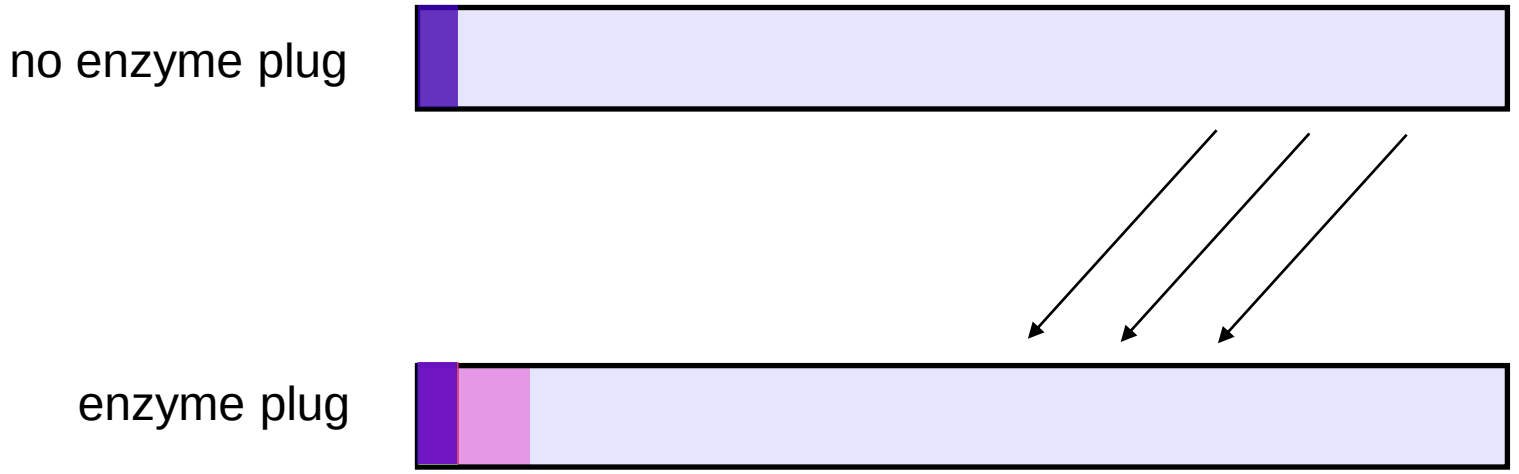
Lu, G., C.L. Crihfield, S. Gattu, L.M. Veltri, and L.A. Holland, Capillary Electrophoresis Separations of Glycans. *Chemical Reviews*, **2018**. 118(17): p. 7867-7885.

Unique Topics to be Addressed

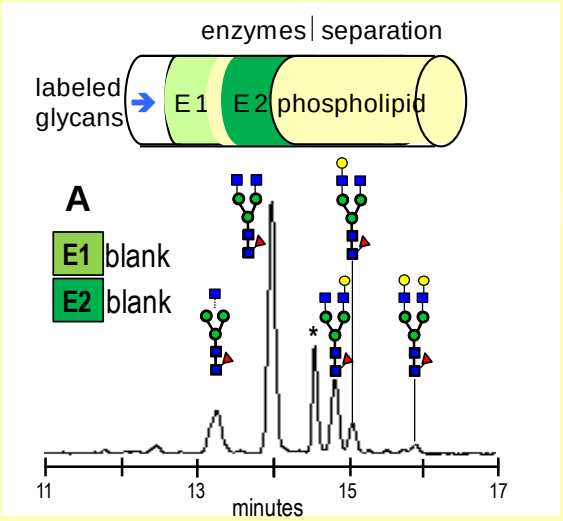
What makes capillary electrophoresis ideal for integrated enzymes and reaction-based analyses?

Enzyme Processing with Nanogels

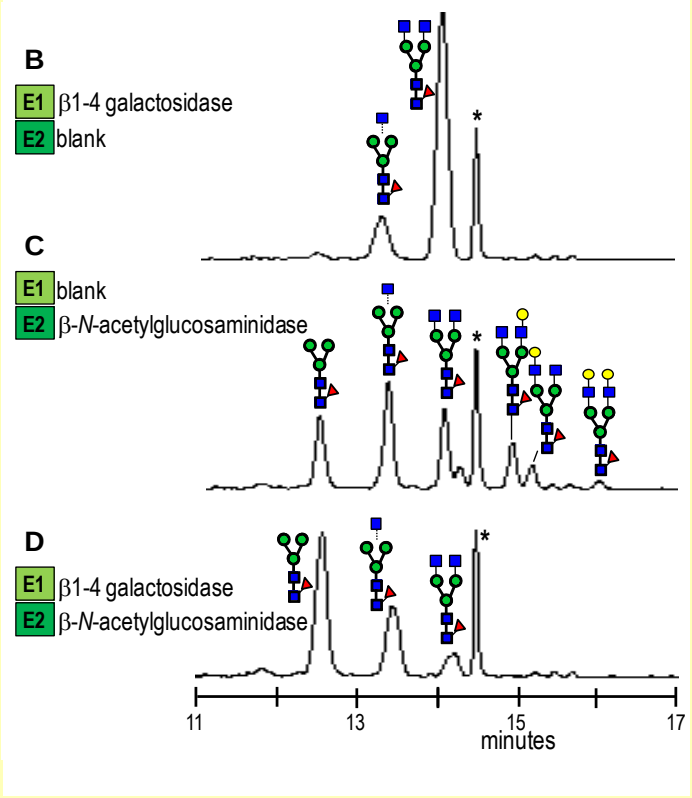
shift in migration time
identifies terminal monomer



Modification with Enzyme Microscale Sequencing



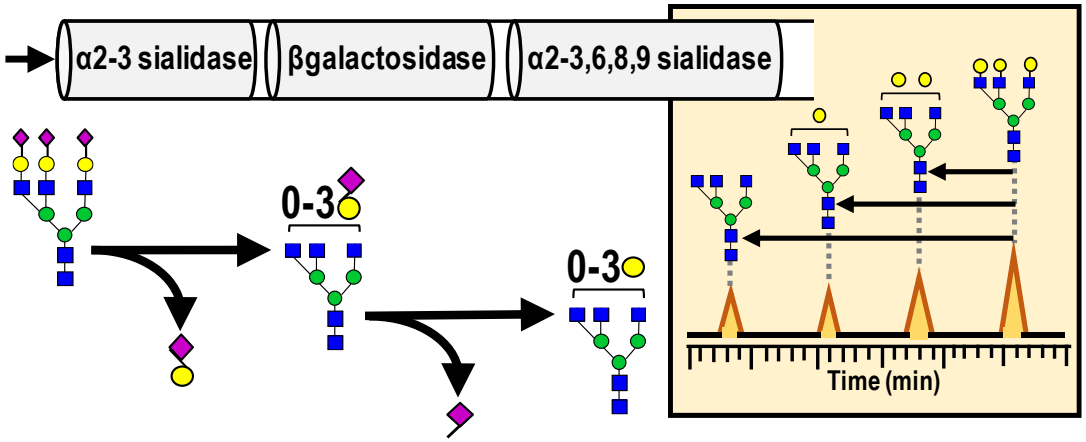
The integration of enzymes in series in a capillary for the hydrolysis and separation of glycans from Trastuzumab.



- Automated process
- nL zones of enzyme
- Pattern in capillary
- Monitoring shifts
- Replace each run

SA Archer-Hartmann, CL Crihfield, LA Holland, **On-Line Enzymatic Sequencing of Glycans from Trastuzumab by Phospholipid Assisted Capillary Electrophoresis**, *Electrophoresis* **2011.**

Nanogel Multi-Enzyme Processing to Determine Sialic Acid Linkage

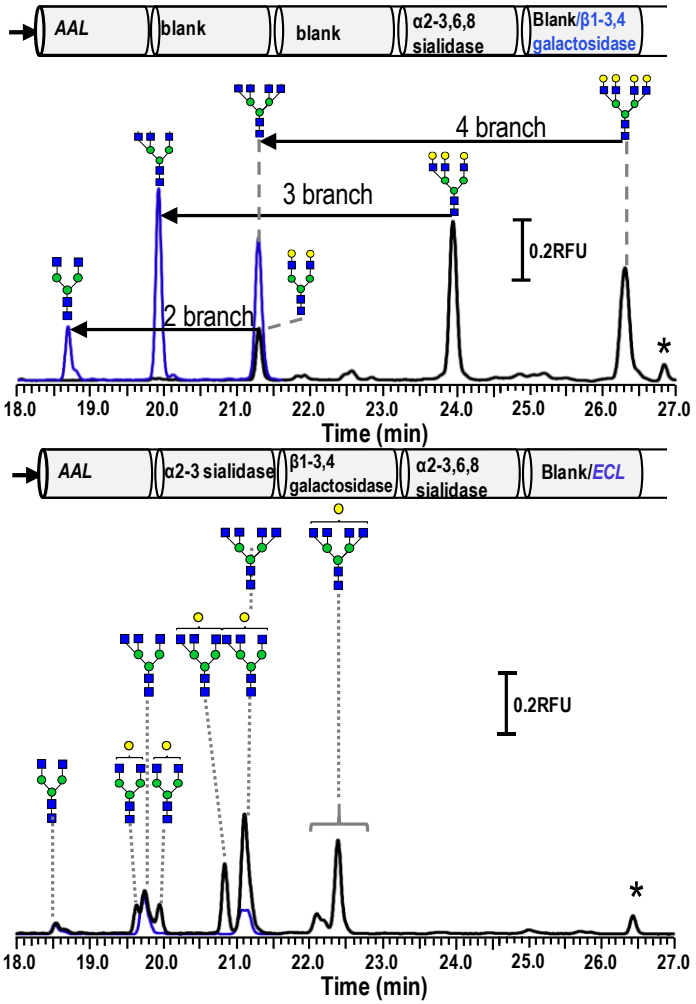


%α2-6sialic acid (◆) = %galactose (○)

- Subtle differences in Sialic acids
- Sialic acids anionic
- Co-cleavage converts 2-3 linkage into an exposed GlcNac
- 2-6 linkage correlates with Gal

Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A. **Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis.** Analytical Chemistry 2020, 92, 1518-1524.

Nanogel Multi-Enzyme Processing to Determine Sialic Acid Linkage



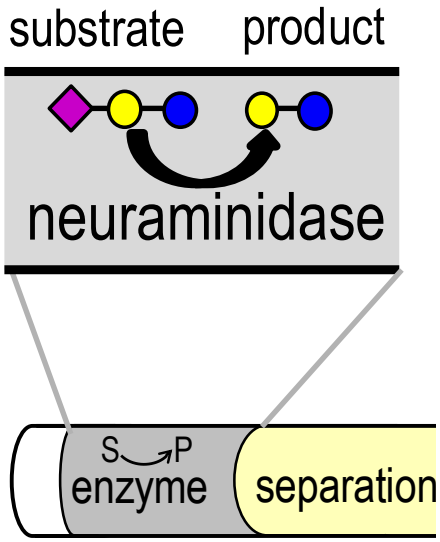
- Human AGP (biomarker)
- Sialic Acid Linkage
- 5 zones of enzymes and lectins (nL)
- No Standards

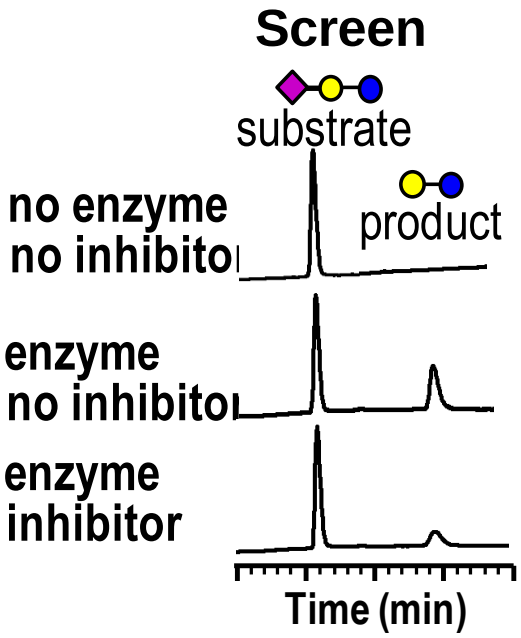
Table 1. Peak area of α2-6 linked sialic from AGP glycans

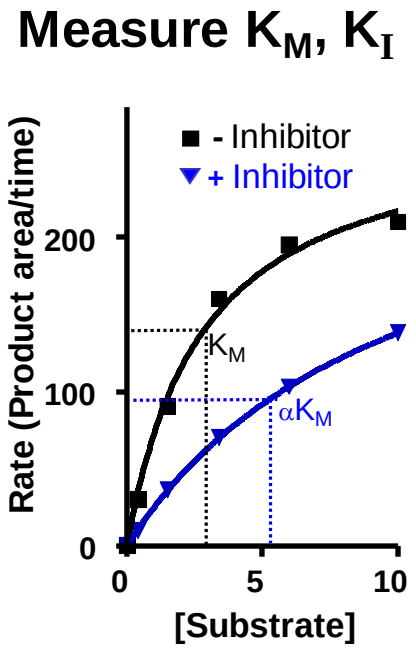
	α2-6 linked sialic acid (◆) area x 10^3 (%RSD)					
Branch	**0 ◆**	**1 ◆**	**2 ◆**	**3 ◆**	**4 ◆**	**Total**
	25(10)	89(7)	0	—	—	**114(12)**
	104(5)	336(8)	0	0	—	**440(9)**
	94(7)	265(3)	0	0	0	**359(8)**

Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A. **Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis.** Analytical Chemistry 2020, 92, 1518-1524.

Nanogel Enzyme Zones Characterize Enzyme Activity

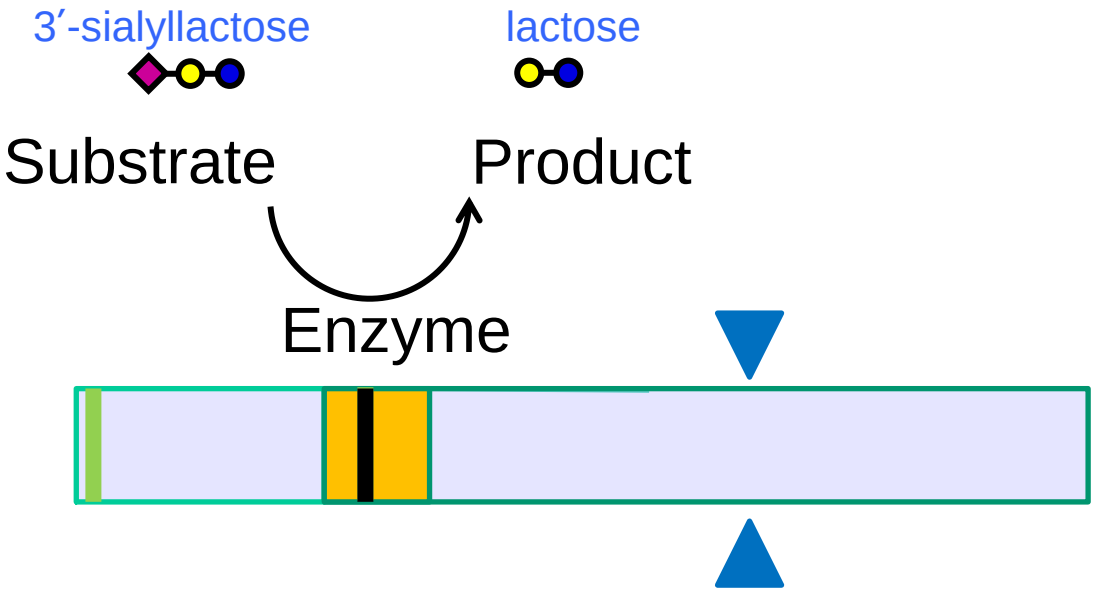


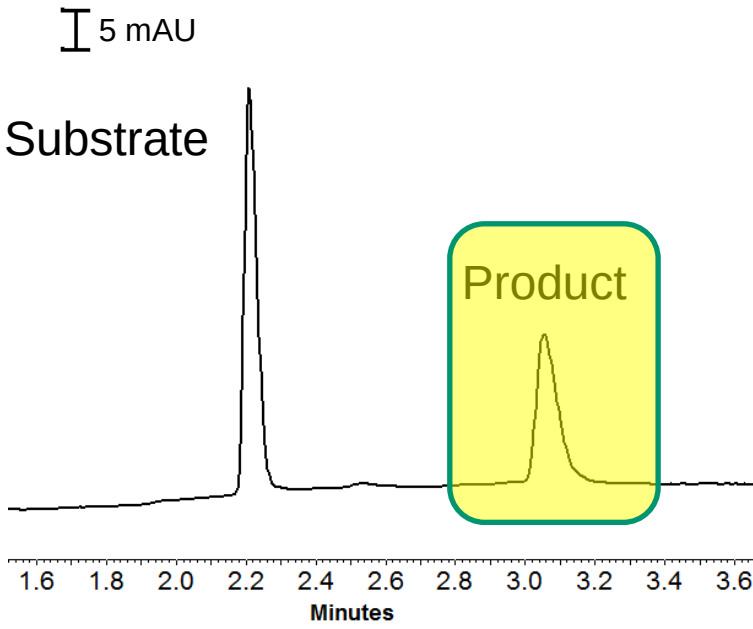




Overview of neuraminidase screening (IC_{50}) and K_I Determinations

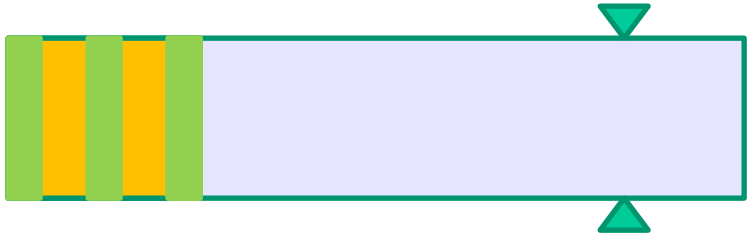
In-capillary Enzyme Processing

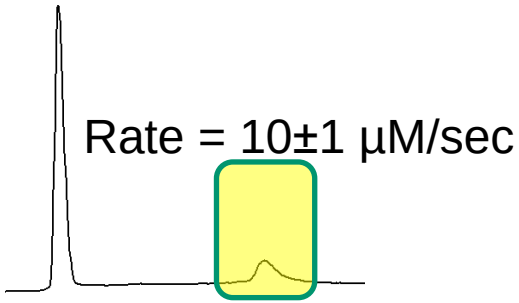




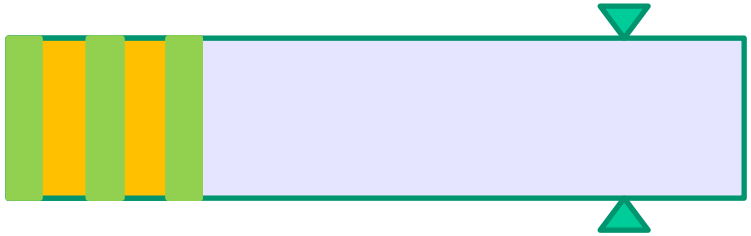
Improved In-capillary Mixing

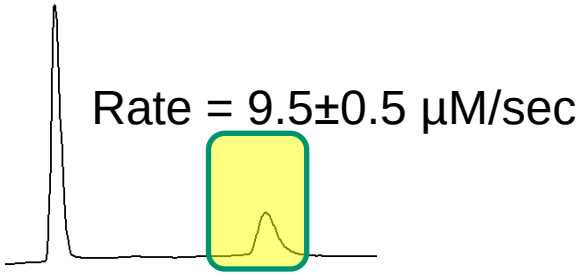
Incubate
40s (3 passes)



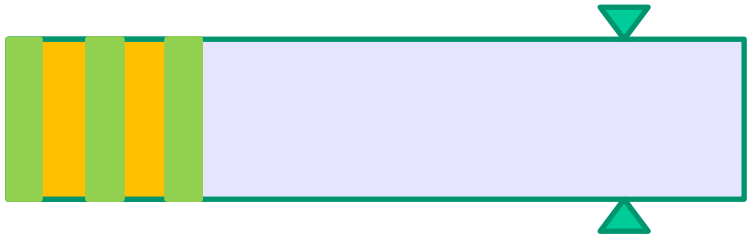


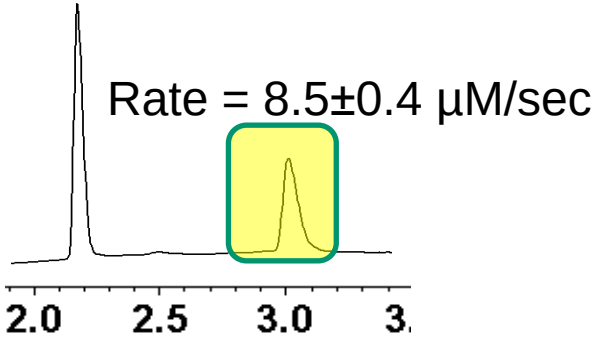
Incubate
60s (5 passes)





Incubate
100s (7 passes)





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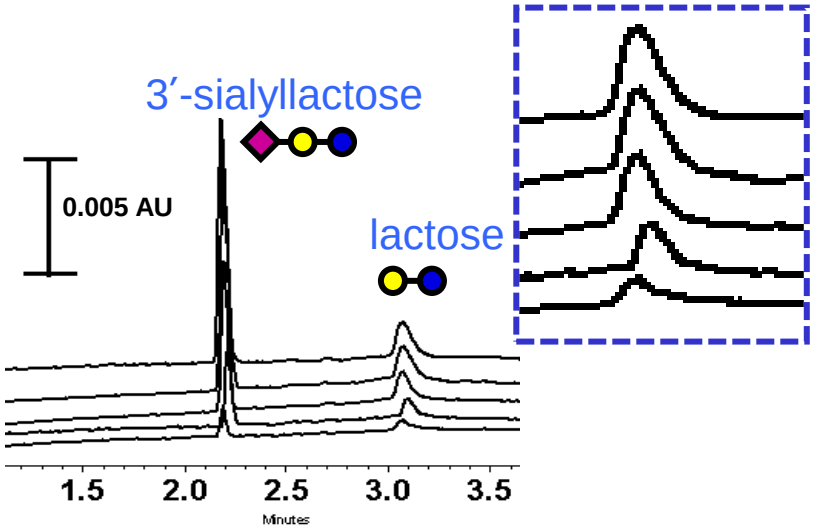
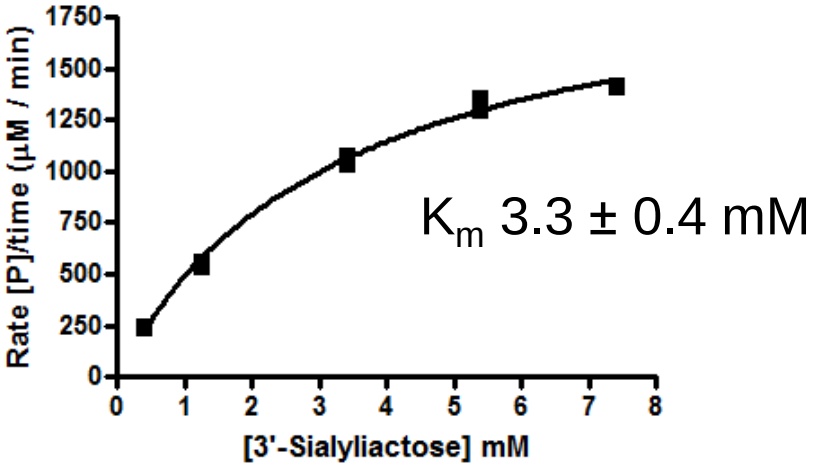
Michaelis-Menten Curve for 3’-Siallylactose

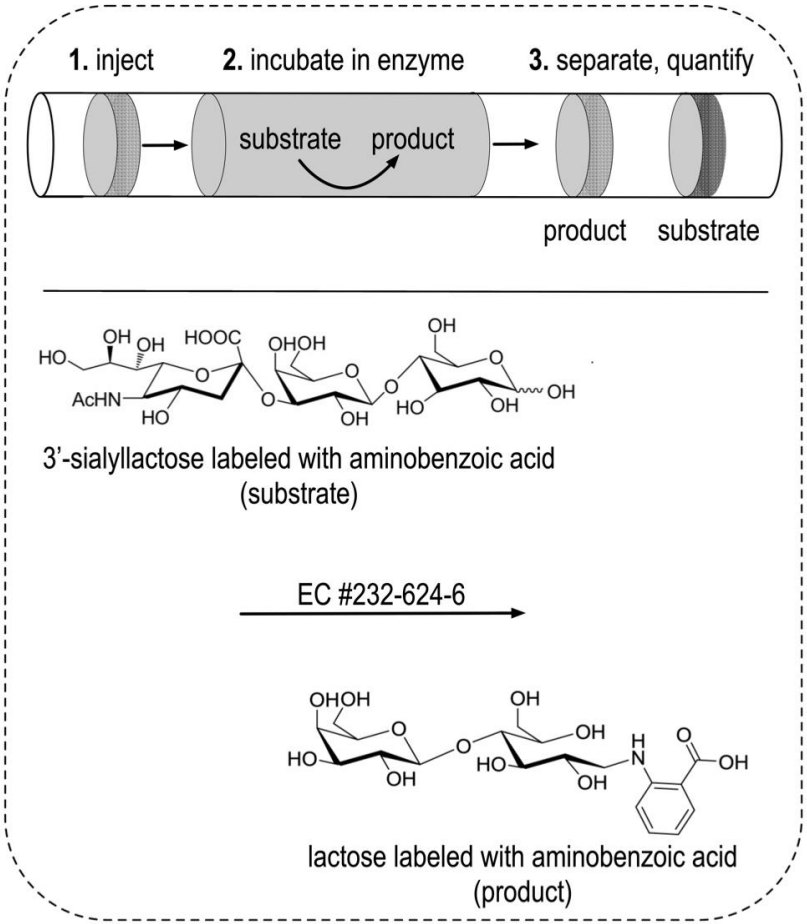




- **[E] is constant**
- **[S] is varied**
- **Velocity = [P]/(incubation time)**

Adapted from Gattu, S., C.L. Crihfield, and L.A. Holland, **Microscale Measurements of Michaelis–Menten Constants of Neuraminidase with Nanogel Capillary Electrophoresis for the Determination of the Sialic Acid Linkage**. *Analytical Chemistry*, 2017. 89(1): p. 929-936.

Enzyme Characterization



2',3',6',8' – Neuraminidase

3'-Siallylactose

K_m = 3.3, V_{max} = 2090 μM/min

6'-Siallylactose

K_m = 2.0, V_{max} = 420 μM/min

3'- Neuraminidase

3'-Siallylactose

K_m = 2.7, V_{max} = 940 μM/min

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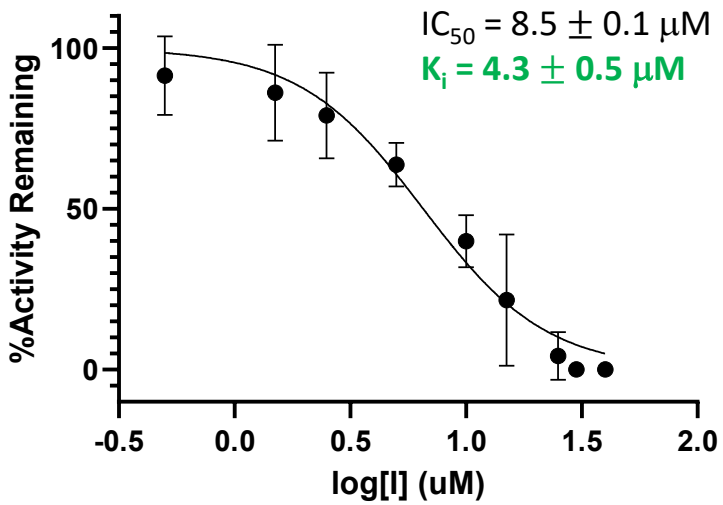
Adapted from Gattu, S., C.L. Crihfield, and L.A. Holland, **Microscale Measurements of Michaelis–Menten Constants of Neuraminidase with Nanogel Capillary Electrophoresis for the Determination of the Sialic Acid Linkage**. *Analytical Chemistry*, 2017. 89(1): p. 929-936.

Quantification of Inhibitor Activity

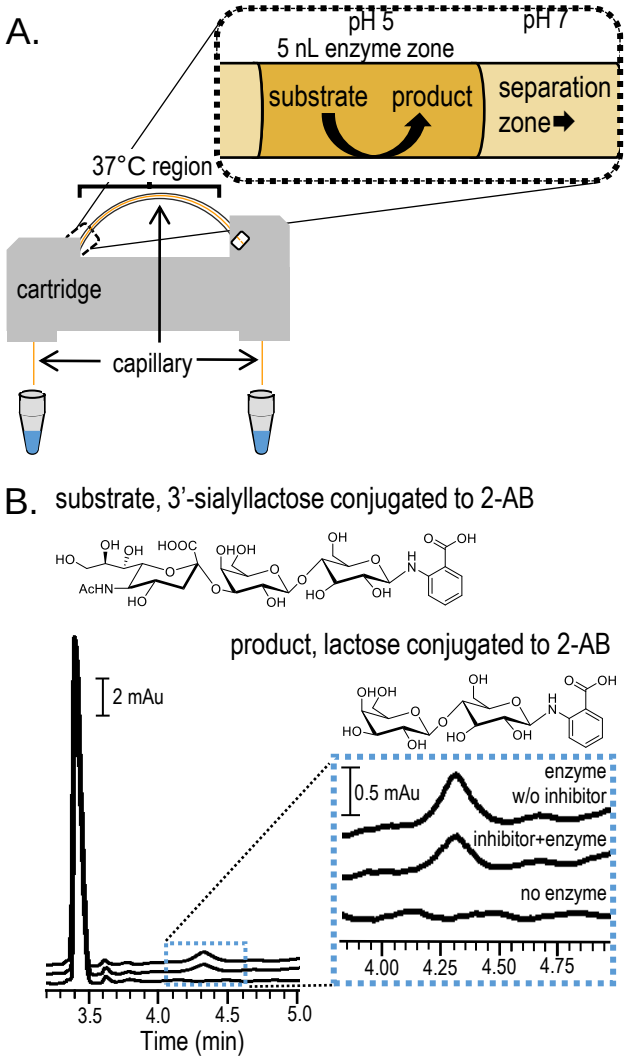
$$K_i = \frac{IC_{50}}{\left(\frac{[S]}{K_m}\right) + 1}$$



- K_I sigmoid (dose-response) curve
- Range of Inhibitor Concentrations Used
- Inhibitor Patterned (40 nL volumes)
- Single enzyme preparation (5 nL consumed per run)
- K_i calculated when K_m known of $[S] << K_m$
- K_I varies with substrate and Inhibitor

Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

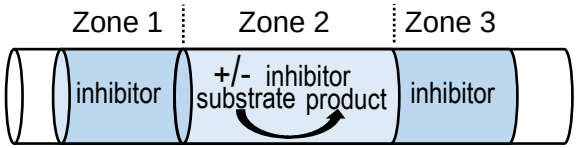
Determination of Inhibitor Activity



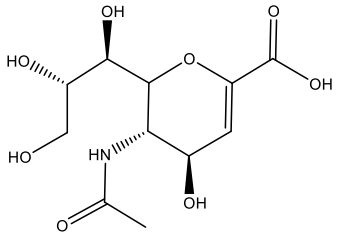
- Rapid determination of enzymatic rates
- Addition of inhibitor enables study of inhibitor activity against enzyme sialic acid cleavage
- CE fast analysis (5 min), small sample, enzyme (5 nL), and inhibitor volumes

Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

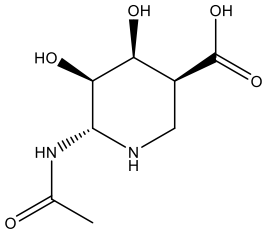
Universal and Programmable Inhibitor Patterning



DANA



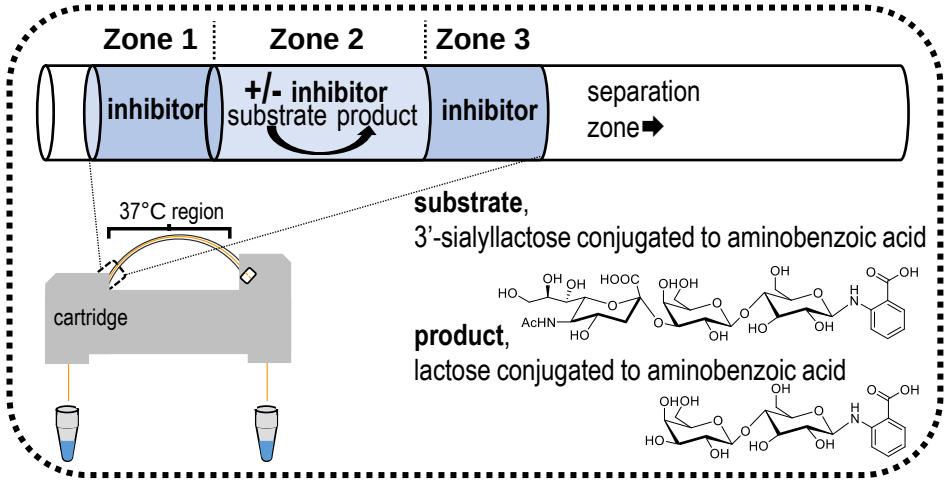
Siastatin B



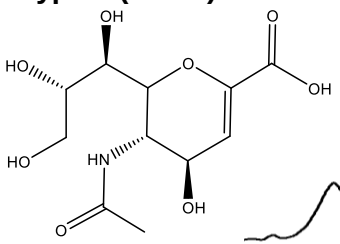
- Universally applicable to different molecular inhibitors
- Adding to enzyme preparation consumes excessive enzyme and requires
- Sandwiching enzyme allows robotics to do sample preparation
- Realize full advantage of automation

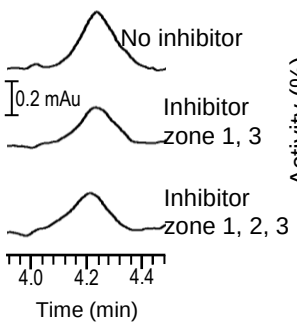
Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

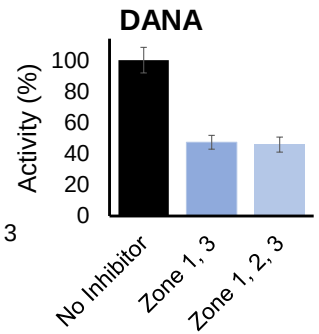
Inhibitor (DANA) and enzyme are mixed in capillary



Inhibitor type 1 (anion)
DANA



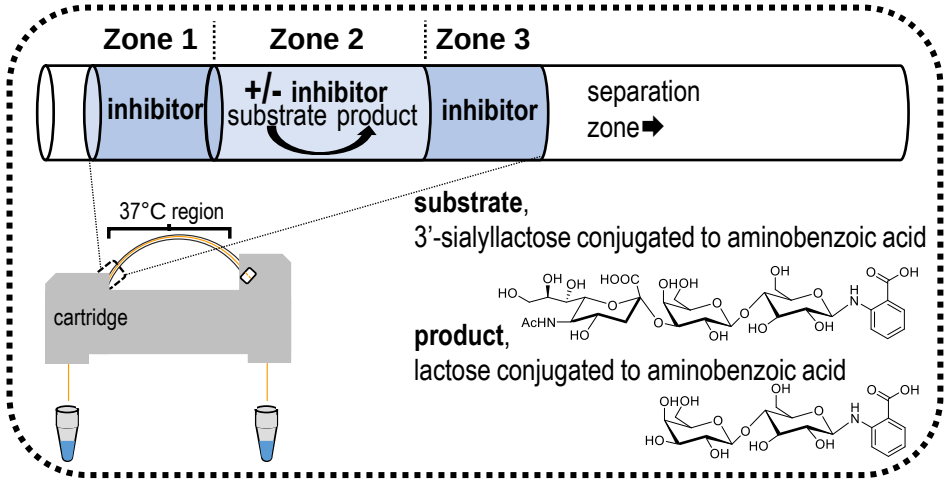




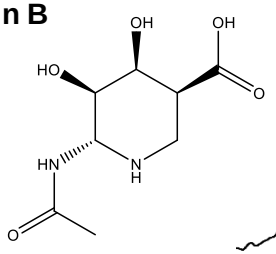
- 3.3 mM sialyllactose
- DANA-transition state analog
- Pattern capillary 1-cm enzyme zone
- Sandwich between inhibitor zones (7-cm/1-cm)
- No statistical difference if in all 3 zones, versus absent from enzyme

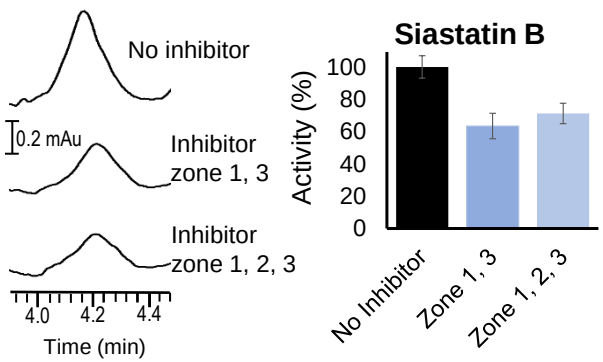
Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

Inhibitor (Siastatin B) and enzyme are mixed in capillary



Inhibitor type 2 zwitterion
Siastatin B

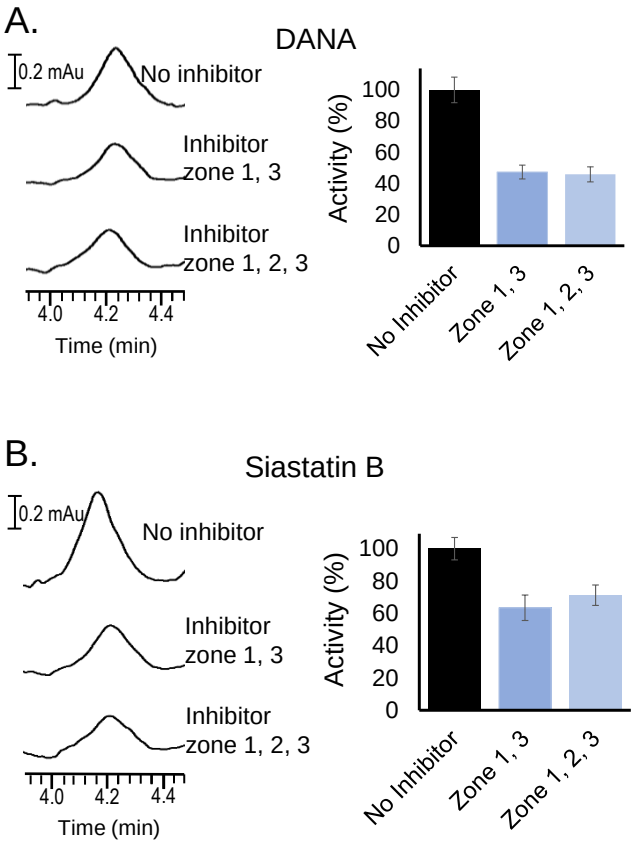




- 3.3 mM sialyllactose
- Siastatin B
- Pattern capillary 1-cm enzyme zone
- Sandwich between inhibitor zones (7-cm/1-cm)
- No statistical difference if in all 3 zones, versus absent from enzyme

Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

Determination of Inhibitor Activity



- Compare conversion with and without bracketing
- No statistical difference between zones 1,2,3 and 1,3
- Equally applicable to neutral and cationic inhibitors

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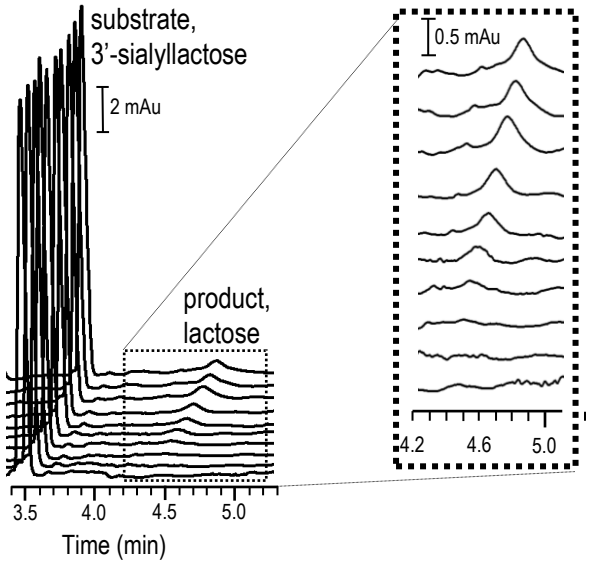
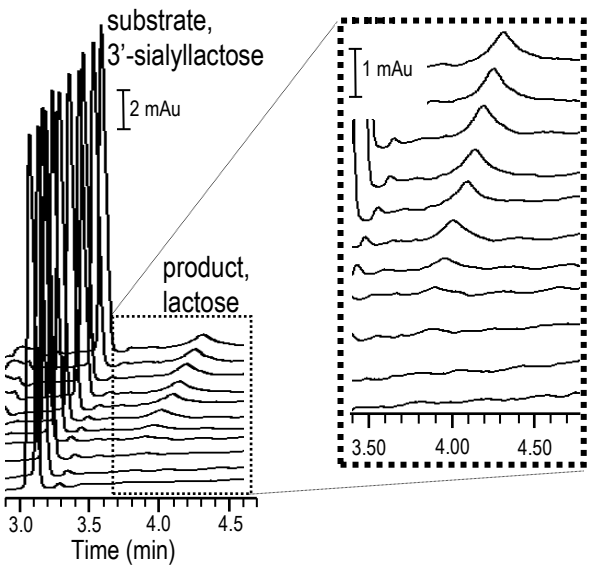
Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

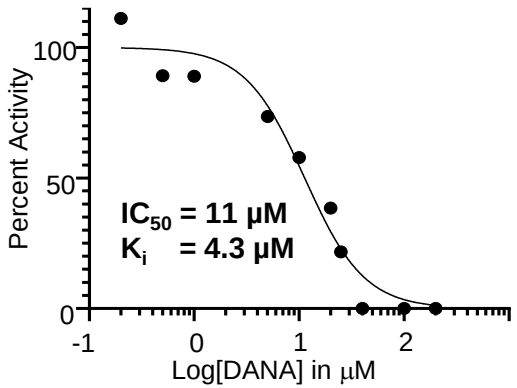
Determination of Inhibitor Activity

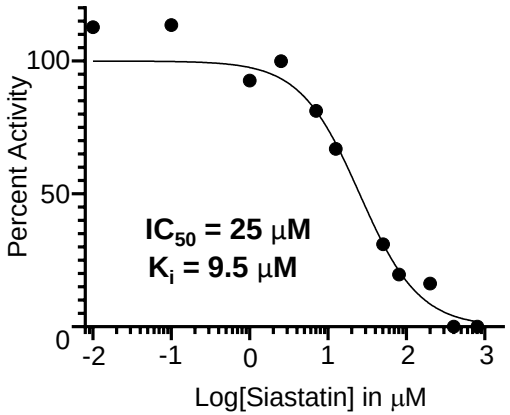
DANA



Siastatin B







- Run in series reagent patterning and conversion are programmed
- Each inhibition measurement is normalized against no inhibitor run (% remaining)
- Normalization every third run (10-15 runs total)

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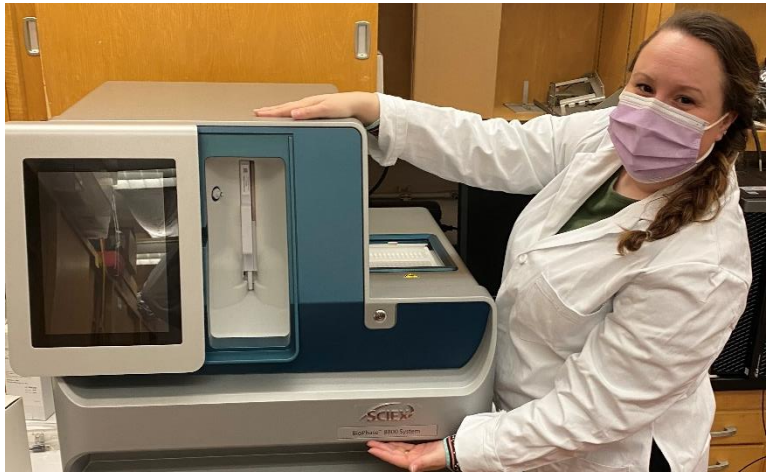
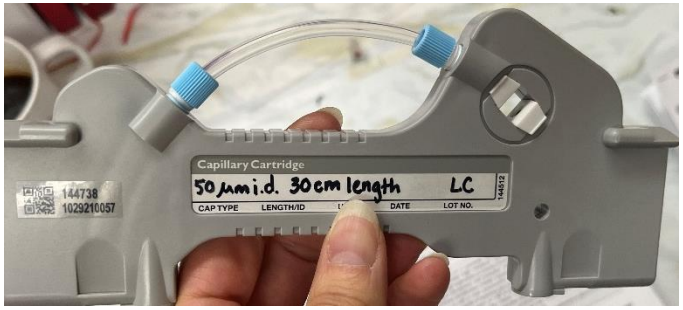
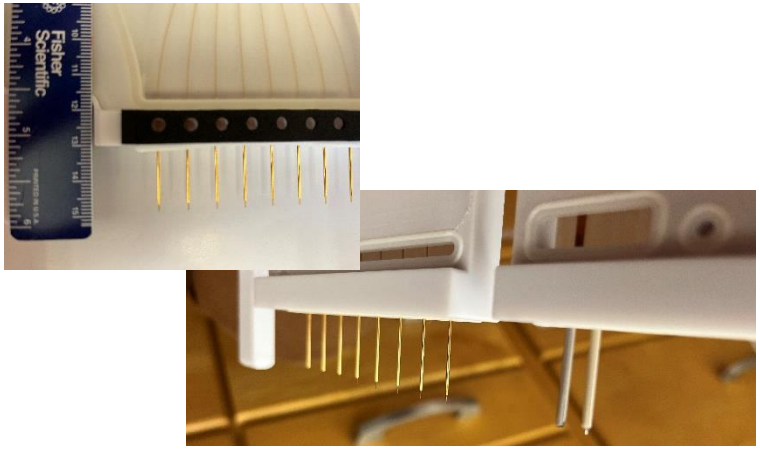
Casto-Boggess, L.D., L.A. Holland, P.A. Lawer-Yolar, J.A. Lucas, and J.R. Guerrette, *Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Analytical Chemistry, 2022.*

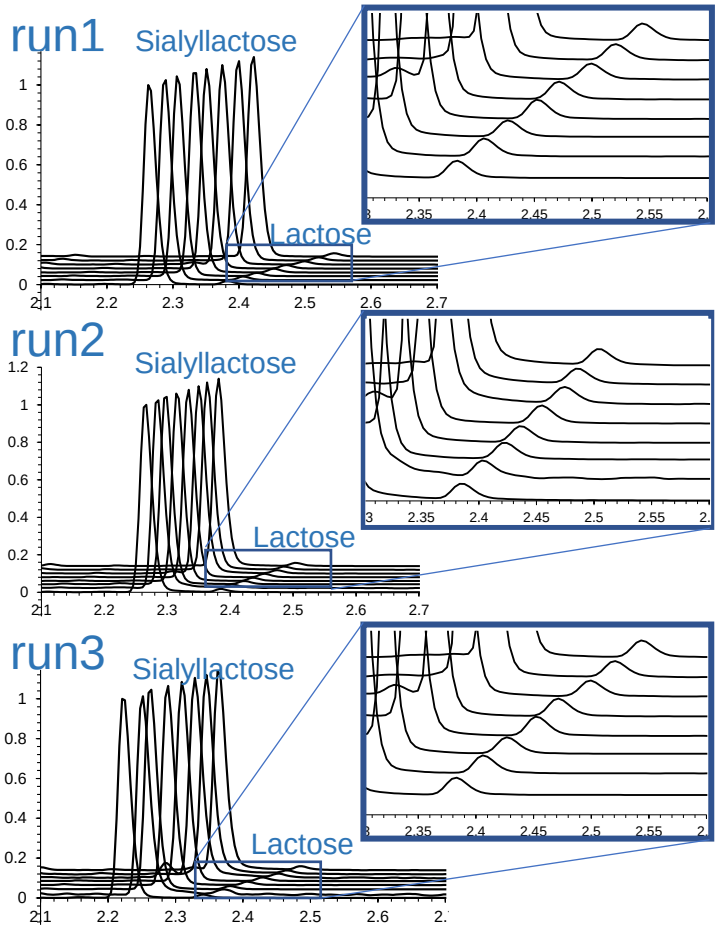
Higher Throughput is Possible





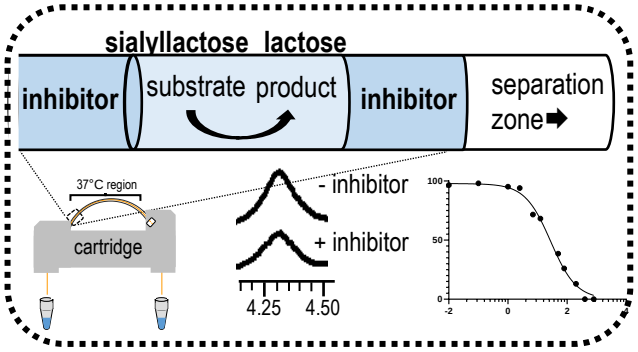


Higher Throughput is Possible

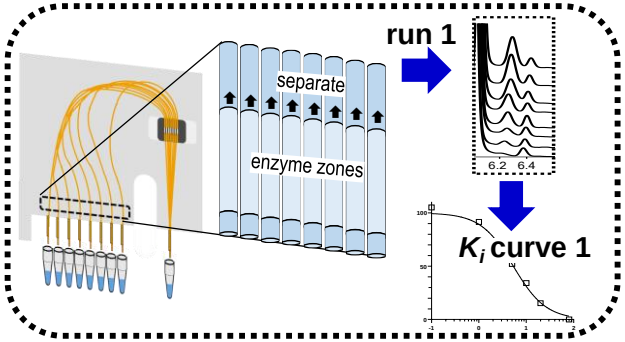


- 3 runs, 24 separations of sialyllactose, lactose
- migration time 1%RSD, 2.26 ± 0.03 and 2.38 ± 0.03
- normalized area (55 ± 6) 10% RSD

Determination of Inhibitor Activity
Series vs Parallel



- Run in series reagent patterning and conversion are programmed
- Each inhibition measurement is normalized against no inhibitor run (% remaining)
- Normalization every third run (10-15 runs total)



- Run in parallel reagent patterning and conversion are programmed but are simultaneous
- Each inhibition measurement is normalized against a single no inhibitor run (% remaining)
- Curves are achieved in a single run of 8 parallel electrophoresis separations

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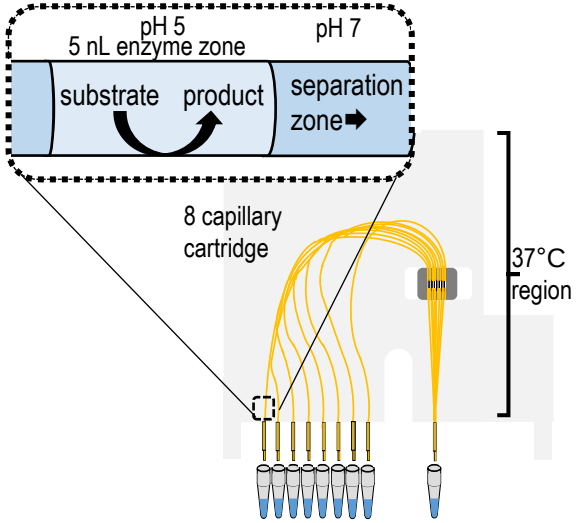
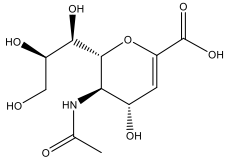
Casto-Boggess, L.D., Holland, L.A. *submitted*

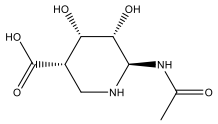
Determination of Inhibitor Activity

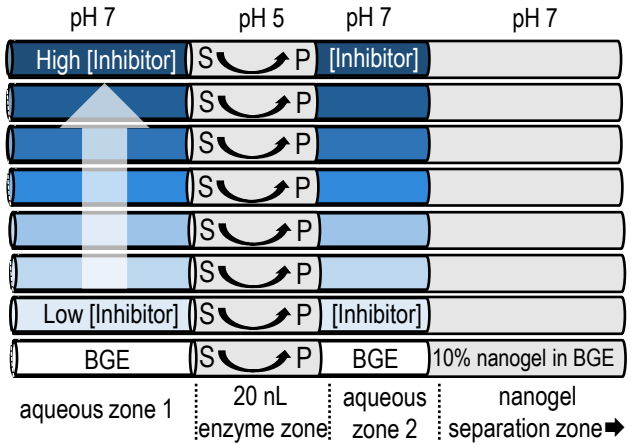


DANA
Anionic Inhibitor



Siastatin B
Neutral Inhibitor





- Run in parallel reagent patterning and conversion are programmed but are simultaneous
- Each inhibition measurement is normalized against a single no inhibitor run (% remaining)
- Curves are achieved in a single run of 8 parallel electrophoresis separations

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Casto-Boggess, L.D., Holland, L.A. *submitted*

Determination of Inhibitor Activity with Fluorescent Substrates

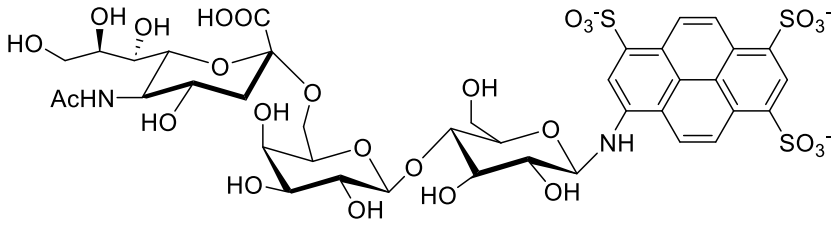
Substrate
6' sialyllactose

EC #232-624-6 →

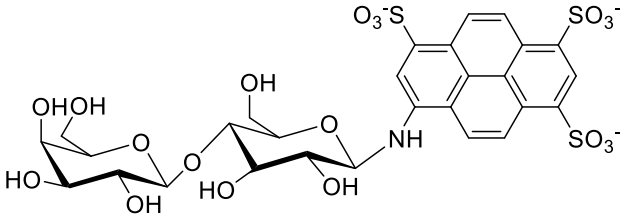
Product
lactose

$$K_i = \frac{IC_{50}}{\left(\frac{[S]}{K_m}\right) + 1}$$

APTS labeled 6' sialyllactose (substrate)



APTS labeled lactose (product)



$K_i = IC_{50}$

- Change label to APTS
- Reduce [Substrate] to nM regime
- K_m no longer required for K_i determinations

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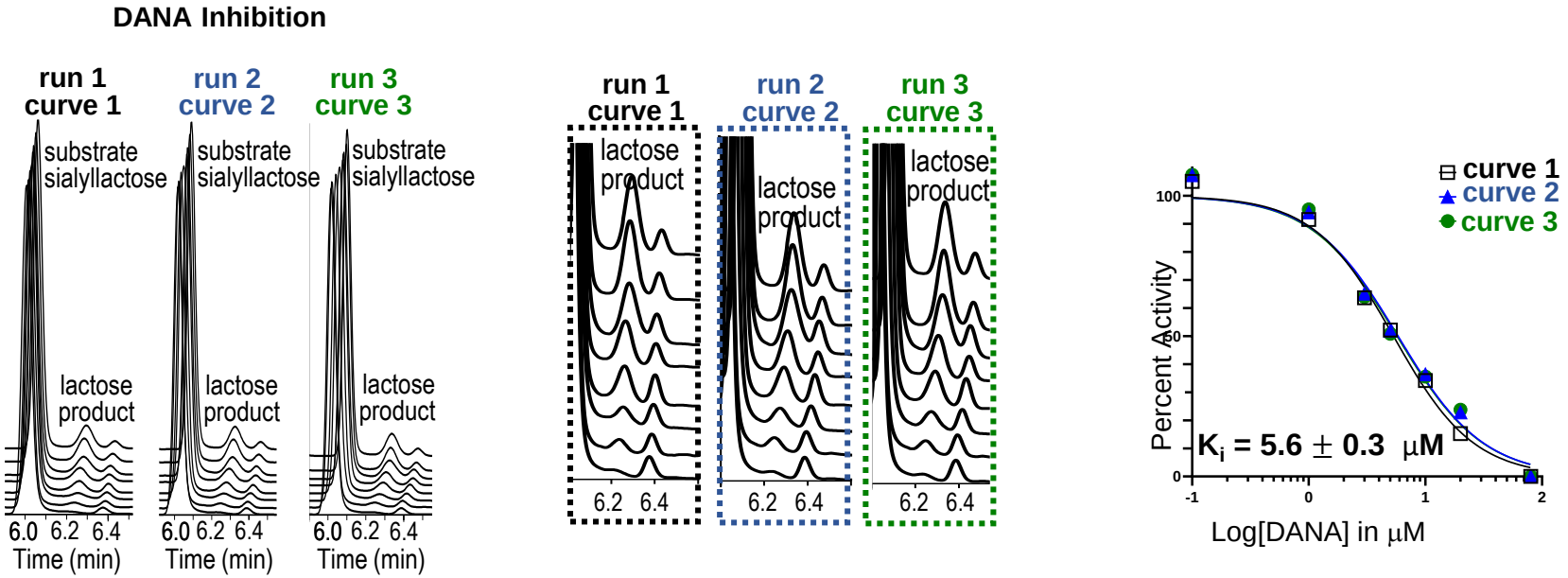
Casto-Boggess, L.D., Holland, L.A. *submitted*

Determination of Inhibitor Activity



- A single run in parallel is performed for a single curve
- K_i values identical to serial analyses

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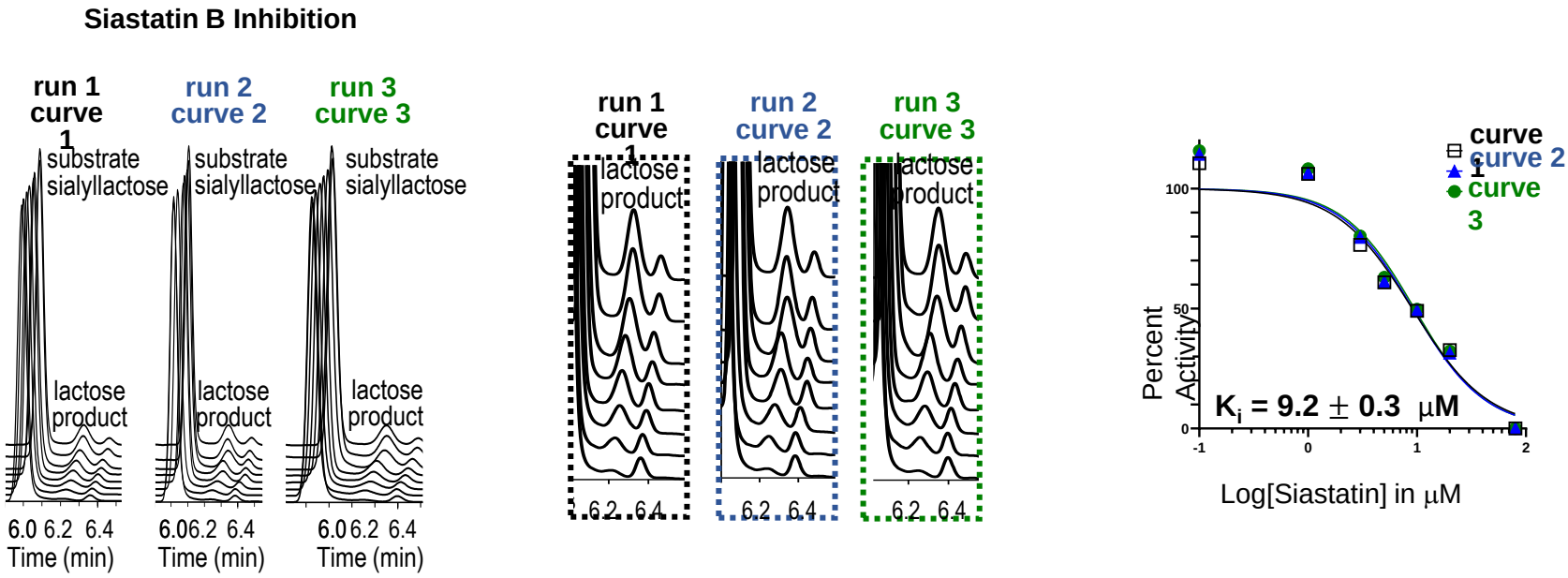
Casto-Boggess, L.D., Holland, L.A. *submitted*

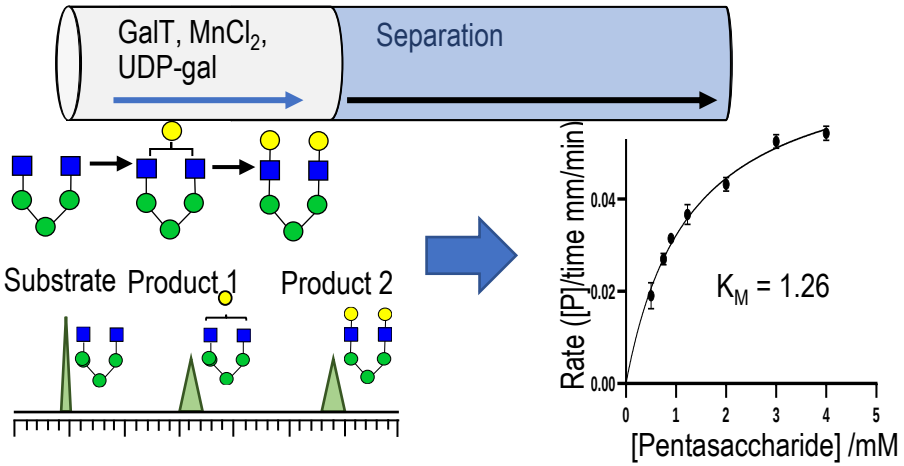
Determination of Inhibitor Activity



- A single run in parallel is performed for a single curve
- K_i values identical to serial analyses

Casto-Boggess, L.D., Holland, L.A. *submitted*

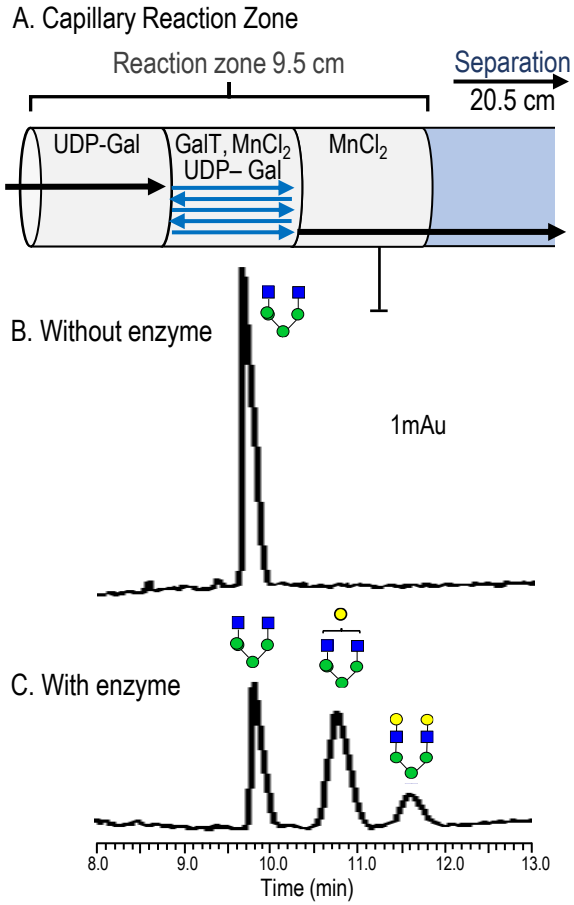
Nanogel Enzyme Processing to Evaluate Enzyme Function



- transferase enzyme
- donor, cofactor, substrate
- activity, preference
- Small volume

Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the B1–4 Galactosyltransferase Michaelis–Menten Constant and Real-Time Addition of Galactose Residues to N-Glycans and Glycoprotein. *Anal. Chem. 2021.*

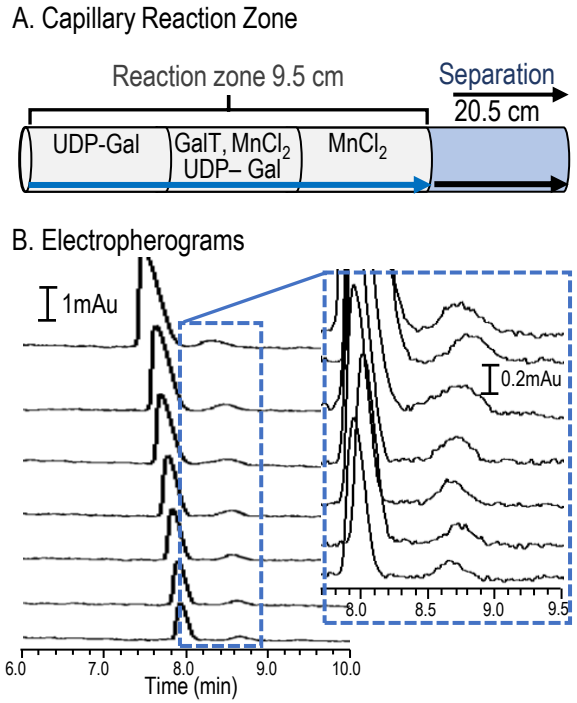
Nanogel Enzyme Processing to Evaluate Enzyme Function

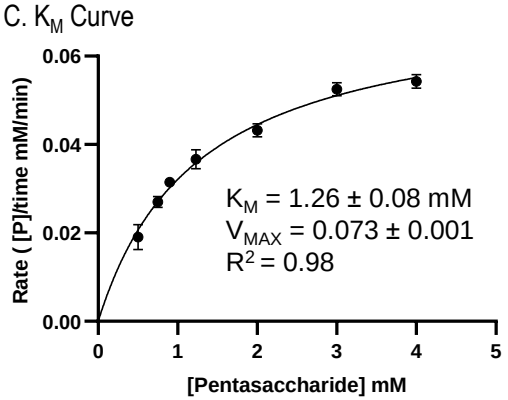


- transferase enzyme
- donor, cofactor, substrate
- Polarity mixing
- 30 min incubation

Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the B1–4 Galactosyltransferase Michaelis–Menten Constant and Real-Time Addition of Galactose Residues to N-Glycans and Glycoprotein. *Anal. Chem. 2021.*

Nanogel Enzyme Processing to Evaluate Enzyme Function

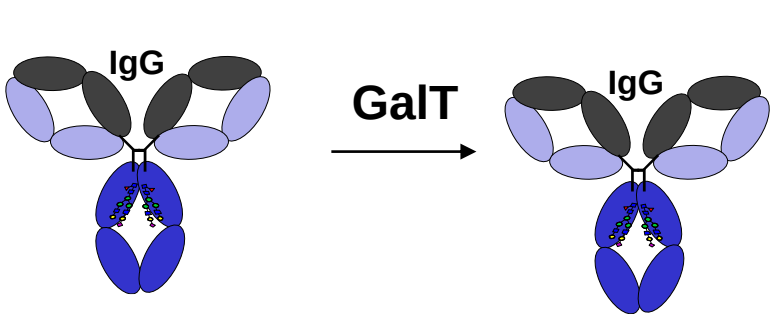


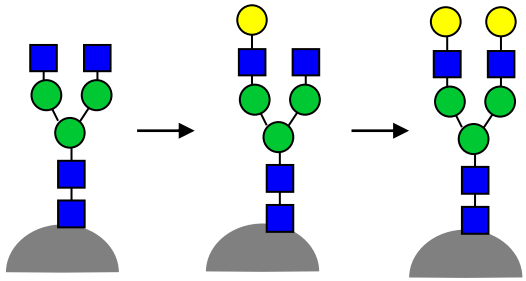


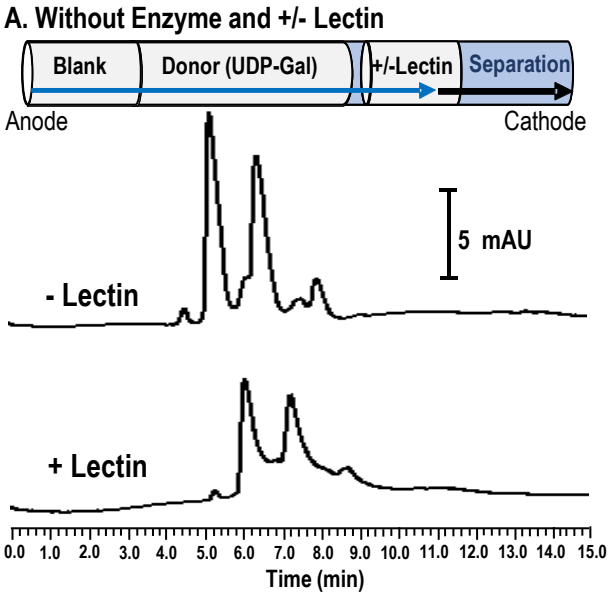
- GalT
- K_m 1.26 mM
- Lit report 1.23 mM

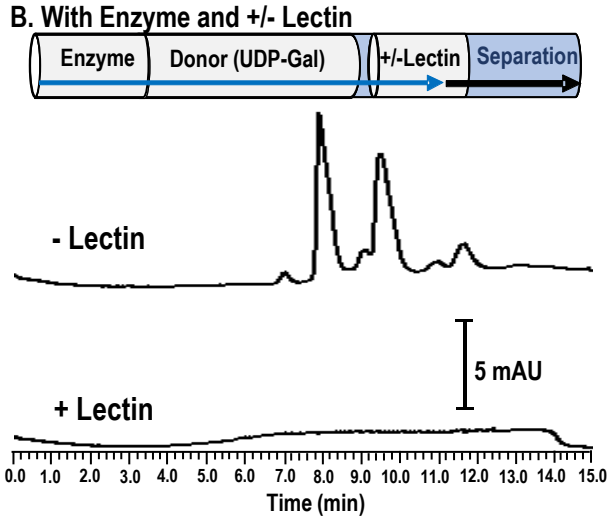
Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the B1–4 Galactosyltransferase Michaelis–Menten Constant and Real-Time Addition of Galactose Residues to N-Glycans and Glycoprotein. *Anal. Chem. 2021.*

Protein Modification









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Significance: Nanogel electrophoresis increases the functionality and throughput of a microscale separation-based assay

Self assembled phospholipid nanogels provide fluid steering and tunable selectivity of biomolecules at a cost of $0.76 for the nanogel.

Innovation: Nanoliter reactions are patterned, processed, and quantified repeatedly. Smart materials enhance functionality at a lower cost and higher throughput.

Questions?
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