Capillary Electrophoresis to Quantify Drug Inhibition of H1N1 and H5N1 Neuraminidases

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Overview of Influenza

Influenza

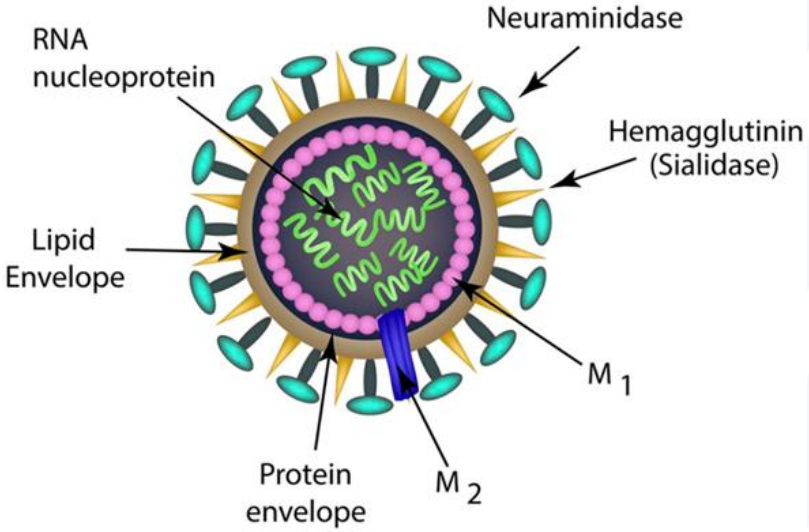
- Type A
- Type B
- Type C
- Type D

Most Severe

H1N1

H5N1

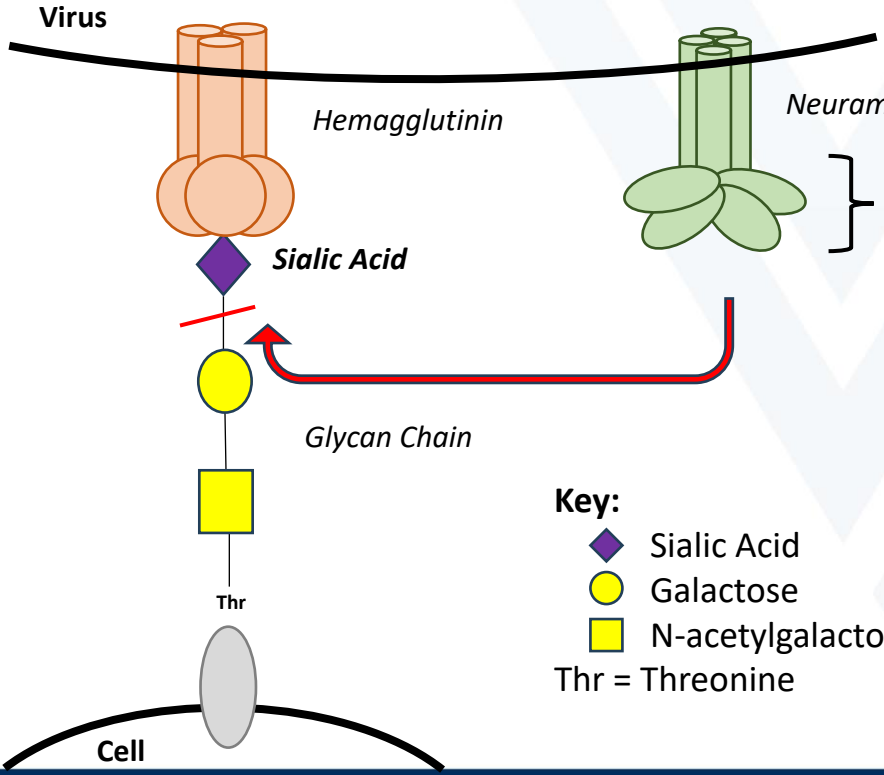
Structure of Influenza A

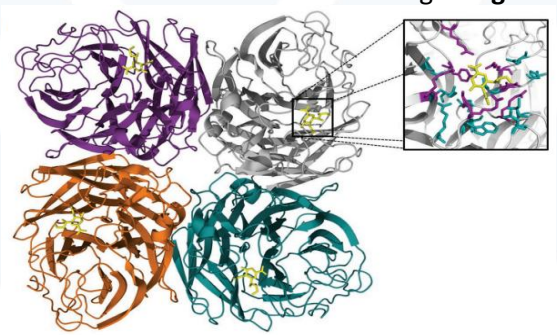


H#N#

1. CDC. Types of Influenza Viruses. Centers for Disease Control and Prevention. https://www.cdc.gov/flu/about/viruses/types.htm.
2. Huffman, B. Cow | Mammal. *Encyclopædia Britannica*; 2018.

Viral Infection of Influenza A





Receptor-binding pocket containing **charged residues**

Key:

- Sialic Acid
- Galactose
- N-acetylgalactosamine (GalNAc)

Thr = Threonine

How can we quantify neuraminidase activity?

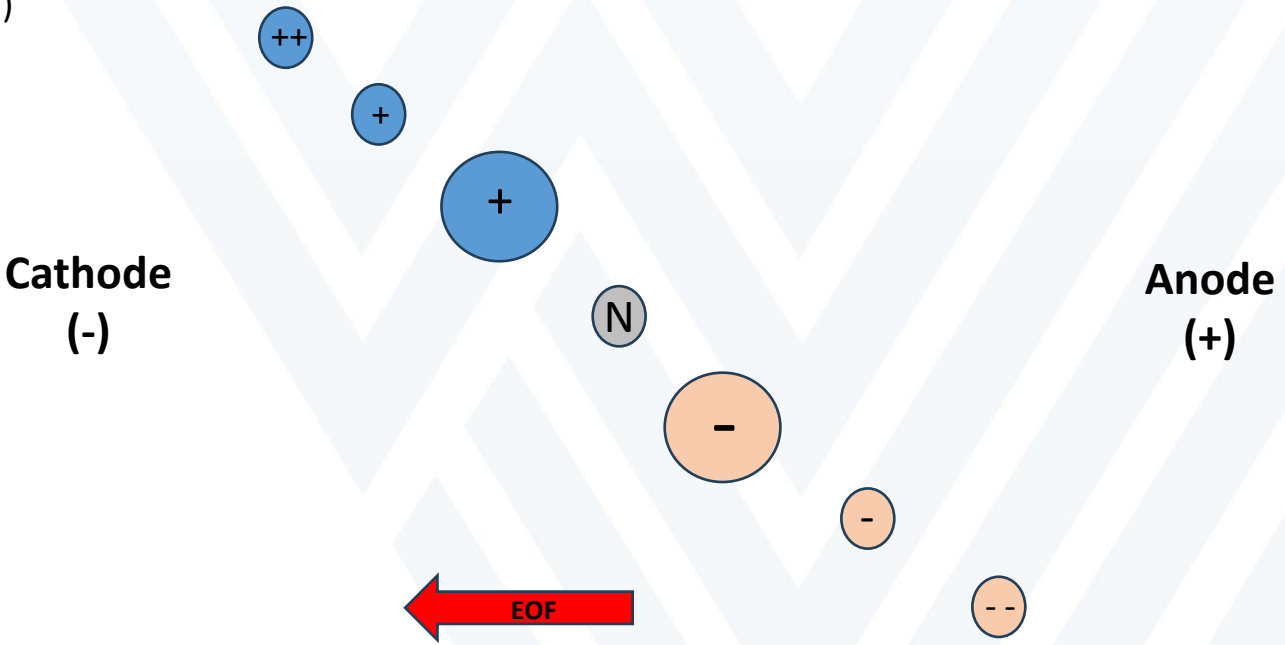
McAuley, J. L.; Gilbertson, B. P.; Trifkovic, S.; Brown, L. E.; McKimm-Breschkin, J. L. Influenza Virus Neuraminidase Structure and Functions. Frontiers in Microbiology 2019, 10 (39). https://doi.org/10.3389/fmicb.2019.00039.

Capillary Electrophoresis

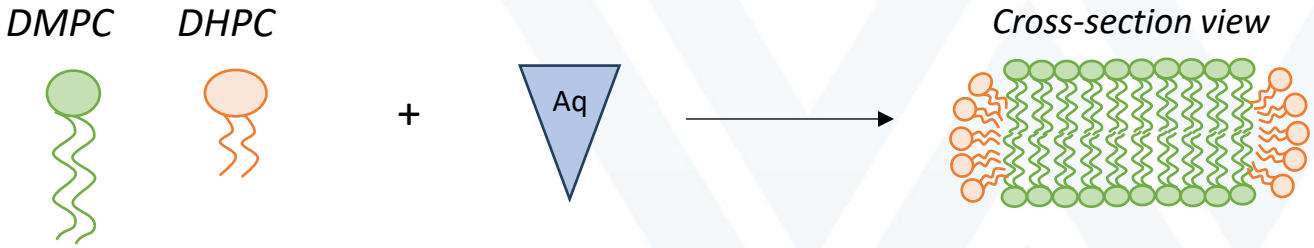
Purpose: Separate analytes based on charge-to-size ratio

Separation dependent on:

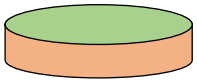
- Electrophoretic mobility (EPH)
- Electroosmotic flow (EOF)



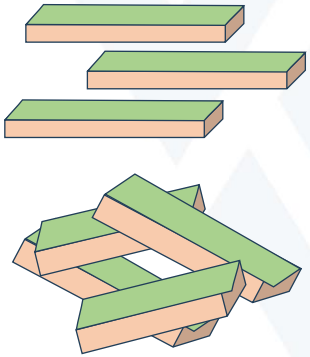
Phospholipids



Under gel phase temp:



Above gel phase temp:



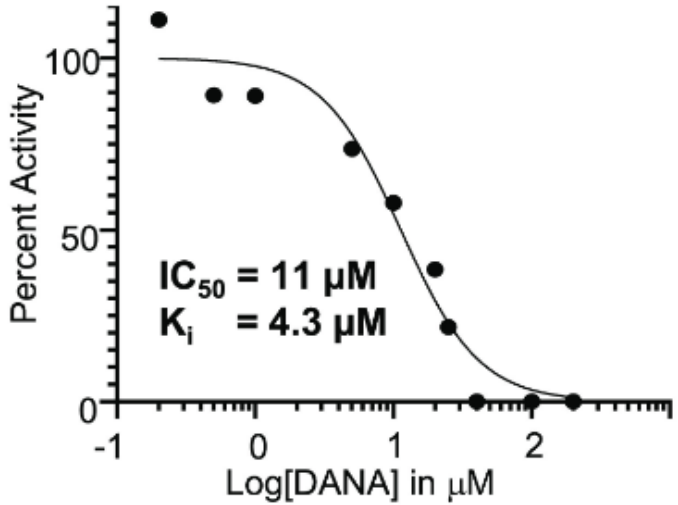
Viscosity is dependent on:

- Temperature
- Hydration (%)
- q – the mole ratio

Applications:

- Suppress EOF
- Improve resolution
- Localize enzymes, substrates and inhibitors

IC_{50} vs K_i Curves



K_i = Dissociation constant
IC_{50} = Inhibition constant

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

When [S] << K_m then K_i = IC_{50}

Casto-Boggess LD, Holland LA, Lawer-Yolar PA, Lucas JA, Guerrette JR. Microscale Quantification of the Inhibition of Neuraminidase Using Capillary Nanogel Electrophoresis. Anal Chem. 2022 Nov 22;94(46):16151-16159. doi: 10.1021/acs.analchem.2c03584. Epub 2022 Nov 7. PMID: 36343965; PMCID: PMC9686991.

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Article

Native Capillary Nanogel Electrophoresis Assay of Inhibitors of Neuraminidases Derived from H1N1 and H5N1 Influenza A Pandemics

Laura N. Taylor, Lisa A. Holland,* and Makenzie T. Witzel

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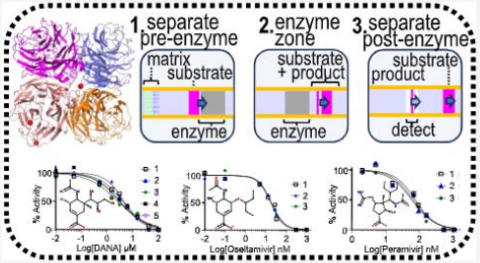
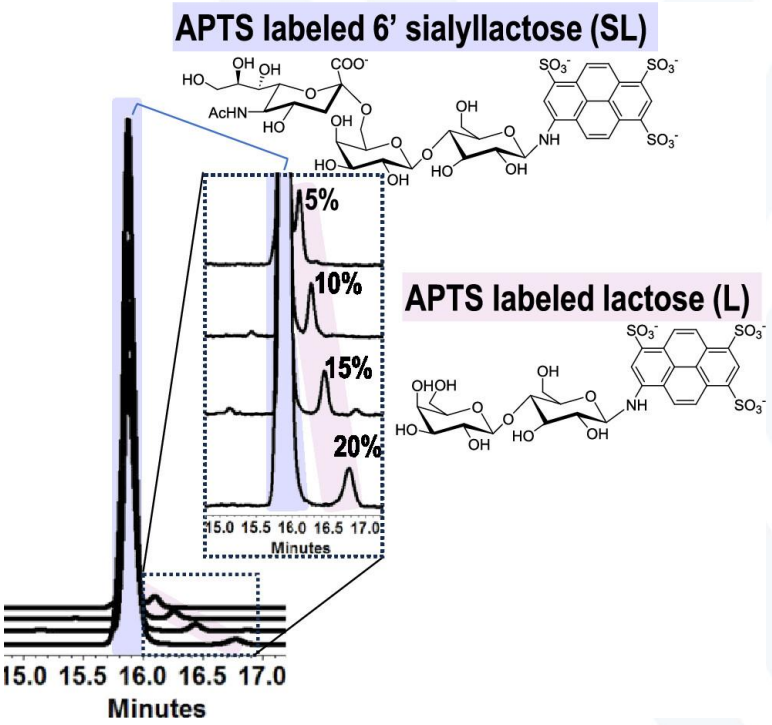
ABSTRACT: Tetrameric neuraminidases cleave the end-capping sialylated monomer from oligosaccharide ligands at the surface of a host cell infected by the influenza A virus. This cleavage releases the replicated virions from the host cell, making drugs that inhibit neuraminidase function effective to treat influenza A infections. A capillary electrophoresis separation-based assay is reported that maintains the native structure of tetrameric viral neuraminidases derived from H1N1 or H5N1 influenza A pandemics which convert, in-real time, a substrate that mimics 6′-sialyllated threonine-linked glycans on human cells. The assay integrates the enzyme reaction with the separation and is operated using a background electrolyte containing 100 mM NaCl with a thermally reversible nanogel in a 10 μm inner diameter fused silica capillary.



In addition to defining the 0.4 nL reaction zone maintained at 37 °C, the nanogel medium resolves the substrate from contaminants as well as the substrate from the product before and after the enzymatic conversion. The enzyme activity is quantifiable based on the percent conversion observed in the presence of a range of inhibitor concentrations. For 1918 H1N1 (A/Brevig Mission/1/18) neuraminidase, the inhibition constant of the transition state analog 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) is 3.5 ± 0.8 μM (n = 5). The inhibition constants for oseltamivir acid (inhibiting compound of Tamiflu) and peramivir (Rapivab) are 18.2 ± 0.5 nM (n = 3) and 67 ± 8 nM (n = 3), respectively. For 2004 H5N1 (A/Vietnam/1203/2004) neuraminidase, which contained a foreign tetramerization domain to maintain the structure, the inhibition constant for peramivir is 5.4 nM.

Altering Nanogel Concentration to Increase Resolution



Resolutions:

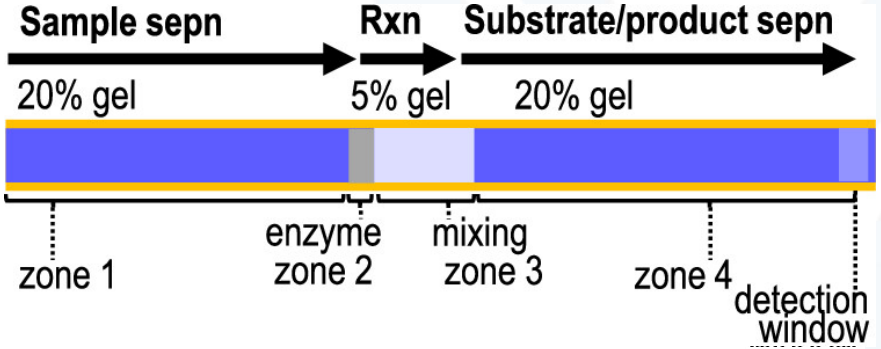
5% - unquantifiable

10% - 2.6 ± 0.1

15% - 3.3 ± 0.2

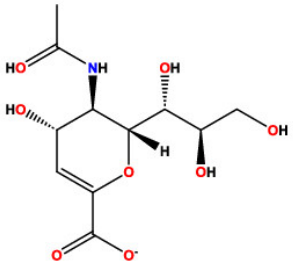
20% - 4.0 ± 0.1

Capillary Patterning on a 10µm ID Capillary

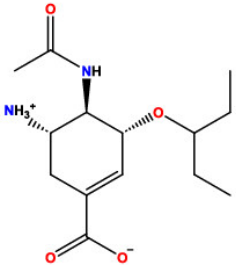


Inhibitors Included in Zones 1, 3, 4

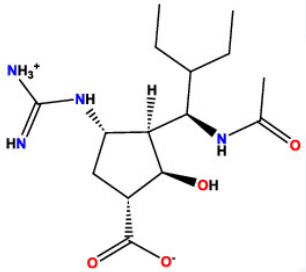
DANA
pKa 3.9,
anionic

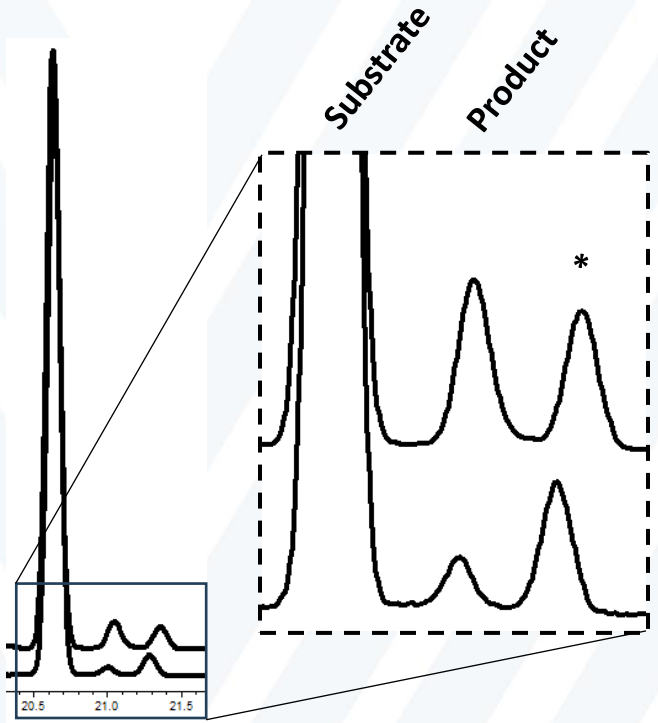


Oseltamivir acid
pKa 6.7
net anionic

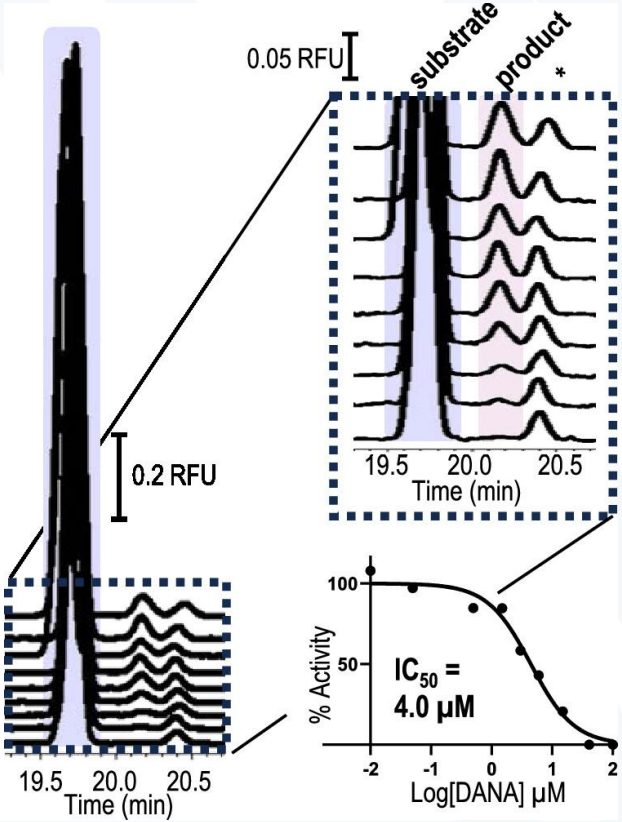


Peramivir
pKa 8.7
net cationic

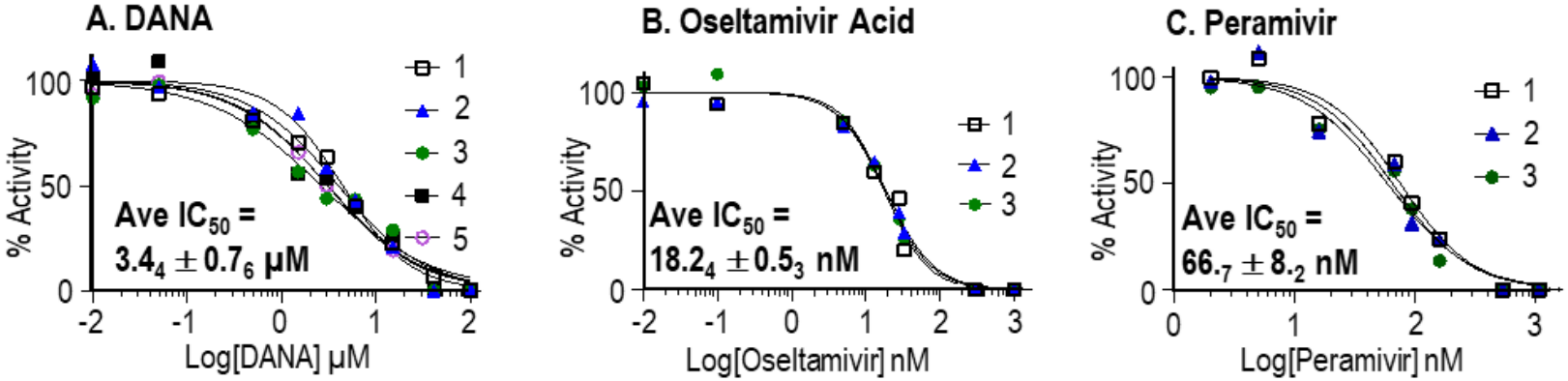




H1N1 Neuraminidase Inhibition with DANA

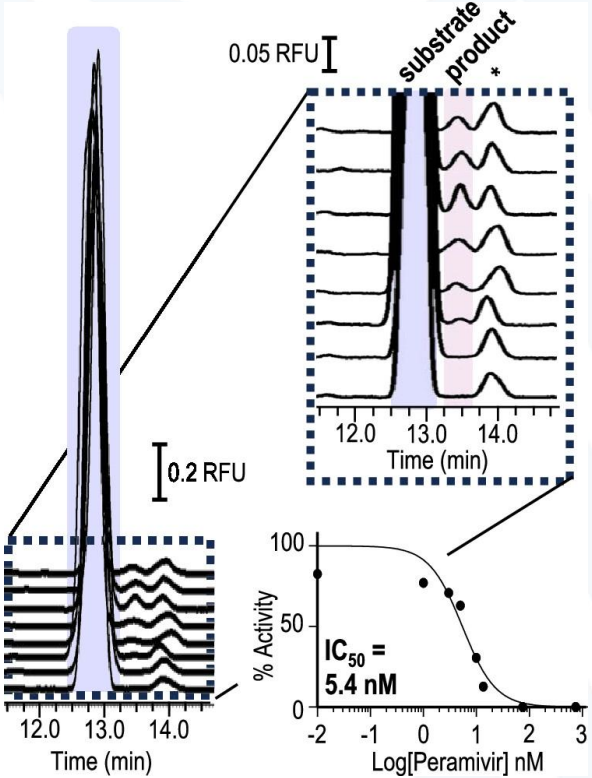


H1N1 IC_{50} Curves of DANA, Oseltamivir Acid, and Peramivir



Oseltamivir Acid is the strongest therapeutic!

H5N1 Neuraminidase Inhibition with Peramivir



Conclusion:

- Measured the effectiveness of neuraminidase inhibitors for H1N1 and H5N1 influenza A pandemics
- Oseltamivir had the stronger therapeutic effects against H1N1 neuraminidase
- Lower IC_{50} value for peramivir with H5N1 when compared to H1N1 neuraminidase

Future Directions:

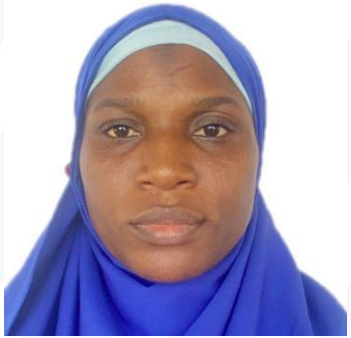
- Evaluate other recombinant multimeric enzymes
- Measure the effectiveness of these inhibitors with 3'sialyllactose

Acknowledgements

Thank you to the Holland Group and NIH!







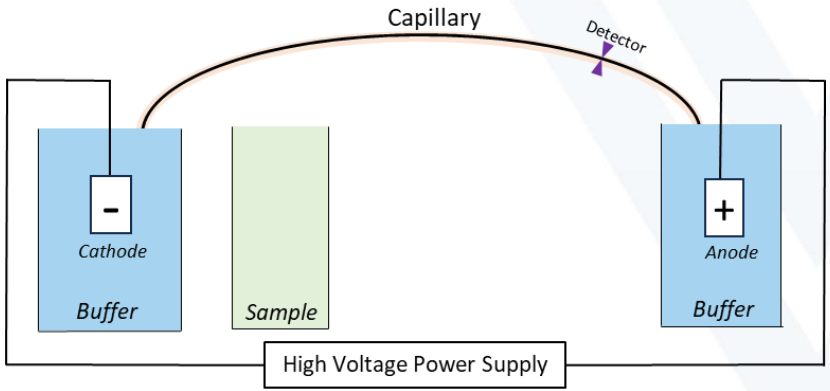
This material is based upon work supported by NIH Grant No. R01GM140560

Questions?

Supporting Information

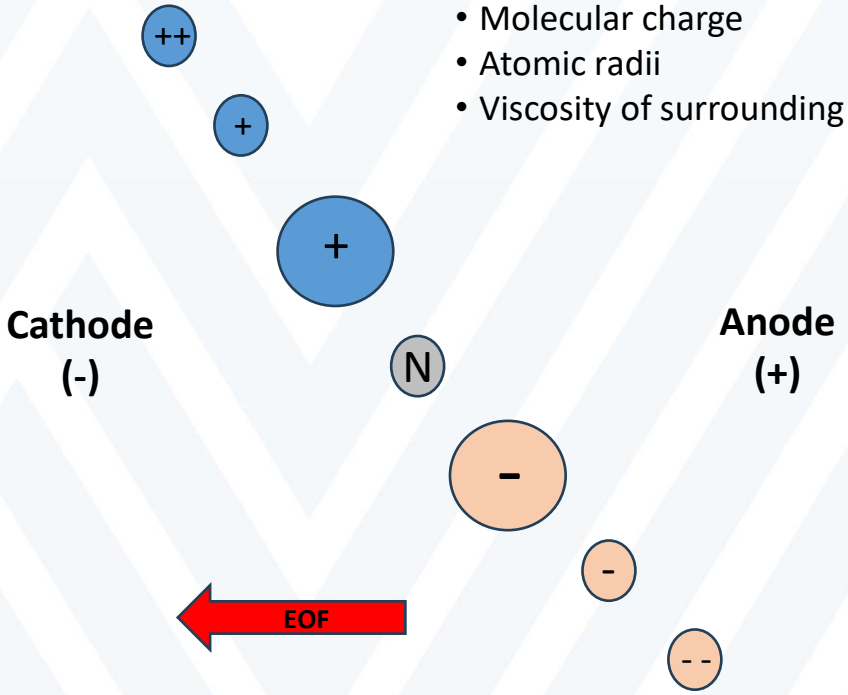
Capillary Electrophoresis

Purpose: Separate analytes based on charge-to-size ratio



Mobility dependent on:

- Molecular charge
- Atomic radii
- Viscosity of surrounding



Advantages:

- Nanoliter reagent volumes
- Automation
- Fast run times
- Separation modalities

Electrophoretic Mobility

Separation of charged species under reverse polarity conditions

Mobility dependent on:

- Molecular charge
- Atomic radii
- Viscosity of surrounding

Cathode
(-)

Anode
(+)

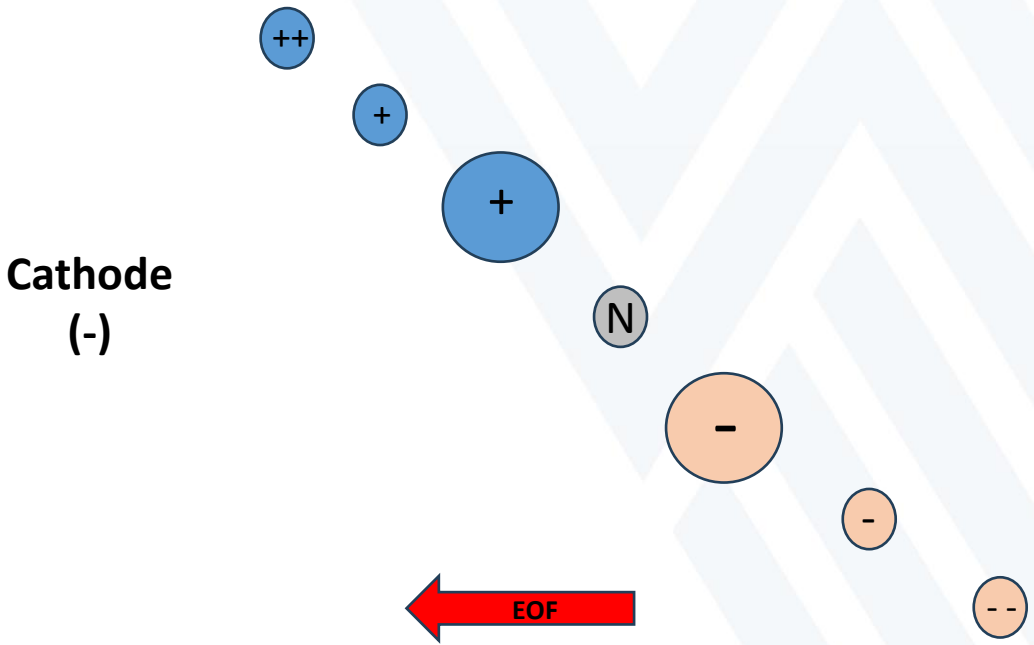
EOF

Electrophoretic Mobility

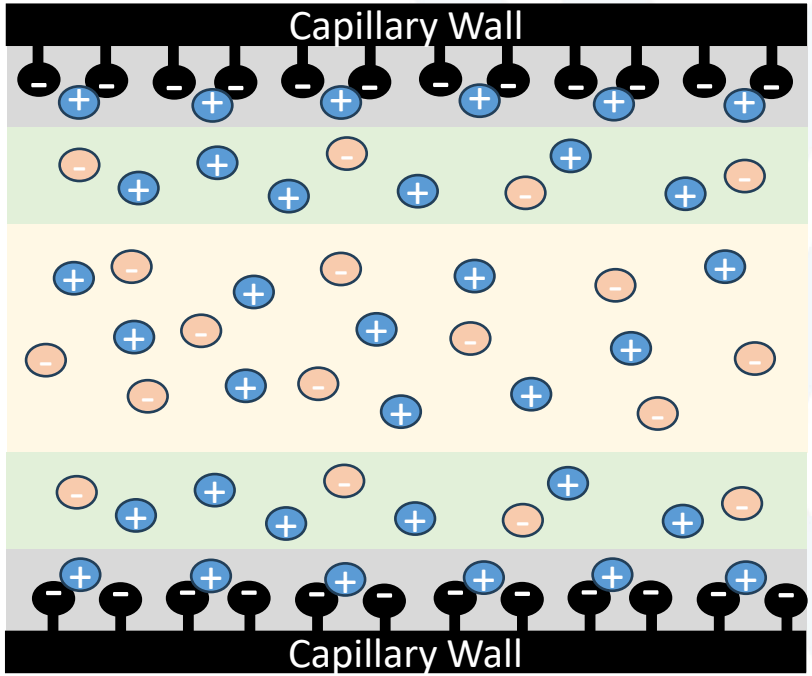
Separation of charged species under reverse polarity conditions

Mobility dependent on:

- Molecular charge
- Atomic radii
- Viscosity of surrounding



Cross-sectional View of a Capillary



Fixed Layer

Diffuse Layer

Electrical Double layer

Bulk Solution

Cathode (-)

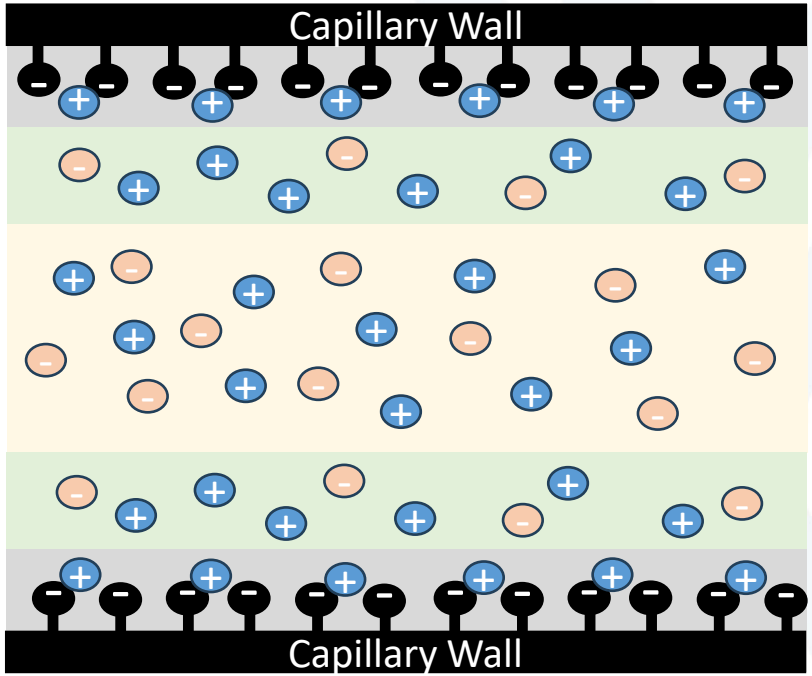
Anode (+)

Separation of analytes occur via:

- Electroosmotic Flow
- Electrophoretic mobility

Electroosmotic Flow (EOF)

Direction of bulk solution flow under reverse polarity conditions



Fixed Layer

Diffuse Layer

Electrical Double layer

Bulk Solution

Cathode (-)

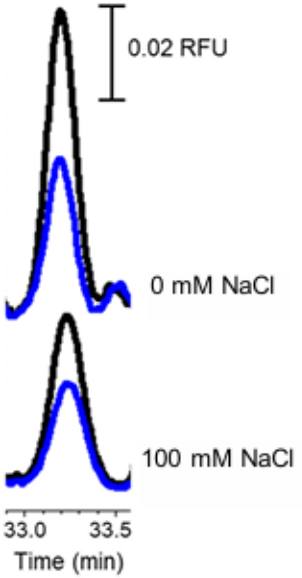
Anode (+)

Effect of 100 mM NaCl on H1N1 Neuraminidase Conversion

Table S1. Effect of 100 mM NaCl on H1N1 Neuraminidase Conversion

	[DANA][a]	6'-SL Area[a]	Lactose Area	Total Area	Conversion (%)[b]	Activity Remaining (%)[c]
0 mM NaCl	No Inhibitor	4475736	64995	4540731	1.43	100
		4695627	81080	4776707	1.70	100
		4653954	79419	4733373	1.68	100
	Average Conversion: *1.6 ± 0.1 (9% RSD)*					
	1 µM	4866239	35922	4902161	0.73	45.74
		5024033	39369	5063401	0.78	48.53
		5050518	38386	5088904	0.75	47.08
	Average % Activity Remaining: *47 ± 1 (3% RSD)*[d]					
100 mM NaCl	No Inhibitor	10964532	85754	11050286	0.78	100
		11283796	96709	11380505	0.85	100
		10437696	86381	10524077	0.82	100
	Average Conversion: *0.82 ± 0.04 (5% RSD)*					
	1 µM	11911443	53981	11965424	0.45	55.32
		11885712	52496	11938208	0.44	53.92
		10375981	44520	10420501	0.43	52.39
	Average Activity Remaining: *54 ± 1 (3% RSD)*[d]					

[a]*Abbreviations: DANA 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA), 6'-sialyllactose (6'-SL)*
[b]*The percent enzyme conversion is calculated as the area of lactose divided by total area.*
[c]*The percent activity remaining is calculated as the percent conversion with inhibitor divided by the average percent conversion without inhibitor.*
[d]*The averages of percent activity remaining for 0 mM NaCl and 100 mM NaCl are statistically different (student's t-test, n = 3, 95 % confidence level).*



% Conversion and Activity Remaining are statistically different

Resolution Achieved with Increased Nanogel Concentration

Table S2A. Resolution Achieved with Increased Nanogel Concentration

		6'-Siallylactose		Lactose		
	% Nanogel	Time (min)	WHM[a] (min)	Time (min)	WHM (min)[a]	Resolution[b]
Set 1	5	15.283	0.077	15.512	0.101	1.52
	10	16.883	0.082	17.267	0.086	2.70
	15	18.550	0.096	19.154	0.105	3.55
	20	20.825	0.114	21.671	0.133	4.04
Set 2	5	15.871	0.082	16.100	0.098	1.50
	10	17.342	0.086	17.717	0.092	2.49
	15	19.400	0.096	19.954	0.101	3.32
	20	21.650	0.122	22.563	0.144	4.05
Set 3	5	16.462	0.084	16.679	-[c]	-[c]
	10	18.058	0.088	18.438	0.091	2.51
	15	20.642	0.106	21.233	0.118	3.11
	20	23.842	0.180	25.087	0.205	3.82

[a]Width at half maximum height (WHM)
[b]Resolution is calculated as 1.18(time lactose- time sialyllactose)/(WHM lactose + WHM sialyllactose).*
[c]The WHM cannot be determined using automatic peak detection with the data processing software.

Table S2B. Summary of Resolution Data in Table S2A

% Nanogel	Resolution (n = 3)
5	1.5[a]
10	2.6 ± 0.1 (5% RSD)
15	3.3 ± 0.2 (7% RSD)
20	4.0 ± 0.1 (3% RSD)

[a]Calculated from n=2

5% vs no 5%

Table S3. The Effect of Patterning 5% with DANA[a]

[DANA][a]	6'-SL Area[a]	Lactose Area	Total Area	% Conversion[b]	% Activity Remaining[c]
No 5% Nanogel included in Pattern					
No Inhibitor	624534	32211	656745	4.90	100
	614241	30388	644629	4.71	100
	706596	37888	744484	5.09	100
***Average % Conversion**: 4.9 ± 0.2 % (4% RSD)[d]*					
3 µM	682369	17456	699825	2.49	50.88
	560643	17520	578163	3.03	61.81
	521397	16024	537421	2.98	60.82
***Average Activity Remaining:** 58 ± 6 % (10% RSD)[e]*					
5% Nanogel included in Pattern					
No Inhibitor	639991	38219	678210	5.64	100
	642027	37951	679978	5.58	100
	680645	44762	725407	6.17	100
***Average % Conversion:** 5.8 ± 0.3 % (6% RSD)[d]*					
3 µM	769311	20425	789736	2.59	44.62
	789244	27986	817230	3.42	59.09
	752558	24661	777219	3.17	54.75
***Average Activity Remaining:** 53 ± 7 % (10% RSD)[e]*					

[a]*Abbreviations: DANA 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA), 6'-sialyllactose (6'-SL)*
[b]*The percent enzyme conversion is calculated as the area of lactose divided by total area.*
[c]*The percent activity remaining is calculated as the percent conversion with inhibitor divided by the average percent conversion without inhibitor.*
[d]*The average conversion is statistically different in the absence and presence of 5% nanogel (student's t-test, n = 3, 95 % confidence level).*
[e]*The average percent activity remaining is statistically the same in the absence and presence of 5% nanogel (student's t-test, n = 3, 95 % confidence level).*

Avg % conversion is statistically different but activity remaining remained the same

DANA Zone Study – H_1N_1

Table S4A. Zone Study of DANA[a]

	6'-SL Area[a]	Lactose Area	Total Area	% Conversion[b]	% Activity Remaining[c]
Set 1 Patterning with 0 µM DANA Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 1	695482	38665	734147	5.27	100.00
Inhibitor in zones 1, 3, and 4	774607	29020	803627	3.61	68.57
	732035	28276	760311	3.72	70.61
	685861	29518	715379	4.13	78.35
Average Activity Remaining: 73 ± 5 (7% RSD)[d]					
Set 2 Patterning with 3 µM DANA Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 2	653877	45634	699511	6.52	100.00
Inhibitor in zones 1, 2, 3, and 4	706407	24160	730567	3.31	50.69
	708419	27993	736412	3.80	58.27
	686604	33505	720109	4.65	71.32
Average Activity Remaining: 60 ± 10 (20% RSD)[d]					

[a] *Abbreviations: DANA 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA), 6'-sialyllactose (6'-SL). A 400 s push step and an additional 200 s 103 kPa (15 psi) 20% nanogel post plug were used after the enzyme zone was patterned to mitigate the effects of nanogel expansion.*

[b] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*

[c] *The percent activity remaining for set 1 vs. set 2 is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor. The runs obtained in the absence of inhibitor for set 1 (No Inhibitor 1) and for set 2 (No Inhibitor 2) were done immediately before the set of replicate runs and used to normalize the percent conversion for each set.*

[d] *The average percent activity remaining with inhibitor in zones 1, 3, and 4 is statistically the same as that obtained with inhibitor in zones 1, 2, 3, and 4 (student's t-test, n = 3, 95 % confidence level).*

Oseltamivir Acid Zone Study – H_1N_1

Table S4B. Zone Study of Oseltamivir Acid[a]

	6'-SL Area[b]	Lactose Area	Total Area	% Conversion[c]	% Activity Remaining[d]
Set 1 Patterning with 0 nM Oseltamivir Acid Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 1	1676202	48886	1725088	2.83	100.00
Inhibitor in zones 1, 3, and 4	1445748	22375	1468123	1.52	53.78
	1273180	23093	1296273	1.78	62.87
	1637451	30445	1667896	1.83	64.41
Average Activity Remaining: 60 ± 6 (10% RSD)[e]					
Set 2 Patterning with 23 nM Oseltamivir Acid Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 2	1378126	55534	1433660	3.87	100.00
Inhibitor in zones 1, 2, 3, and 4	1175277	26392	1201669	2.20	56.70
	1176301	28019	1204320	2.33	60.06
	1088437	27799	1116236	2.49	64.29
Average Activity Remaining: 60 ± 4 (6% RSD)[e]					

[a] *For this study 5 μL of the enzyme stock supplied by the manufacturer was diluted up to 10 μL to a final composition of 5% nanogel in 100 mM NaCl, 5 mM CaCl2, and 50 mM Tris buffered to pH 7.5 yielding 0.11 mg/mL H1N1*

[b] *Abbreviation: 6'-sialyllactose (6'-SL)*

[c] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*

[d] *The percent activity remaining for set 1 vs. set 2 is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor. The runs obtained in the absence of inhibitor for set 1 (No Inhibitor 1) and for set 2 (No Inhibitor 2) were done immediately before the set of replicate runs and used to normalize the percent conversion for each set.*

[e] *The average percent activity remaining with inhibitor in zones 1, 3, and 4 is statistically the same as that obtained with inhibitor in zones 1, 2, 3, and 4 (student's t-test, n = 3, 95 % confidence level).*

Peramivir Zone Study – H_1N_1

Table S4C. Zone Study of Peramivir

	6'-SL Area[a]	Lactose Area	Total Area	% Conversion[b]	% Activity Remaining[c]
Set 1 Patterning with 0 nM Peramivir Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 1	1444514	20533	1465047	1.40	100.00
Inhibitor in zones 1, 3, and 4	1477388	15822	1493210	1.06	75.60
	1372309	15143	1387452	1.09	77.87
	1297081	15084	1312165	1.15	82.02
Average Activity Remaining: 78 ± 3 (4% RSD)[d]					
Set 2 Patterning with 16 nM Peramivir Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 2	1435546	24088	1459634	1.65	100.00
Inhibitor in zones 1, 2, 3, and 4	1271472	14447	1285919	1.12	68.08
	1225498	15447	1240945	1.24	75.43
	1241341	16335	1257676	1.30	78.70
Average Activity Remaining: 74 ± 5 (7% RSD)[d]					

[a] *Abbreviation: 6'-sialyllactose (6'-SL)*

[b] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*

[c] *The percent activity remaining for set 1 vs. set 2 is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor. The runs obtained in the absence of inhibitor for set 1 (No Inhibitor 1) and for set 2 (No Inhibitor 2) were done immediately before the set of replicate runs and used to normalize the percent conversion for each set.*

[d] *The average percent activity remaining with inhibitor in zones 1, 3, and 4 is statistically the same as that obtained with inhibitor in zones 1, 2, 3, and 4 (student's t-test, n = 3, 95 % confidence level).*

The Effect of 6SL Concentration on Conversion

Table S5A. Effect of 6'-SL Concentration on Conversion[a]

	[6'-SL] nM	6'-SL Area	Lactose Area	Total Area	Conversion (%)[b]
Set 1	26	893575	29588	923163	3.21
	200	5510063	166783	5676846	2.94
Set 2	26	848271	28992	877263	3.30
	200	6529284	204338	6733622	3.03
Set 3	26	839410	29987	869397	3.45
	200	5495406	202696	5698102	3.56
Set 4	26	874625	33790	908415	3.72
	200	5848298	218632	6066930	3.60

[a]*Abbreviation: 6'-sialyllactose (6'-SL). SL is reconstituted in 1.5 mM TRIS buffered to pH 7.5.*
[b]*The percent enzyme conversion is calculated as the area of lactose divided by total area.*

Table S5B. Summary of Table S5A

[6'-SL] in nM[a]	Conversion (%)[b]
26	3.4 ± 0.2 (7% RSD)
200	3.3 ± 0.3 (10% RSD)

[a]*Abbreviation: 6'-SL 6'-sialyllactose*
[b]*The percent enzyme conversion is calculated as the area of lactose divided by total area. The average conversion with 26 nM 6'-SL is statistically the same as that obtained 200 nM 6'-SL (student's t-test, n = 4, 95 % confidence level).*

The Effect of the Post Plug Injection Size on Area and Conversion

Table S6A. Effect of Post Injection Plug Size on Area and Conversion

	Post injection plug time (seconds)[a]	6'-SL Area[b]	Lactose Area	Total Area	Conversion (%)[c]
Set 1	17.9	694372	10627	704999	1.51
	13.9	788574	15011	803585	1.87
	9.90	414203	7996	422199	1.89
Set 2	17.9	756224	14801	771025	1.92
	13.9	730246	14596	744842	1.96
	9.90	330055	7072	337127	2.10
Set 3	17.9	809137	16466	825603	1.99
	13.9	734773	16289	751062	2.17
	9.90	491506	10192	501698	2.03
Set 4	17.9	698710	15086	713796	2.11
	13.9	749709	14685	764394	1.92
	9.90	375906	7691	383597	2.00

[a] *After the sample injection is complete a post plug of nanogel is introduced at 103 kPa (15 psi) for the times specified in the Table.*
[b] *Abbreviation: 6'-sialyllactose (6'-SL)*
[c] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*

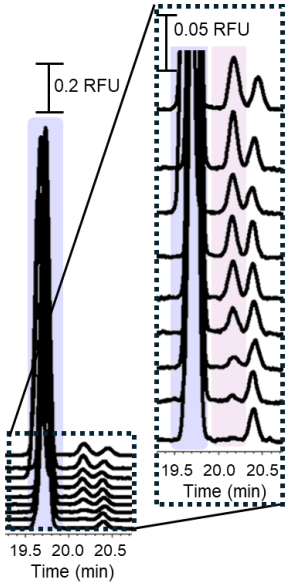
Table S6B Summary of Post Fill Plug Injections in Table S6A

Post injection plug time (seconds)	6'-SL Area x 10^5 (n=4)[a,b]	Conversion (%) (n=4)[c]
17.9	7.4 ± 0.5 (7%)	1.9 ± 0.3 (10% RSD)
13.9	7.5 ± 0.3 (4%)	2.0 ± 0.1 (7% RSD)
9.9	4.0 ± 0.7 (20%)	2.01 ± 0.08 (4% RSD)

[a] *Abbreviation: 6'-sialyllactose (6'-SL)*
[b] *The average peak area at 17.9 s is statistically the same as that obtained 13.9 s (student's t-test, n = 4, 95 % confidence level). The average peak area at 9.9 s is statistically different from that obtained 13.9 s or at 17.9 s (student's t-test, n = 4, 95 % confidence level).*
[c] *The percent enzyme conversion is calculated as the area of lactose divided by total area. The average percent conversions obtained at 17.9 s, 13.9 s, and 9.9 s are statistically the same (student's t-test, n = 4, 95 % confidence level).*

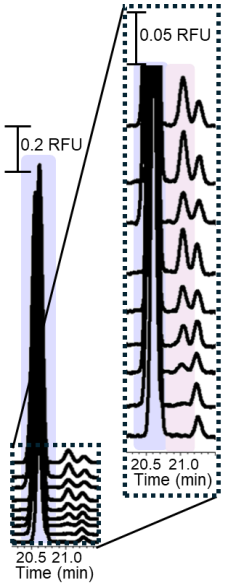
All DANA IC_{50} curve electropherograms

Curve 1:



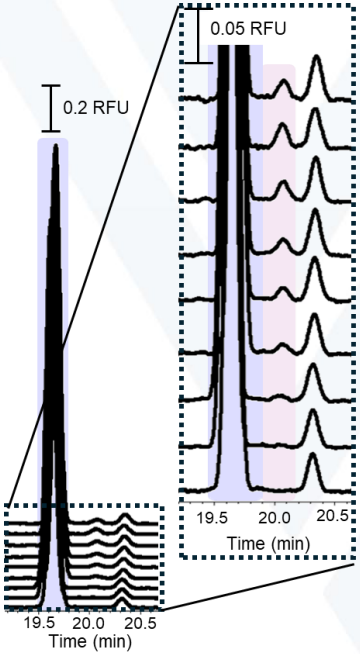
IC_{50} = 4.00 µM

Curve 2:



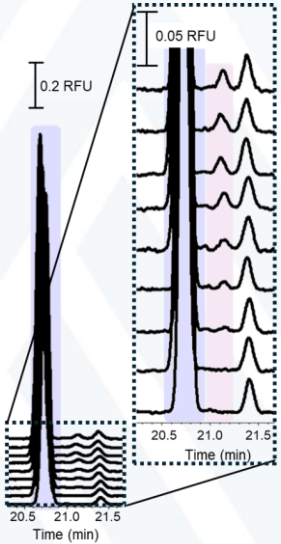
IC_{50} = 4.45 µM

Curve 3:



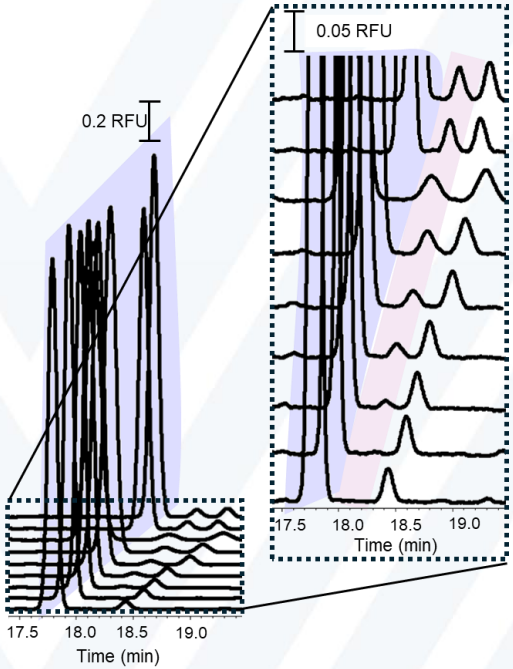
IC_{50} = 2.61 µM

Curve 4:



IC_{50} = 3.10 µM

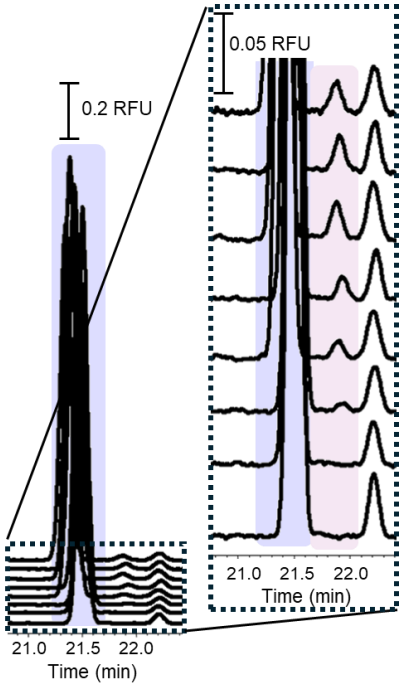
Curve 5:



IC_{50} = 3.07 µM

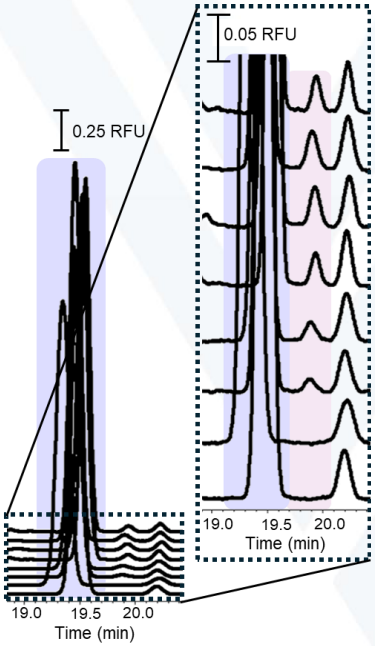
All Oseltamivir Acid - IC_{50} curve electropherograms

Curve 1:



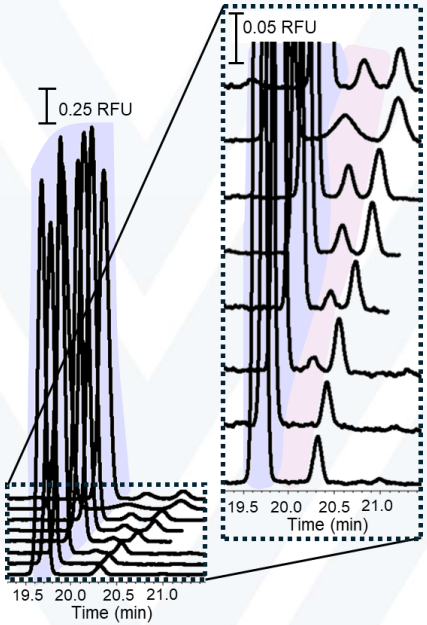
IC_{50} = 17.91 nM

Curve 2:



IC_{50} = 18.86 nM

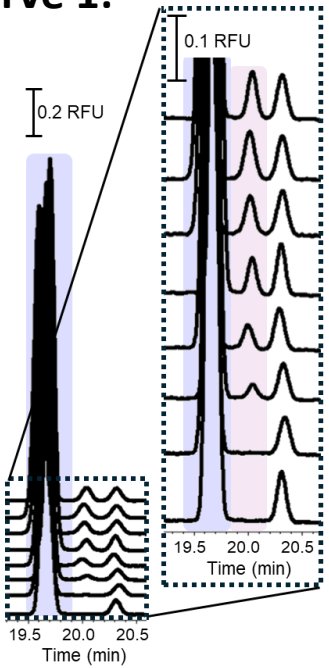
Curve 3:



IC_{50} = 17.97 nM

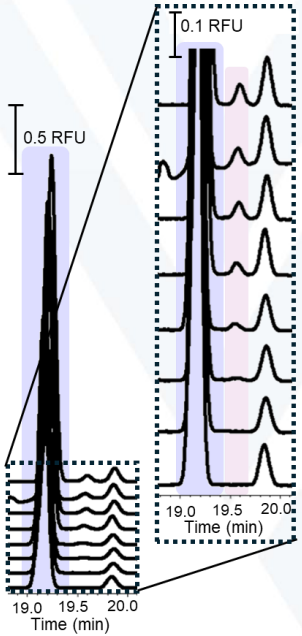
All Peramivir - IC_{50} curve electropherograms

Curve 1:



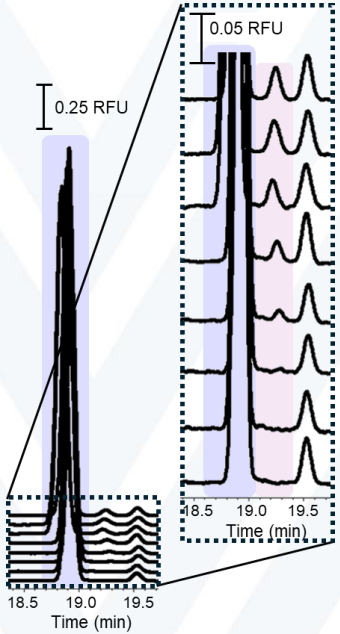
IC_{50} = 75.61 nM

Curve 2:



IC_{50} = 65.25 nM

Curve 3:



IC_{50} = 59.33 nM

Resolution Achieved with Increased Nanogel Concentration - MES

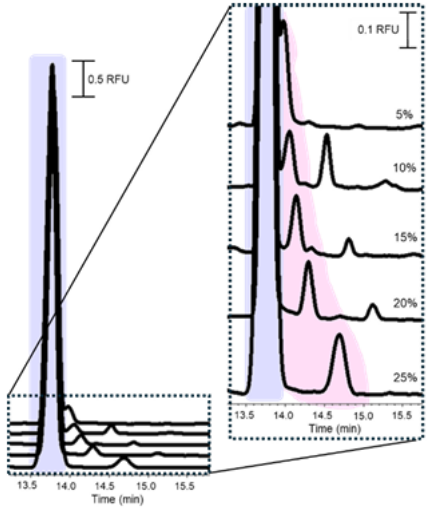
Table S10A. Resolution Achieved with Increased Nanogel Concentration

	% Nanogel	6'-Siallylactose Time (min)	6'-Siallylactose WHM[a] (min)	Lactose Time (min)	Lactose WHM[a] (min)	Resolution[b]
Set 1	5	13.800	0.144	13.979	-[c]	-[c]
	10	14.733	0.130	14.996	0.138	1.16
	15	15.825	0.121	16.192	0.125	1.76
	20	17.600	0.119	18.129	0.121	2.60
	25	20.808	0.158	21.738	0.183	3.22
Set 2	5	14.104	0.142	14.371	-[c]	-[c]
	10	15.217	0.128	15.492	0.133	1.24
	15	16.300	0.122	16.679	0.130	1.77
	20	18.125	0.126	18.688	0.128	2.62
	25	20.967	0.158	21.817	0.159	3.16
Set 3	5	14.200	0.140	14.521	-[c]	-[c]
	10	15.300	0.124	15.575	0.134	1.26
	15	16.529	0.115	16.917	0.125	1.91
	20	18.450	0.121	19.012	0.126	2.68
	25	22.387	0.168	23.321	0.169	3.27
Set 4	5	14.375	0.139	14.750	-[c]	-[c]
	10	15.350	0.123	15.625	0.130	1.28
	15	16.637	0.114	17.025	0.132	1.86
	20	18.667	0.121	19.229	0.118	2.77
	25	22.392	0.155	23.363	0.188	3.34

[a] *Width at half maximum height (WHM)*
[b] *Resolution is calculated as 1.18*(time lactose- time sialyllactose)/(WHM lactose + WHM sialyllactose).*
[c] *The WHM cannot be determined using automatic peak detection with the data processing software.*

Table S10B. Summary of resolution data in Table S10A

% Nanogel	Resolution (n = 4)
10	1.24 ± 0.05 (4% RSD)
15	1.83 ± 0.07 (4% RSD)
20	2.67 ± 0.08 (3% RSD)
25	3.25 ± 0.08 (2% RSD)



0mM NaCl – H_5N_1

Table S11A. Effect of 0 mM NaCl on H5N1 Neuraminidase Conversion[a]

6'-SL Area (SL)	Lactose Area (L_p)	Lactose Cont. (L_c)	Total Area (L_p+L_c)	Total Area (SL+L_p+L_c)	Total % Lactose (L_p+L_c)	Conversion to Lactose (%) (L_p)[b]	Activity Remain (%)[c]
No enzyme, no inhibitor (*blank*)							
4375159	0	91083	91083	4466242	2.04	*NA*	*NA*
Enzyme, no inhibitor (*0 mM Peramivir*)							
2973382	359593	59349	418942	3392324	12.35	10.31[d]	100
3748684	380483	81050	461533	4210217	10.96	8.92[e]	100
4054543	424928	85774	510702	4565245	11.19	9.15[f]	100
Average L_p Conversion: *9.5 ± 0.7 (8% RSD)*[g]							
Enzyme and inhibitor (*1 nM Peramivir*)							
3601295	231372	84317	315689	3916984	8.06	6.02[d]	58.39[d]
3976748	283406	88036	371442	4348190	8.54	6.50[e]	72.88[e]
4207257	290917	92828	383745	4591002	8.36	6.32[f]	69.08[f]
Average Activity Remaining: *67 ± 8 (10% RSD)*[h]							

[a]*Abbreviation: 6'-sialyllactose (6'-SL), 6'-sialyllactose substrate (SL), lactose product (L_p), lactose contaminant present in sample prior to enzyme reaction (L_c).*

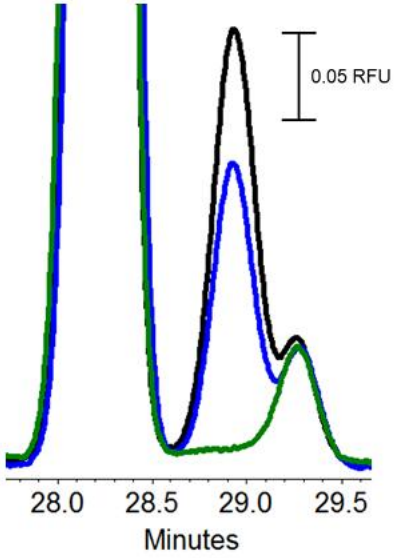
[b]*The calculation of the percent enzyme conversion (i.e. Conversion to Lactose, L_p) is modified for this study because the peaks for enzymatically converted lactose and lactose contaminant in the sample are not baseline resolved. For this calculation, the percent contribution of the contaminant peak area (L_c) is measured in the absence of enzyme (i.e.* ***blank****) and calculated from the blank as a percentage and this value is then subtracted from the enzyme runs. For the blank, the percent lactose contaminant is calculated as the L_c area divided by the total as follows (Area,L_c)/(Area,SL+ Area,L_c). This value is the amount of lactose contaminant (% area L_c) that is subtracted from the analyses done with enzyme where the amount of lactose product and lactose contaminant (% Area of L_p+L_c) which is measured as the unresolved peak areas of lactose and contaminant observed following the enzyme reaction. The calculation of this corrected percent conversion to lactose product is as follows: (% total lactose in the presence of enzyme)-(%lactose contaminant in the blank) = (%lactose product formed from enzyme). The area calculations are as follows: ((L_p+L_c)/(SL+L_p+L_c))-((L_c)/(SL+L_c)) = (L_p)/(SL+L_p+L_c)*

[c]*The percent activity remaining is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor.*

[d,e,f]*Indicates which no inhibitor run was used for the calculation of activity remaining.*

[g]*The averages of percent conversion for 0 mM NaCl (Table S11A) and 100 mM NaCl (Table 11SB) are statistically different (student's t-test, n = 3, 95 % confidence level).*

[h]*The averages of percent activity remaining for 0 mM NaCl (Table S11A) and 100 mM NaCl (Table 11SB) are statistically the same (student's t-test, n = 3, 95 % confidence level).*



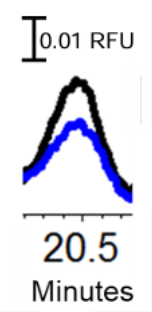
% Conversion is statistically different where as activity remaining is the same

100mM NaCl – H_5N_1

Table S11B. Effect of 100 mM NaCl on H5N1 Neuraminidase

[Peramivir] nM	6'-Sialyllactose Area	Lactose Area	Total Area	Conversion (%)[a]	Activity Remaining (%)[b]
No Inhibitor	10391417	74902	10466319	0.72[c]	100
	9094367	52915	9147282	0.58[d]	100
	9535653	44403	9580056	0.46[e]	100
Average Conversion: *0.6 ± 0.1 (20% RSD)*[f]					
1 nM	9929082	52382	9981464	0.52[c]	73.33[c]
	8471695	32307	8504002	0.38[d]	65.67[d]
	10352801	33483	10386284	0.32[e]	69.55[e]
Average Activity Remaining: *70 ± 4 (6% RSD)*[g]					

[a] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*
[b] *The percent activity remaining is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor.*
[c,d,e] *Indicates which no inhibitor run was used for the calculation of activity remaining.*
[f] *The averages of percent conversion for 0 mM and 100 NaCl (Table S11A,B) are statistically different (student's t-test, n = 3, ρ = 0.05).*
[g] *The averages of percent activity remaining for 0 mM and 100 NaCl (Table S11A,B) are statistically the same (student's t-test, n = 3, ρ = 0.05).*



% Conversion is statistically different where as activity remaining is the same

Peramivir Zone Study – H_5N_1

Table S12 Zone Study of Peramivir and H5N1

	6'-SL Area[a]	Lactose Area	Total Area	% Conversion[b]	% Activity Remaining[c]
Set 1 Patterning with 0 nM Peramivir Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 1	6882183	559134	7441317	7.51	100.00
Inhibitor in zones 1, 3, and 4	7798460	353419	8151879	4.34	57.70
	7694725	317538	8012263	3.96	52.74
	7841602	321466	8163068	3.94	52.41
Average Activity Remaining: 54 ± 3 (5% RSD)[d]					
Set 2 Patterning with 5.4 nM Peramivir Inhibitor in the Enzyme Stock (Zone 2)					
No Inhibitor 2	8029965	423786	8453751	5.01	100.00
Inhibitor in zones 1, 2, 3, and 4	9478088	208191	9686279	2.15	42.88
	9354311	323222	9677533	3.34	66.63
	6308774	227408	6536182	3.48	69.40
Average Activity Remaining: 60 ± 10 (20% RSD)[d]					

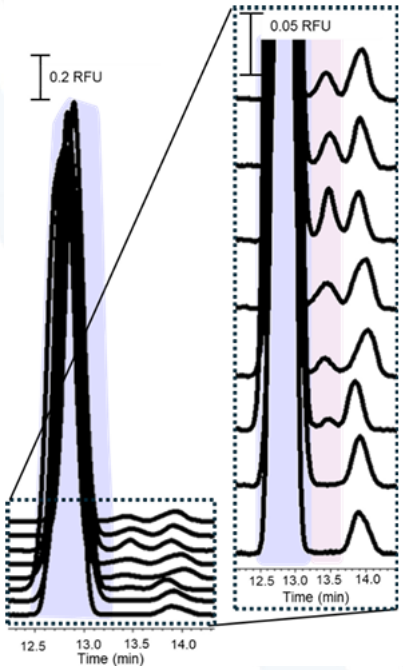
[a] *Abbreviations: 6'-SL 6'-sialyllactose*

[b] *The percent enzyme conversion is calculated as the area of lactose divided by total area.*

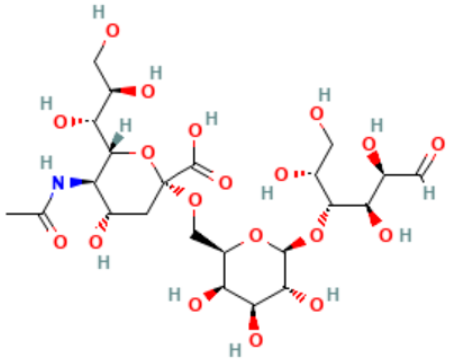
[c] *The percent activity remaining for set 1 vs. set 2 is calculated as the percent conversion with inhibitor divided by the percent conversion without inhibitor. The runs obtained in the absence of inhibitor for set 1 (No Inhibitor 1) and for set 2 (No Inhibitor 2) were done immediately before the set of replicate runs and used to normalize the percent conversion for each set.*

[d] *The average percent activity remaining with inhibitor in zones 1, 3, and 4 is statistically the same as that obtained with inhibitor in zones 1, 2, 3, and 4 (student's t-test, n = 3, 95 % confidence level).*

All Peramivir - IC50 curve electropherograms – H5N1

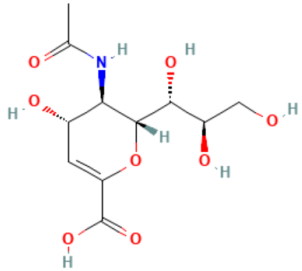


Substrate & Inhibitors

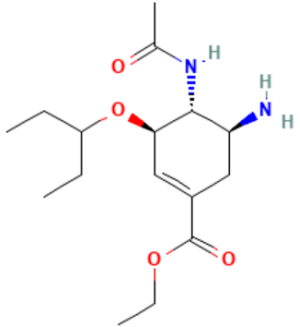


A. 6’-Siallylactose

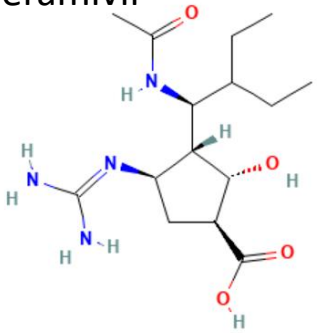
B. DANA



C. Oseltamivir Acid



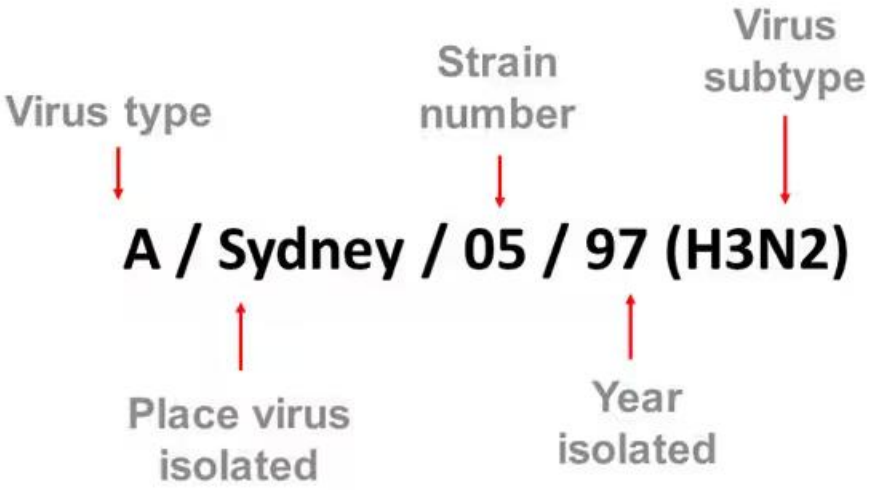
D. Peramivir



A.) PubChem. *6’-Sialyllactose*. pubchem.ncbi.nlm.nih.gov. https://pubchem.ncbi.nlm.nih.gov/compound/6_-Sialyllactose#section=2D-Structure
B.) 2-Deoxy-2,3-dehydro-n-acetyl-neuraminic acid | C11H17NO8 | CID 65309 - PubChem (nih.gov)
C. Oseltamivir | C16H28N2O4 | CID 65028 - PubChem (nih.gov)
d. Peramivir | C15H28N4O4 | CID 154234 - PubChem (nih.gov)

Naming Influenza Viruses

strain A/Brevig Mission/1/1918 H1N1

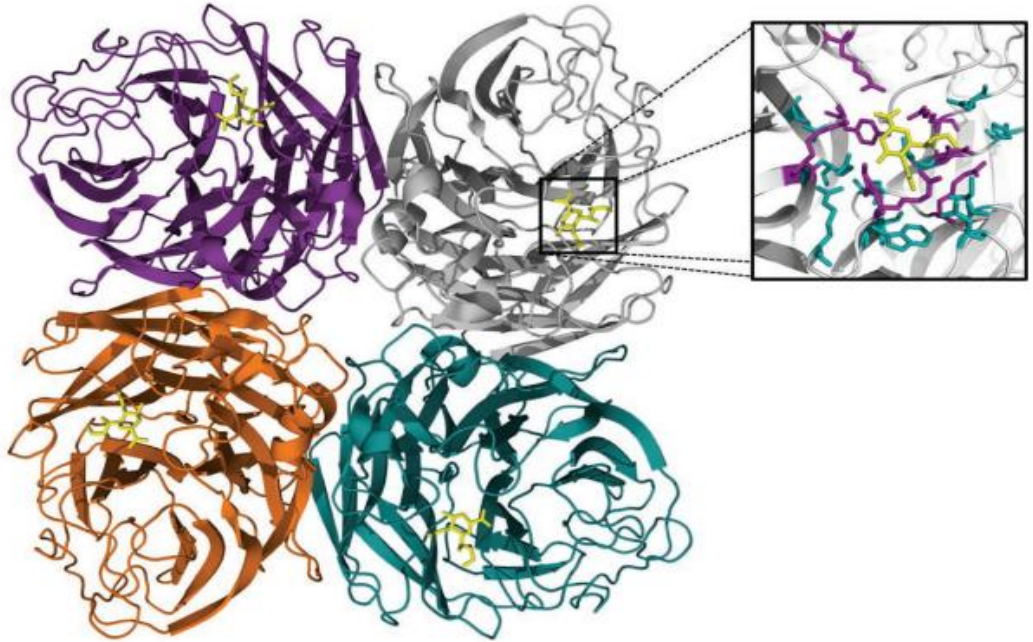


https://www.cdc.gov/flu/about/viruses/types.htm

Residues within catalytic site

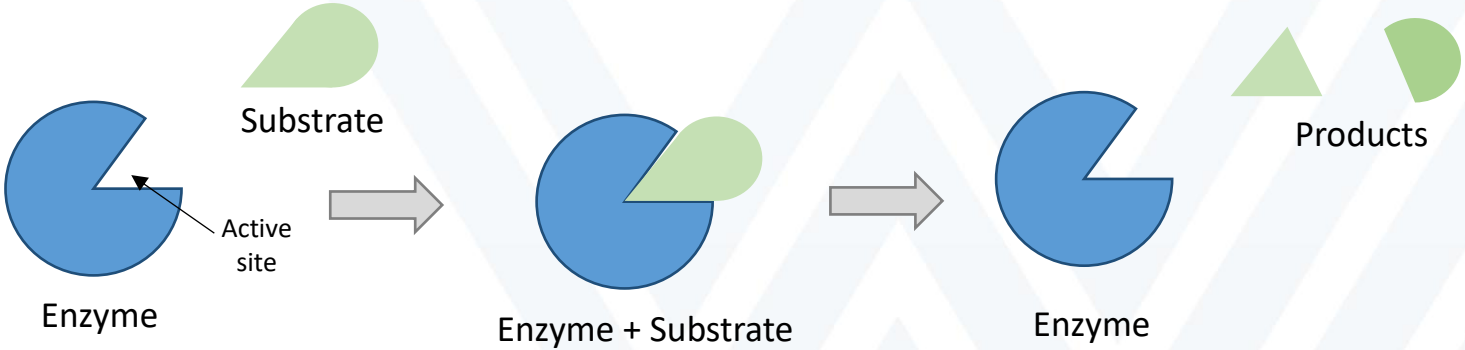
Charged residues such as:

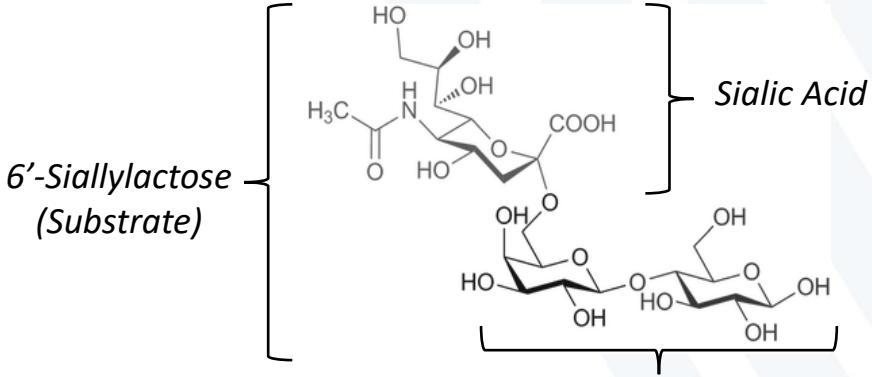
- Arg 118 (+)
- Asp 151(-)
- Arg 152 (+)
- Arg 224 (+)
- Glu 276 (-)
- Arg 292 (+)
- Arg 371 (+)
- Tyr406 (hydrophobic side chain)



McAuley, J. L.; Gilbertson, B. P.; Trifkovic, S.; Brown, L. E.; McKimm-Breschkin, J. L. Influenza Virus Neuraminidase Structure and Functions. Frontiers in Microbiology 2019, 10 (39). https://doi.org/10.3389/fmicb.2019.00039.

Enzymatic Reactions





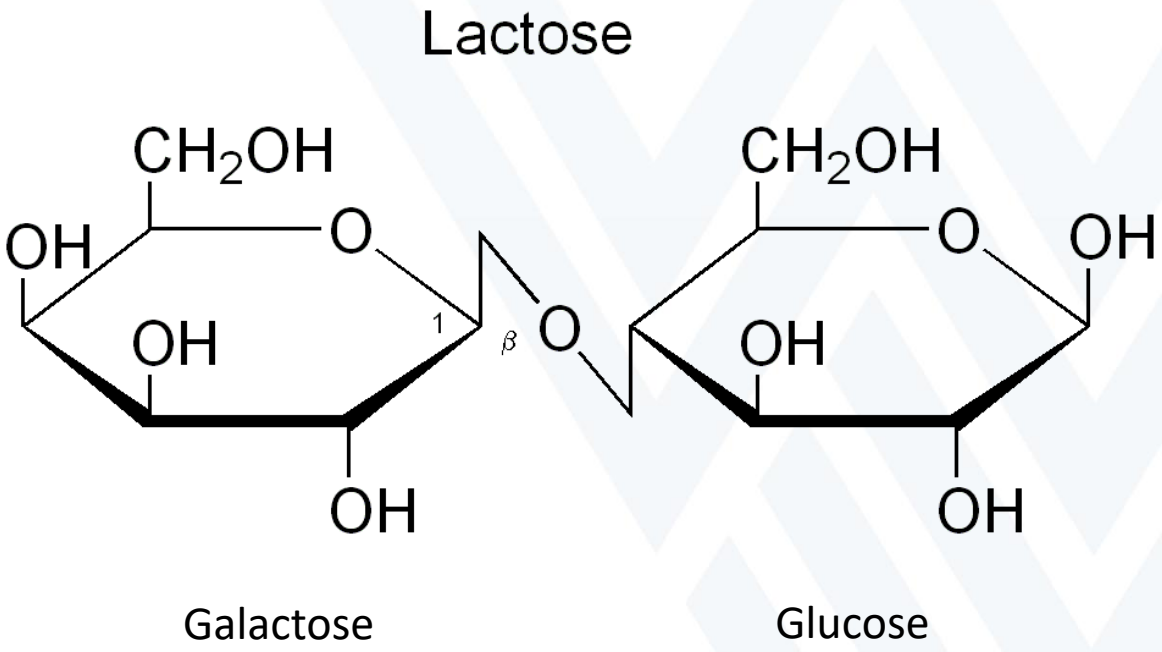
6'-Siallylactose (Substrate)

Sialic Acid

Lactose *(Fluorescently tagged product)*

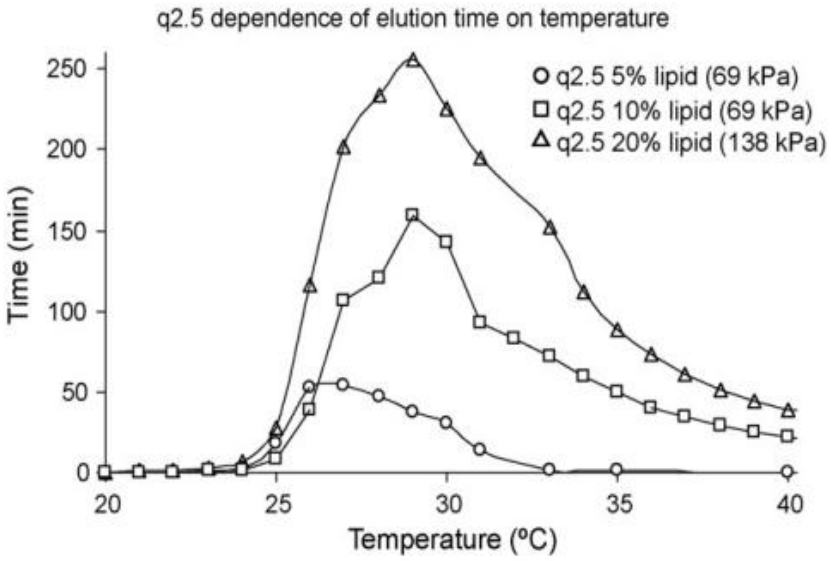
Pandey, R.P., Kim, D.H., Woo, J. *et al.* Broad-spectrum neutralization of avian influenza viruses by sialylated human milk oligosaccharides: *in vivo* assessment of 3'-sialyllactose against H9N2 in chickens. *Sci Rep* **8**, 2563 (2018). https://doi.org/10.1038/s41598-018-20955-4

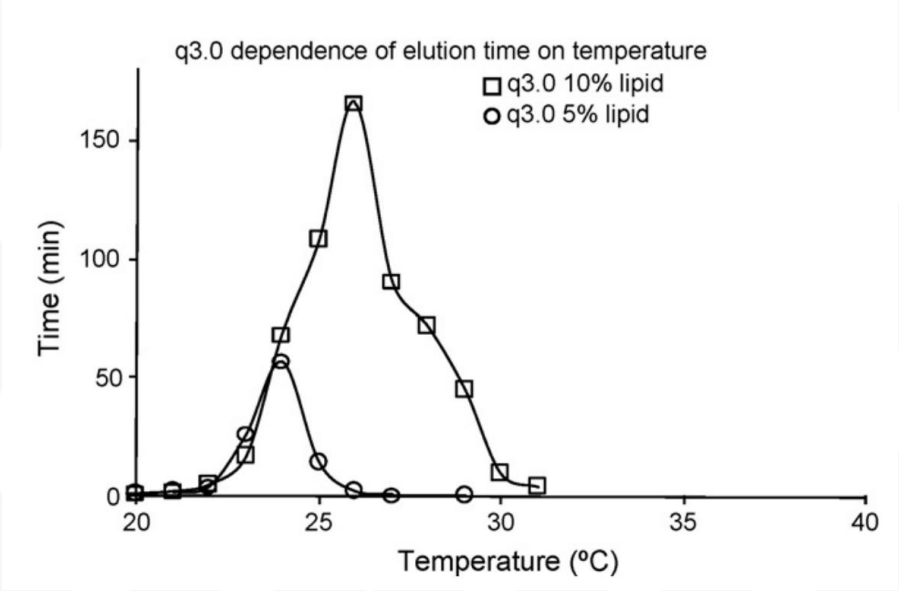
Lactose



Wobble. *simplified lactose chemical structure*. Wikimedia Commons. https://commons.wikimedia.org/wiki/File:Lactose(lac).png.

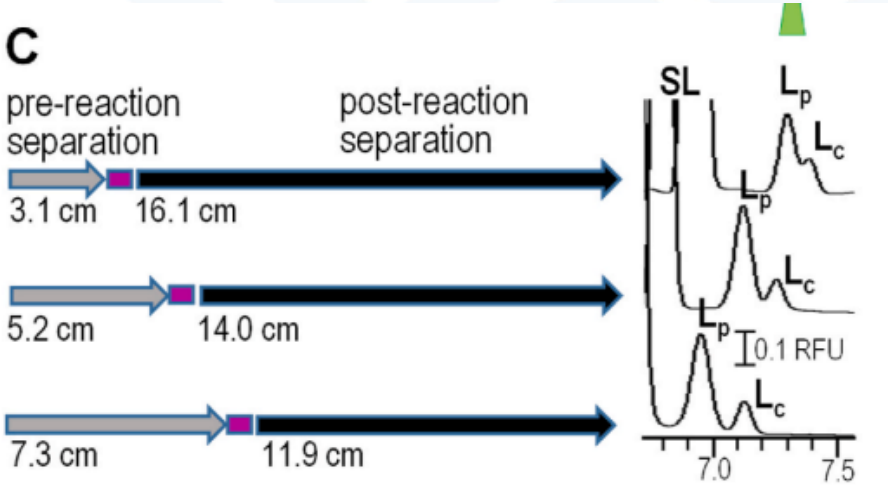
Nanogel





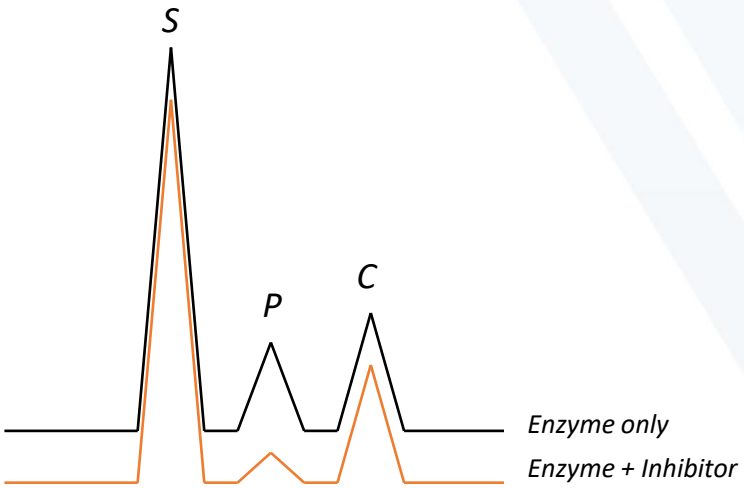
Theron J. Pappas, Lisa A. Holland, Fluid steering in a microfluidic chip by means of thermally responsive phospholipids, Sensors and Actuators B: Chemical, Volume 128, Issue 2, 2008, Pages 427-434, ISSN 0925-4005

Push studies



Casto-Boggess, L. D.; Holland, L. A. Fluorescent Parallel Electrophoresis Assay of Enzyme Inhibition. *Analytica Chimica Acta* **2024**, *1296*, 342268. https://doi.org/10.1016/j.aca.2024.342268.

% Conversion & % Activity Remaining Calculations



This is a drawling… Not real data!!

Equations:

1.) Enzyme Only

$$\%\ Conversion = \frac{P_{area}}{S_{area} + Parea}$$

2.) Enzyme + Inhibitor

$$\%\ Conversion = \frac{P_{area}}{S_{area} + Parea}$$

3.) % Activity Remaining

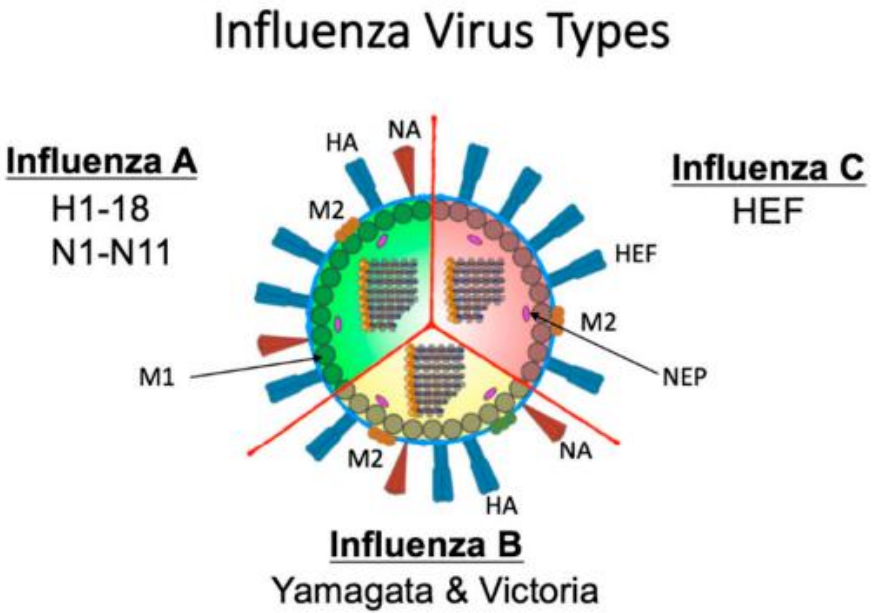
$$\%\ Activity\ Remaining = \frac{(Inhibitor\ \%\ conversion)}{(Enzyme\ \%\ conversion)} * 100$$

4.) % Inhibition

$$\%\ \text{Inhibiton} = 100 - \%\ \text{Activity Remining}$$

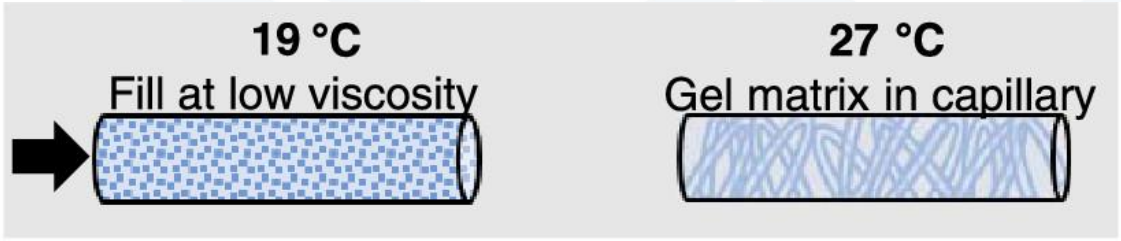
RFU = *relative fluorescence units*

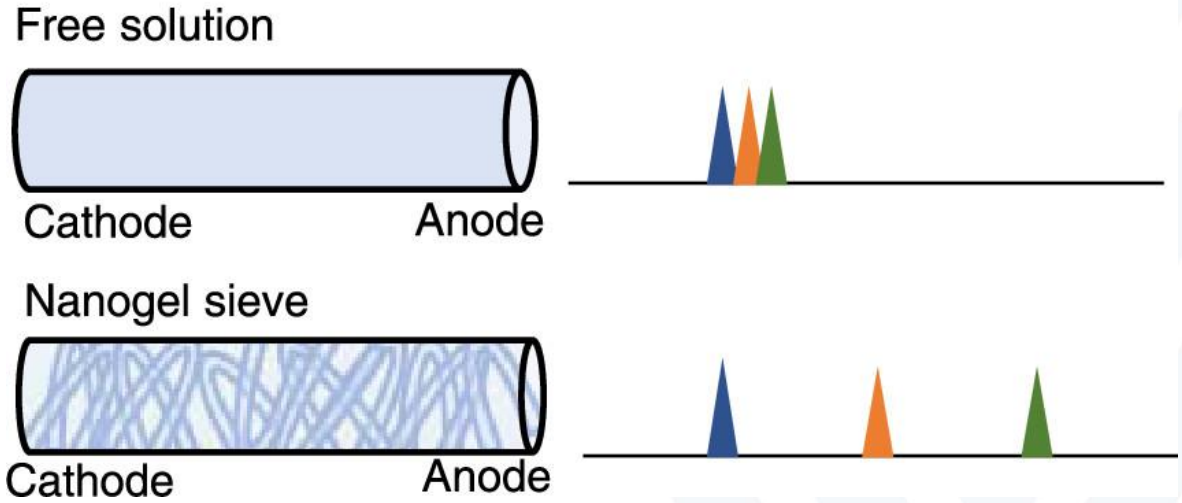
Influenza Types



Francis, Magen & King, Morgan & Kelvin, Alyson. (2019). Back to the Future for Influenza Preimmunity—Looking Back at Influenza Virus History to Infer the Outcome of Future Infections. Viruses. 11. 122. 10.3390/v11020122.

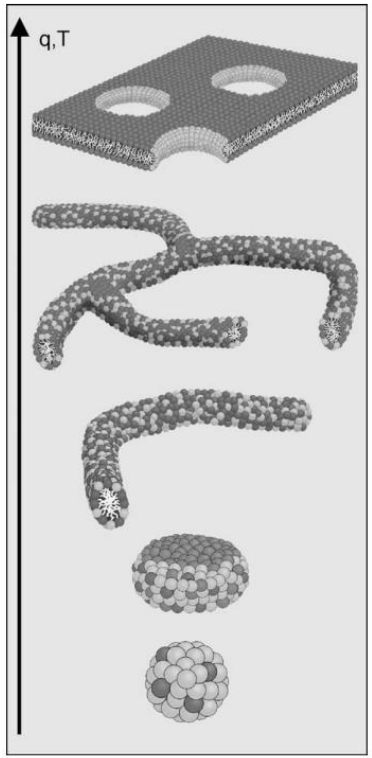
Sieving





Crihfield, C. L.; Holland, L. A. Protein Sieving with Capillary Nanogel Electrophoresis. *Analytical Chemistry* **2020**, *93* (3), 1537–1543. https://doi.org/10.1021/acs.analchem.0c03865.

Structure of Lipids



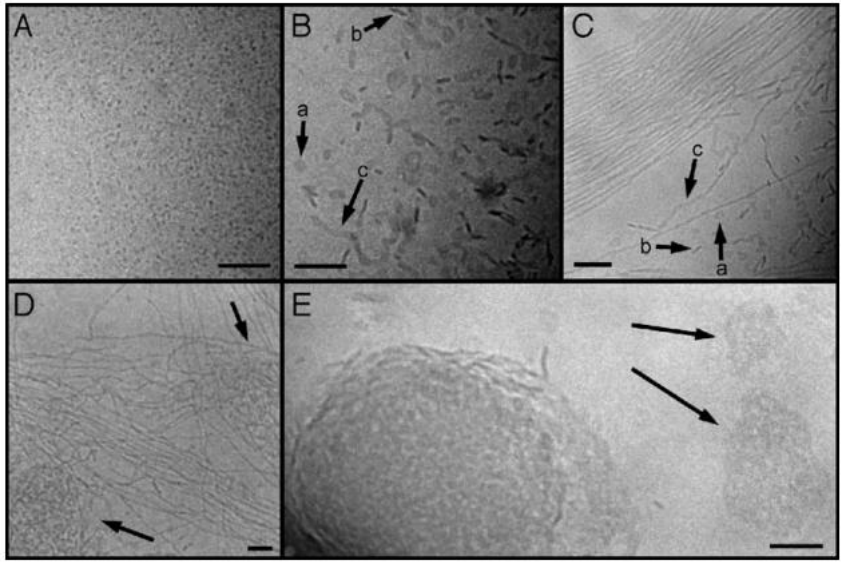
Lorens van Dam; Karlsson, G.; Edwards, K. Direct Observation and Characterization of DMPC/DHPC Aggregates under Conditions Relevant for Biological Solution NMR. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2004**, *1664* (2), 241–256. https://doi.org/10.1016/j.bbamem.2004.06.005.

Structure of Lipids

Using a Cryo-TEM

q=2.2
C = elongated distorted dics

q=3
C = quasi cylindrical micelle

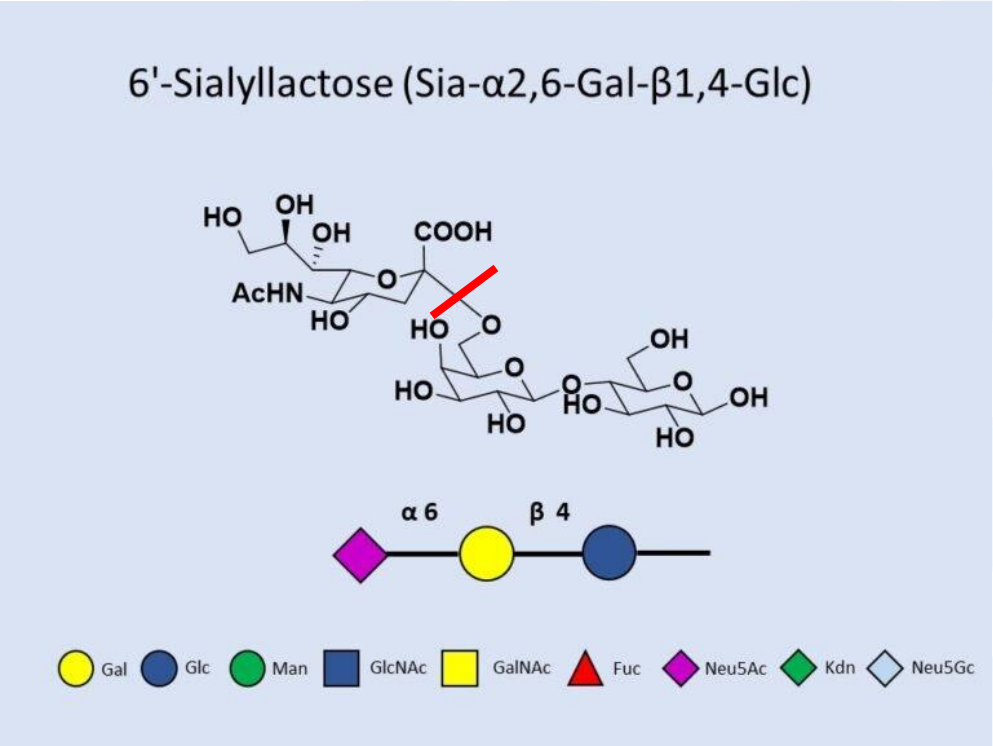


Arrows:
A = disc face
B = edge

Lorens van Dam; Karlsson, G.; Edwards, K. Direct Observation and Characterization of DMPC/DHPC Aggregates under Conditions Relevant for Biological Solution NMR. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2004**, *1664* (2), 241–256. https://doi.org/10.1016/j.bbamem.2004.06.005.

6’-SL

Alpha-keto acid sugars with a nine-carbon backbone

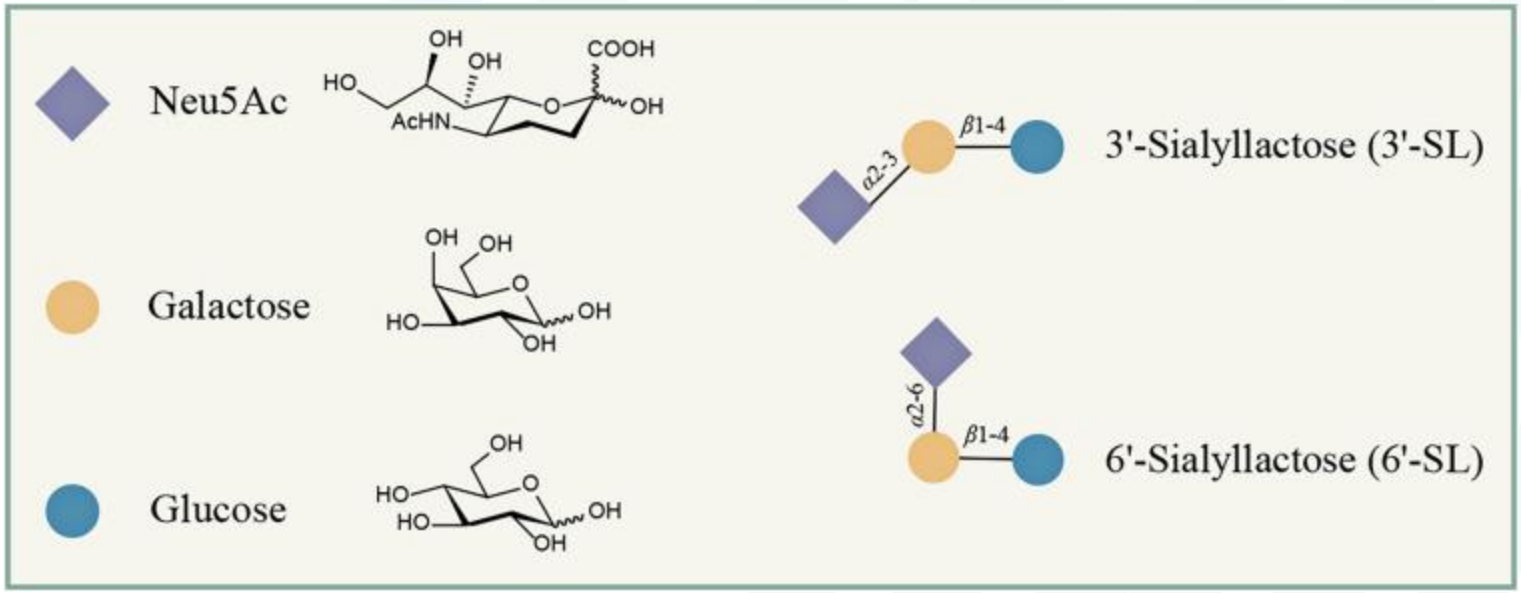


They play essential roles in

- Cell recognition
- Immune response
- Cell signaling
- Host-pathogen interactions

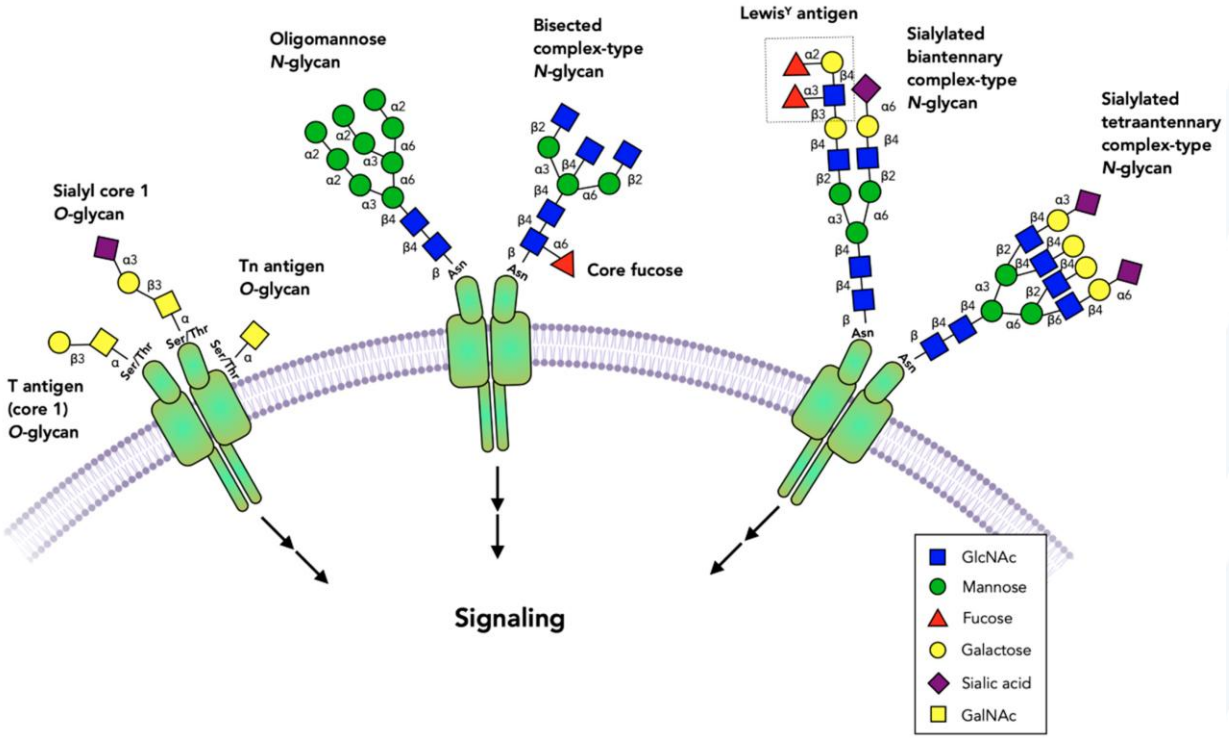
https://akonscientific.com/product/6-sialyllactose/

6’-SL



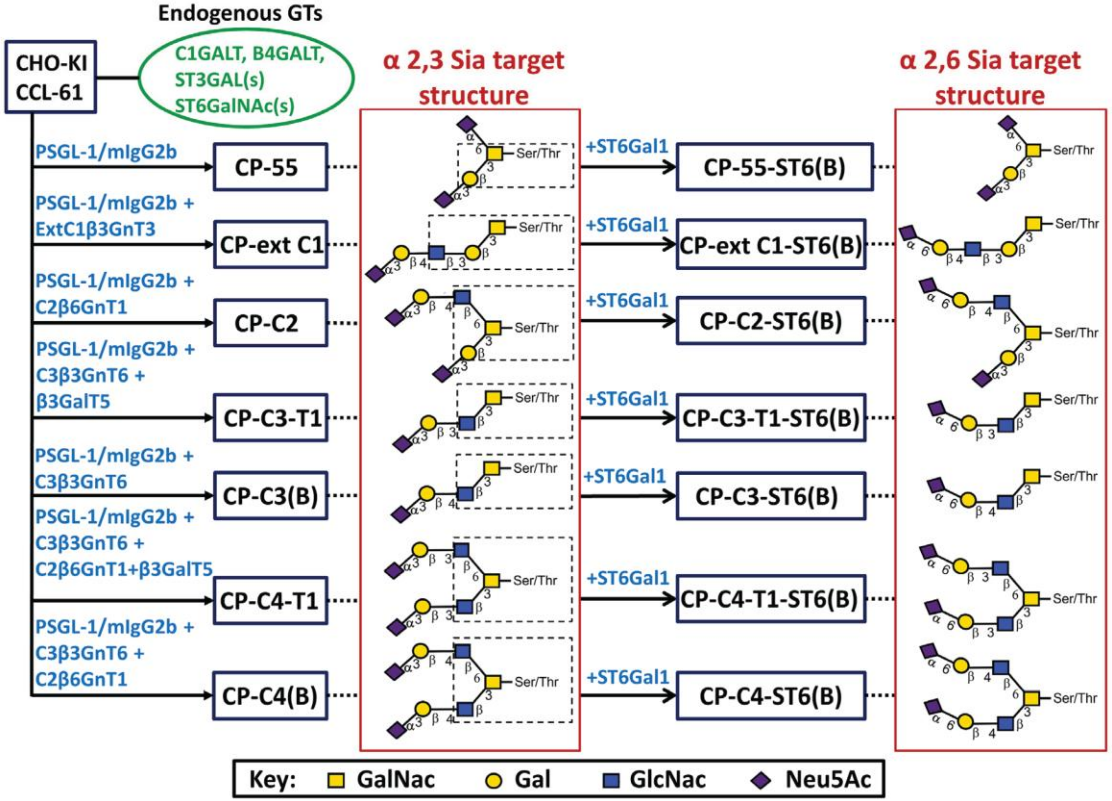
Zhu, Y.; Zhang, J.; Zhang, W.; Mu, W. Recent Progress on Health Effects and Biosynthesis of Two Key Sialylated Human Milk Oligosaccharides, 3’-Sialyllactose and 6’-Sialyllactose. *Biotechnology Advances* **2023**, *62*, 108058–108058. https://doi.org/10.1016/j.biotechadv.2022.108058.

O and N linked glycans



Gao, Y.; Luan, X.; Melamed, J.; Brockhausen, I. Role of Glycans on Key Cell Surface Receptors That Regulate Cell Proliferation and Cell Death. *Cells* **2021**, *10*, 1252. https://doi.org/10.3390/cells10051252

O and N linked glycans



Cherian, R. M.; Jin, C.; Liu, J.; Karlsson, N. G.; Holgersson, J. A Panel of Recombinant Mucins Carrying a Repertoire of Sialylated O-Glycans Based on Different Core Chains for Studies of Glycan Binding Proteins. *Biomolecules* **2015**, *5* (3), 1810–1831. https://doi.org/10.3390/biom5031810.