

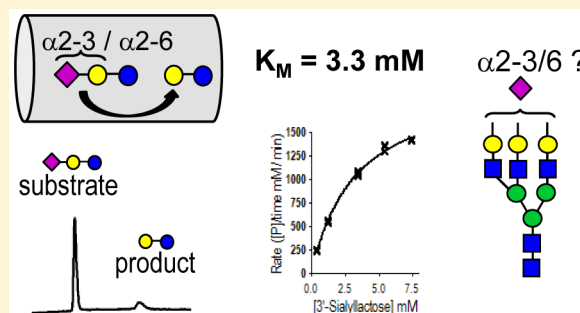
Microscale Measurements of Michaelis–Menten Constants of Neuraminidase with Nanogel Capillary Electrophoresis for the Determination of the Sialic Acid Linkage

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S Supporting Information

ABSTRACT: Phospholipid nanogels enhance the stability and performance of the exoglycosidase enzyme neuraminidase and are used to create a fixed zone of enzyme within a capillary. With nanogels, there is no need to covalently immobilize the enzyme, as it is physically constrained. This enables rapid quantification of Michaelis–Menten constants (K_M) for different substrates and ultimately provides a means to quantify the linkage (i.e., 2-3 versus 2-6) of sialic acids. The fixed zone of enzyme is inexpensive and easily positioned in the capillary to support electrophoresis mediated microanalysis using neuraminidase to analyze sialic acid linkages. To circumvent the limitations of diffusion during static incubation, the incubation period is reproducibly achieved by varying the number of forward and reverse passes the substrate makes through the stationary fixed zone using in-capillary electrophoretic mixing. A K_M value of 3.3 ± 0.8 mM (V_{max} 2100 \pm 200 μ M/min) was obtained for 3'-sialyllactose labeled with 2-aminobenzoic acid using neuraminidase from *Clostridium perfringens* that cleaves sialic acid monomers with an α 2-3,6,8,9 linkage, which is similar to values reported in the literature that required benchtop analyses. The enzyme cleaves the 2-3 linkage faster than the 2-6, and a K_M of 2 ± 1 mM (V_{max} 400 \pm 100 μ M/min) was obtained for the 6'-sialyllactose substrate. An alternative neuraminidase selective for 2-3 sialic acid linkages generated a K_M value of 3 ± 2 mM (V_{max} 900 \pm 300 μ M/min) for 3'-sialyllactose. With a knowledge of V_{max} the method was applied to a mixture of 2-3 and 2-6 sialyllactose as well as 2-3 and 2-6 sialylated triantennary glycan. Nanogel electrophoresis is an inexpensive, rapid, and simple alternative to current technologies used to distinguish the composition of 3' and 6' sialic acid linkages.



Sialic acids, the common name for *N*-acetylneuraminic acids, are the terminal monomer on the nonreducing end of glycans. Sialic acids and enzymes associated with their synthesis or catabolism are involved in a number of cellular processes¹ and are implicated in physiological dysfunctions including cancer,^{2–5} antibody function,⁶ and inflammation.⁷ In proteins, asparagine-linked glycans contain a common core structure,⁸ such that can be capped by sialic acids that are adjacent to a galactose residue. These sialic acid–galactose sequences are linked from carbon 2 on the sialic acid to carbon 3 or 6 on the adjacent monomer. The position of this linkage is relevant to cancer,⁹ and as a result, the linkage chemistry is monitored. Although there is a critical need for routine sialic acid determinations, the challenges of linkage analysis, isomerization, and data interpretation make structural assignment difficult using current analytical technologies.

Oligosaccharides have been identified using benchtop sequencing with enzymes, but this requires a considerable amount of exoglycosidase and substantial incubation time.^{10–14} The cost, stability, and sample preparation related to the use of the enzyme are limiting factors. Ultimately, separation strategies that reduce the sample handling and sample volume are critical. Microflow systems can efficiently screen chemical processes, are

more stream-lined to develop and optimize reactions, and are used to predict the success of scaling up a method. There are a few barriers to rapidly assessing enzymatic processing on the microscale, which include the cost and lifetime of enzyme preparations. These barriers can be overcome with immobilized enzymes, which can have enhanced performance¹⁵ and have increased stability. Methods of covalent enzyme immobilization require mild derivatization conditions and extensive optimization, and this has led to the development of new strategies to physically confine enzymes without covalent modification.^{16,17}

To realize the full potential of enzymes in chemical assays, enzymes must be manipulated on the microscale without immobilization. In addition, the rate of enzyme catalysis must be established for different reaction conditions because the enzyme rate is specific for each substrate, and it is dependent upon the conditions used for the enzymatic reaction. Precise knowledge of enzyme activity can be quantified as Michaelis–Menten constants (K_M), and it is important to harnessing enzymes for biomolecule recognition, sequencing, and assess-

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ment. With this information, enzyme-based microscale analyses have the potential to shed light on the relationship between the biomolecular structure of the substrate and enzyme function. However, new analytical tools are required that stabilize enzyme performance and consume small quantities of enzyme in faster analyses.

Capillary electrophoresis has been adapted to utilize enzymes to improve detection limits and separation specificity with electrophoretically mediated microanalysis, commonly referred to as EMMA.¹⁸ This method relies upon differences in electrophoretic mobility of enzyme, substrate, and product for the analysis. EMMA is rapid, consumes nanoliter volumes of enzyme, and is reported extensively^{19–22} as a method to enhance detection limits or provide indirect detection. While not as prevalent, the use of EMMA to determine K_M is feasible if the enzyme remains in the native state, the enzyme does not adsorb to the capillary surface, and the electrophoretic differences of analyte, enzyme, and product facilitate separation. Significant optimization is required to ensure that the incubation conditions are compatible with the electrophoresis, and exquisite strategies to address this optimization have been described.^{23–25} These barriers are overcome with the use of phospholipid nanogels, which self-assemble to form a thermally responsive material that is suitable as a replaceable gel to sieve DNA in capillary electrophoresis,^{26,27} as a viscosity switch to close and open channels in microfluidics,^{28,29} and as a viscous additive to improve capillary electrophoresis separations of oligosaccharides.^{30–32} Nanogels are biocompatible materials that immobilize and pattern enzymes in microscale channels.

This paper outlines a new approach to EMMA that utilizes nanogels to physically constrain an enzyme in a separation capillary. Reconstitution of the neuraminidase enzyme in nanogel at low concentration results in a longer lifetime upon storage. This provides a means to cost-effectively use a single enzyme stock solution to deliver the subnanoliter volumes required for the capillary method. An electrophoretic mixing method is developed to circumvent diffusion limitation of static incubation. For the first time, nanogels are harnessed to precisely quantify the Michaelis–Menten constants of an enzyme with different degrees of stereospecificity in the presence of substrates with different linkage positions. The trisaccharide sialyllactose, which is *O*-(*N*-acetyl- α -neuraminosyl)-[2 \rightarrow 3(or 2 \rightarrow 6)]-*O*- β -D-galactopyranosyl-(1 \rightarrow 4)-*D*-glucose, is used as a model substrate to characterize neuraminidase. The different catalysis rates obtained for sialic acid residues with 2–3 or 2–6 linkages are used to analyze the linkage position of a mixture of 2–3 and 2–6 sialyllactose and in the sialylated triantennary glycan substrate mixture that contained both Gal β (1–3)GlcNAc and Gal β (1–4)GlcNAc. Similar results are achieved when a specific and nonspecific enzyme are used. The approach extends the nanogel separation method to general as well as specific enzymes.

MATERIALS AND METHODS

Preparation and Derivatization of Standards. Enzyme studies with an anticipated K_M in the mM range require that oligosaccharide substrate concentrations in the mM range be used to obtain a Michaelis–Menten curve. For the K_M studies, the substrate was labeled with a chromophore detected by UV–visible absorbance. The chromophore 2-aminobenzoic acid was used as a reagent to label the oligosaccharides as reported previously.³³ The reaction was performed with excess label to ensure 100% labeling efficiency to prevent any bias in the K_M

measurement. If the labeling reaction was not complete, then the concentration of the labeled substrate that was UV-absorbing would have been less than the total concentration of substrate (i.e., the combination of labeled and unlabeled substrate) and would confound the measurement of K_M . A ratio of 1400 nmol 2-aminobenzoic acid:200 nmol sialyllactose (7:1) was achieved by reacting 1 μ L volume of 0.2 M oligosaccharide dissolved in water with 1 μ L volume of 1.39 M 2-aminobenzoic acid and 23 μ L volume of 1 M sodium cyanoborohydride at 65 $^{\circ}$ C for 2 h in 0.5 M acetic acid (glacial acetic acid diluted with methanol). Once the reaction was complete, the reaction mixture was evaporated to dryness on a Savant SpeedVac concentrator (Thermo Scientific, Waltham, MA). Excess 2-aminobenzoic acid was removed from the labeling reaction using a Discovery DPA-6S solid phase extraction cartridge (50 mg packing material, Supelco, Bellefonte, PA) with slight modifications to a previously established literature procedure.³¹ Specifically, once the sample was loaded in the extraction cartridge, the 2-aminobenzoic acid was eluted using 10 mL of 95% acetonitrile, 5% aqueous 1 mM triethylamine, and the retained sugars were eluted from the cartridge using 3 mL of aqueous 25 mM triethylamine. To ensure that all of the sialyllactose was labeled and recovered from the purification process, it was compared against a second reaction performed with excess sialyllactose as described in the [Supporting Information](#).

Studies designed to distinguish the linkage position of sialyllactose or glycan were accomplished with substrate concentrations in the nM range. For these linkage position studies the substrate was labeled with a chromophore detected by fluorescence. The fluorescently conjugated oligosaccharides and glycans were labeled as previously described³⁴ with slight modifications. Glycan labeling was accomplished using 100 mM 8-aminopyrene-1,3,6-trisulfonic acid in 20% acetic acid for a reaction of 7 nmol glycan:250 nmol dye in a total reaction volume of 5 μ L. The labeled glycan sample was purified using a 1 kDa molecular weight cutoff filter (#MCP001C41, Pall Corporation, Ann Arbor, MI). Sialyllactose labeling was accomplished using 100 mM 8-aminopyrene-1,3,6-trisulfonic acid in 20% acetic acid for a reaction of 5 nmol oligosaccharide:250 nmol dye in a total reaction volume of 5 μ L. The labeled sialyllactose samples were purified using the DPA-6S column as described for labeling with 2-aminobenzoic acid. Once purified, the samples were dried in a SpeedVac concentrator before reconstituting to 100 μ L in water and storing at -20 $^{\circ}$ C.

Neuraminidase in powder was reconstituted to a concentration of 33.6 mUnits/ μ L in 50 mM potassium phosphate with the pH adjusted to 5.2 using 1 M sodium hydroxide. The appropriate volume of master stock (i.e., less than or equal to 0.52 μ L) was diluted with 10% nanogel to a final volume of 50 μ L. For both neuraminidase enzymes, 1 Unit was defined as the amount of enzyme required to produce 1 μ mol of methylumbelliferone in 1 min at 37 $^{\circ}$ C, pH 5.0 from 2'-(4-methyl-umbelliferyl)- α -D-*N* acetylneuraminic acid.^{35,36}

Capillary Electrophoresis. Analyses were performed using a P/ACE MDQ or MDQ Plus (Sciex, Redwood City, CA) configured by the manufacturer with laser-induced fluorescence detection (3 mW air cooled argon ion laser or 3 mW solid state laser, with λ_{ex} = 488 nm, λ_{em} = 520 nm) and a photodiode array or UV–visible absorbance detector (monitored at 214 nm). A 25 μ m internal diameter, 360 μ m outer diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ) was used for

separation. Each day capillaries were prepared as previously reported.³² Unless otherwise noted, the background electrolyte was 100 mM 3-(*N*-morpholino)propanesulfonic acid buffered to pH 7. Phospholipids were prepared as described previously, aliquoted, and stored at $-20\text{ }^{\circ}\text{C}$.^{31,32,37} The phospholipid preparation, which was $q = 2.5$ (i.e., [DMPC]:[DHPC] = 2.5) and 10% phospholipid by weight, had low viscosity below the gel phase transition temperature and was easily introduced in the capillary at a temperature of $19\text{ }^{\circ}\text{C}$ or lower. Prior to each run, the capillary was held at $19\text{ }^{\circ}\text{C}$ and prepared as previously reported³¹ with slight modification as described in the [Supporting Information](#). Data collection and analysis were performed using 32 Karat Software version 7.0 (MDQ) or 10.2 (MDQ Plus). The Michaelis–Menten curves were fit using GraphPad Prism version 4.03 (San Diego, CA).

RESULTS AND DISCUSSION

Patterning Enzyme in Capillary. The nanogel fixed enzyme zone was achieved by creating a pseudostationary enzyme plug in phospholipid nanogel, as depicted in [Figure 1](#).

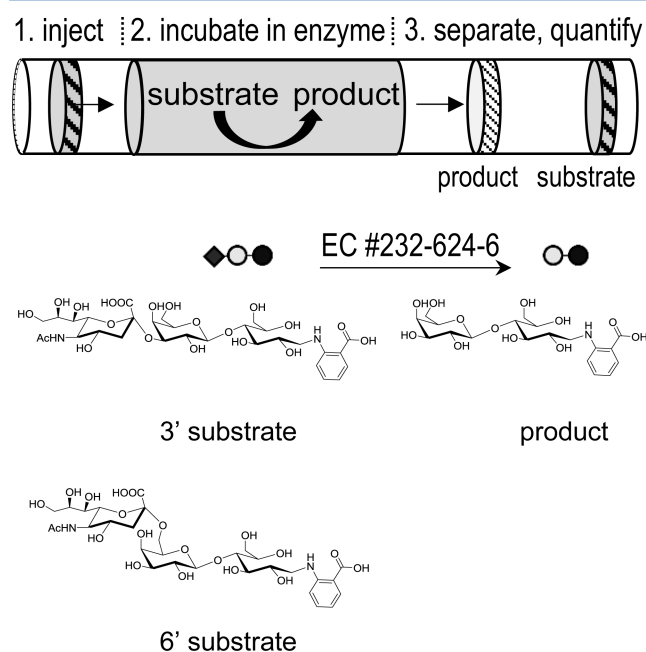


Figure 1. Depiction of electrophoretic migration of substrate in-capillary containing enzyme in a fixed zone. The 3'- or 6'-sialyllactose was incubated in enzyme and converted to lactose. The nonreducing end of the oligosaccharide was labeled with a chromophore (e.g., 2-aminobenzoic acid, for UV-absorbance detection).

Enzyme prepared in the nanogel was introduced into the capillary at $19\text{ }^{\circ}\text{C}$, which was a temperature that maintained fluid-like viscosity of the material.^{28,29} At this temperature, the enzyme was positioned in the thermally controlled region of the capillary to ensure that the desired temperature was maintained during analyses. The temperature was then increased to $37\text{ }^{\circ}\text{C}$ to form a viscous gel²⁸ that maintained the enzyme position within the capillary and supported the enzyme reaction. Two model substrates, sialyllactose with different linkages shown in [Figure 1](#), were used. Once the fixed enzyme zone was patterned in the capillary, as seen in [Figure 1](#), the substrate was electrokinetically driven into the enzyme zone. Following incubation, substrate was then separated from product ([Figure 1](#)). The catalysis rate of neuraminidase was

quantified by monitoring the conversion of substrate to the lactose product ([Figure 1](#)). The separation of sialyllactose and lactose did not require the use of phospholipid nanogel to resolve these analyte peaks. Therefore, the fixed enzyme zone was embedded in an aqueous solution of 100 mM background electrolyte buffered to pH 7. The position of the enzyme zone within the capillary was established by migrating the substrate to a particular position, incubating substrate with enzyme, and then separating and quantifying the peak areas of the substrate and enzymatically generated product. The substrate positions that generated larger product peak area coincided with the position of the enzyme zone, as summarized in [Figure S-1](#) in the [Supporting Information](#). With the position of the enzyme zone established, the impact of nanogel on the enzyme catalysis rate was determined.

Effect of Phospholipid Nanogel on Enzyme Performance. Proteins are reconstituted in a variety of additives to maintain structure and function.^{38–41} These additives influence both stability and activity in a complex manner.⁴² Phospholipids interact with proteins through different mechanisms.⁴³ Physical constraint of enzymes with lipids has been reported using edge stabilized phospholipid nanodiscs to study enzyme catalysis of membrane protein.^{44,45} Although phospholipids are used to eliminate nonspecific adsorption of proteins to surfaces,^{46–48} they are underexplored as an additive for soluble proteins.

To evaluate the effect of phospholipid nanogel on neuraminidase, the enzyme performance was monitored either in a traditional aqueous solution of 50 mM potassium phosphate adjusted to a pH of 5.2 with sodium hydroxide or in nanogel comprised of phospholipid dissolved in the same aqueous solution. The preparations of neuraminidase were diluted to a concentration of 350, 250, or 150 $\mu\text{Units}/\mu\text{L}$, and the enzyme activity was evaluated by quantifying the rate of conversion of sialyllactose substrate to lactose. The rates summarized in [Figure 2](#) demonstrated that in the presence of nanogel the enzyme retained high activity for the 30-day period regardless of concentration. The stability of the enzyme diluted in the aqueous buffer was concentration-dependent. The 350 $\mu\text{Units}/\mu\text{L}$ did not change ([Figure 2A](#)). However, the 250 $\mu\text{Units}/\mu\text{L}$ ([Figure 2B](#)) and 150 $\mu\text{Units}/\mu\text{L}$ ([Figure 2C](#)) enzyme solutions decreased in activity until there was no detectable activity on days 3 and 1, respectively. The reaction velocity of 350, 250, and 150 $\mu\text{Units}/\mu\text{L}$ enzyme was 1100 ± 50 , 830 ± 50 , and 500 ± 40 , respectively. At the highest concentration of 350 $\mu\text{Units}/\mu\text{L}$, the reaction velocity in nanogel was 1.5 times higher than the reaction velocity in aqueous electrolyte ($750 \pm 60\text{ }\mu\text{M}$ lactose/min). For the lower enzyme concentrations, the reaction velocity in nanogel as compared to that obtained in aqueous electrolyte on day 1 was 1.7 ($500 \pm 10\text{ }\mu\text{M}$ lactose/min) and 2.5 ($200 \pm 10\text{ }\mu\text{M}$ lactose/min) times higher. These findings were in agreement with literature evidence that lipid monolayers improve the performance of exoglycosidase enzymes, including galactosidase^{49–51} and neuraminidase.⁵² The improvement in stability observed at the lower enzyme concentration may be due to molecular crowding. The compaction of the enzyme by molecules in the solution maintained enzyme in a folded state and impacted the enzyme rate if the effective concentration of active protein was increased.^{53,54}

Optimizing Incubation Times in the Fixed Enzyme Zone. When enzyme catalysis was performed in-capillary using static incubations, the measured velocity ([Table 1](#)) decreased

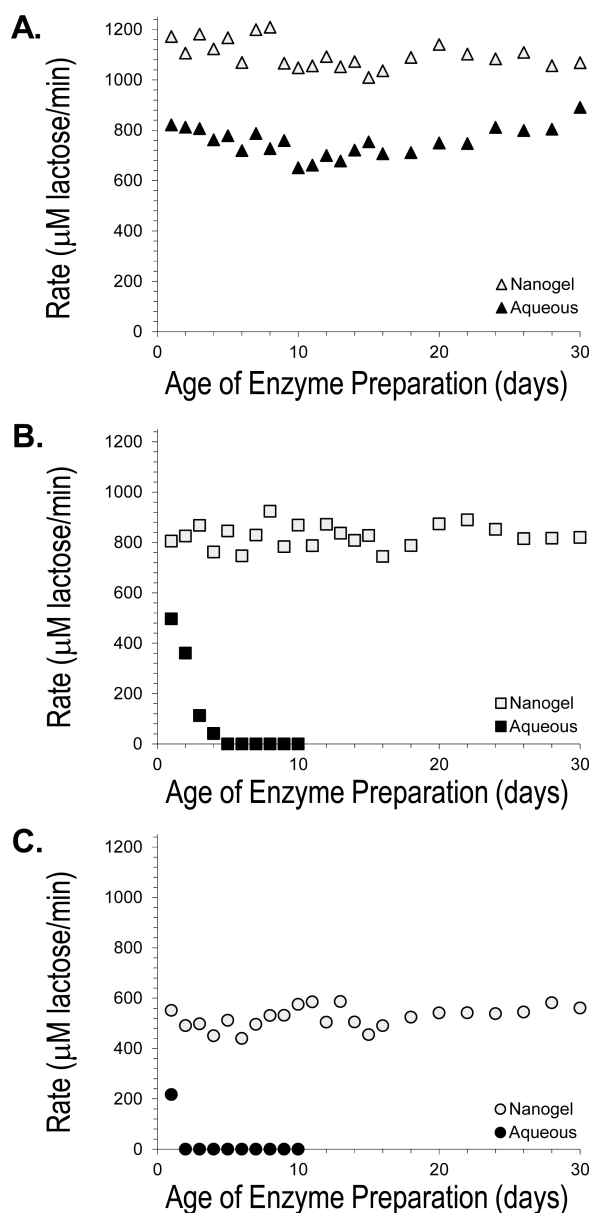


Figure 2. Plot of enzyme activity. Substrate was incubated in α -2,3,6,8,9 neuraminidase suspended in phospholipid nanogel (10% lipid with [DMPC]/[DHPC] = 2.5 in 50 mM potassium phosphate pH adjusted to 5.2 with sodium hydroxide) or in the same aqueous solution devoid of phospholipid at an enzyme concentration of 350 μ Units/ μ L (2A), 250 μ Units/ μ L (2B), and 150 μ Units/ μ L (2C). Substrate (5.4 mM 3'-sialyllactose) was incubated in enzyme for 2 min at 37 °C and separated. Separations were performed at 37 °C in a 25 μ m i.d. capillary, with an effective length of 30 cm and $E = 500$ V/cm (reverse polarity).

with increasing incubation time. This diffusion-limited interaction between substrate and enzyme hindered the quantitative determination of enzyme performance (i.e., K_M values) because enzyme rates must be consistent regardless of incubation time. In nanogel electrophoresis, the limitations of diffusion-based transport of substrate and product were overcome by electrophoretic mixing. In this electrophoretic mixing approach, the sustained contact between substrate and enzyme was achieved by alternating the polarity of the applied voltage to reversibly drive the substrate through the enzyme zone as depicted in Figure 3. The number of passes and length of each

Table 1. Effect of Substrate Delivery on Rate^a

Time, s	static, ^b μ M/s	mixed, ^c μ M/s
40	13.9 \pm 0.6	9.9 \pm 0.9
60	11.6 \pm 0.3	9.5 \pm 0.5
100	8.4 \pm 0.3	8.5 \pm 0.4
200	7.3 \pm 0.1	8.4 \pm 0.5
Ave ^d	10 \pm 3 (30%)	9.1 \pm 0.7 (9%)

^aData are averages ($n = 3$) using 5.4 mM 3'-sialyllactose labeled with 2-aminobenzoic acid and 336 μ Units/ μ L α -2,3,6,8,9 neuraminidase in nanogel at 37 °C. ^bIncubation performed with $E = 0$ V/cm after driving the substrate to the center of the fixed enzyme zone. ^cPerformed by electrophoresing substrate through the enzyme with $E = 250$ V/cm for multiple forward (F) and reverse (R) passes of: 40 s (20F-10R-10F), 60 s (20F-20R-20R), 100 s ([20F-20R]₂-20F), or 200 s ([20F-20R]₄-20F-10R-10F). ^dData are averages of rates at four incubation times.

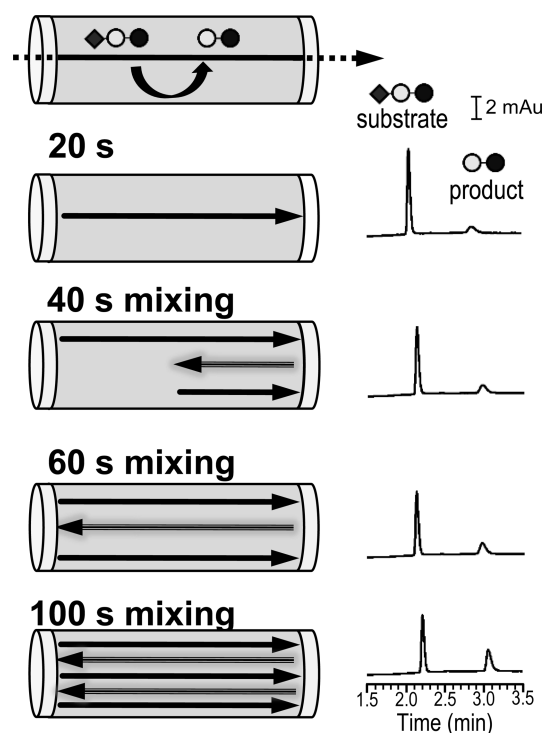


Figure 3. Conceptual diagrams of multipass electrophoretic mixing for control of the incubation time in the enzyme. The mixing duration was determined by the total time the substrate passes through the zone. Mixing may require that passes of different times are combined as in the case of a 40 s incubation. The positions of the arrows in the zone are conceptual and do not imply that the substrate traversed from the top of the zone to the bottom with each successive pass. The electropherograms were obtained using substrate of 5.4 mM 3'-sialyllactose and 336 μ Units/ μ L of α -2,3,6,8,9 neuraminidase that was suspended in phospholipid nanogel. Experimental conditions are as described in Figure 2.

pass were selected to achieve the desired incubation time. The process of mixing in the fixed zone was feasible when the position of the enzyme was profiled in the capillary to determine the zone boundaries as summarized in Figure S-1 in the Supporting Information. The rate of enzyme catalysis increased with the electrophoretic velocity due to the increased probability of enzyme–substrate collision. An Ohm's law plot was performed (Figure S-2 in the Supporting Information) to confirm that this was not due to Joule heating. As summarized

in Table 1, electrophoretic mixing generated catalysis with a precision of 9% relative standard deviation for four different electrophoretic sweep times as compared to 30% relative standard deviation for four different static incubation times.

Determination of Michaelis–Menten Constants for Neuraminidase. The in-capillary enzymatic method was well-suited to characterize the relationship between the substrate concentration and the performance of the rate of enzyme production. The velocity of product formation was calculated as product concentration/incubation time and then plotted to determine the Michaelis–Menten constant. An accurate measurement of enzyme performance required that the classical rules derived for K_M determinations were followed.⁵⁵ In particular, no more than 10% of the substrate could be converted to product because the presence of product inhibits the rate of reaction. An additional consideration was that the amount of product formed must fall within the linear range of quantification for the method of detection. A K_M curve fit using nonlinear regression required 2 points at or near saturation and an additional 3 points distributed across the region where the velocity changes significantly with substrate concentration.

The application of nanogel for in-capillary determination of enzyme activity was demonstrated with a neuraminidase that cleaves sialic acid with an α 2-3, 2-6, 2-8, or 2-9 linkage. Each determination utilized nanogel enzymolysis with mixing at an enzyme concentration of 336 $\mu\text{Units}/\mu\text{L}$ prepared in nanogel. Incubations were performed at 37 °C. Electrophoretic mixing was performed during the incubation period to obviate the time-related dependence of velocity on incubation time observed in static incubations. A set of five electropherograms was obtained with 3'-sialyllactose at concentrations of 0.40, 1.25, 3.4, 5.4, and 7.4 mM (Figure 4A). The peak area for the lactose product obtained in each separation was quantified (Figure 4A inset). The rate of enzymatic conversion was calculated as the concentration of lactose produced over the incubation time. These determinations were performed in triplicate; 15 values were plotted as shown in Figure 4B and fit using nonlinear regression to obtain the K_M of 3.3 mM with a standard deviation of 0.8 mM.

Literature values have been reported for neuraminidase from *Clostridium perfringens* with related sialyllactose substrate. A K_M value of 2.4 with unlabeled 3'-sialyllactose was obtained using potassium acetate buffered at pH 4.5, 37 °C, quantifying the product concentration with thiobarbituric-facilitated colorimetric detection with the alkali-Ehrlich method.⁵⁶ A K_M value of 2.2 ± 0.3 mM with unlabeled 3'-sialyl-*N*-acetylglucosamine was obtained using 50 mM potassium phosphate at a pH adjusted to 5.16 with sodium hydroxide, 37 °C, quantifying the product with anion exchange chromatography coupled to electrochemical detection.⁵⁷ K_M values are difficult to compare given that slight differences exist in the hydrolysis reaction conditions. In addition, the substrate used in this work was labeled with 2-aminobenzoic acid. In light of these differences in reactions, the experimental K_M value was similar, as it was approximately 1.5 times higher than these literature values.

The applicability of nanogel for enzyme characterization was extended to study changes in the rate of catalysis for different substrates and different neuraminidase enzymes. The activity and specificity of α 2-3,6,8,9 neuraminidase on 6'-sialyllactose, a substrate with different linkage positions, were examined using the nanogel capillary electrophoresis. The results demonstrated the utility of the method to screen enzymes of different specificity for the conversion of different substrate molecules.

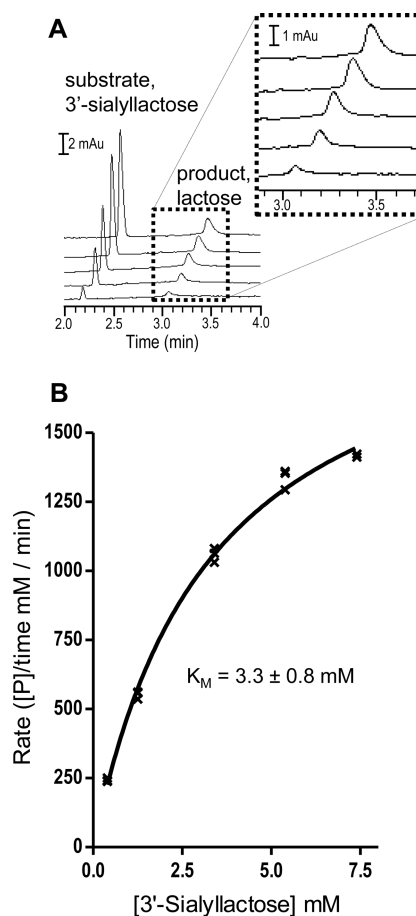


Figure 4. (A) Electropherograms of 3'-sialyllactose substrate and the lactose product generated after enzymatic reaction. The traces were offset in time by 0.1 min for visualization from the lower to upper traces at the following 3'-sialyllactose concentrations: 0.40 mM, 1.25 mM, 3.4 mM, 5.4 mM, and 7.4 mM. The increase in the area of lactose with increasing 3'-sialyllactose concentration is depicted in the inset. The Michaelis–Menten curve (part B) was generated by plotting the substrate concentration versus the rate of product formation for 3'-sialyllactose with 336 $\mu\text{Units}/\mu\text{L}$ α 2-3,6,8,9 neuraminidase in nanogel. Experimental conditions are as described in Figure 2.

The values in Table S-1 in the Supporting Information were obtained under identical pH, ionic strength, substrate concentration, and enzyme concentration. The K_M value from the Michaelis–Menten curve for the general neuraminidase acting on 6'-sialyllactose was quantified as 2 ± 1 mM (Figure S-3 in the Supporting Information). A similar K_M value of 1.2 mM was reported for general neuraminidase and 6'-sialyllactose with slightly different reaction conditions (i.e., 100 mM sodium/potassium phosphate pH adjusted to 5.4, 37 °C).⁵⁸ The K_M value was 3 ± 2 mM for the specific neuraminidase cleaving sialic acid from 3-sialyllactose, as shown in Figure S-4 in the Supporting Information. Incubation of the α 2-3 neuraminidase with 6'-sialyllactose resulted in no production of lactose, confirming that the specific enzyme was effective at cleaving only 3' sialic acid residues.

The K_M value was independent of enzyme concentration, while V_{max} was dependent upon neuraminidase concentration. Curves were obtained at the same enzyme concentration of 336 $\mu\text{Units}/\mu\text{L}$. For this enzyme, the manufacturer reported that the rate was greater for 2-3 than 2-6.⁵⁹ The V_{max} values (Table S-1 in the Supporting Information), which describe the rate of

enzyme catalysis when fully saturated by substrate, indicated that the catalytic rate of the general enzyme (i.e., α 2-3,6,8,9 neuraminidase) was five times faster for 2-3 substrate when compared to the 2-6 substrate. Furthermore, for conversion of 2-3 sialic acid substrate, the general enzyme was twice as fast as the 3' specific enzyme. These findings were utilized to apply neuraminidase to rapidly distinguish the sialic acid linkage.

Differentiating the Sialic Acid Linkage with Neuraminidase. With knowledge of the enzyme catalysis, the application of a fixed zone of neuraminidase to distinguish sialyllactose linkage position was demonstrated. A nanogel separation (Figure 5 trace A) of the 6' and the 3'-sialyllactose

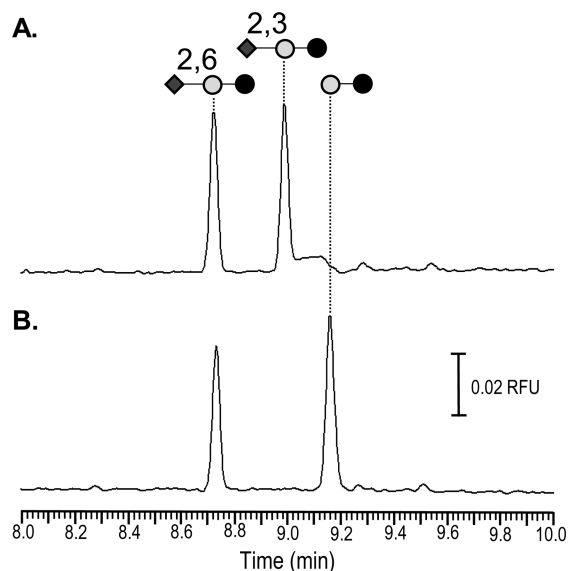


Figure 5. Electropherograms of 3' and 6'-sialyllactose to demonstrate the use of α 2-3 neuraminidase to determine substrate linkages. The separation of 3'- and 6'-sialyllactose in trace A was obtained in the absence of enzyme. Trace B incorporated a fixed zone of α 2-3 neuraminidase (loaded at 69 kPa for 7 s) suspended in phospholipid nanogel (10% lipid with [DMPC]/[DHPC] = 2.5 in 50 mM potassium phosphate pH adjusted to 5.2 with sodium hydroxide) at a concentration of 8 mUnits/ μ L to distinguish 2-3 from 2-6 sialyllactose by cleaving all the sialic acid on the 3'-sialyllactose. Separations and incubations were performed at 37 °C in a 25 μ m i.d. capillary filled with nanogel, with an effective length of 50 cm and $E = 400$ V/cm (reverse polarity).

in the absence of enzyme revealed that the 2-6 and the 2-3 sialyllactose linkages were separated. When a mixture of 2-3 and 2-6 sialyllactose was subjected to a fixed enzyme zone of 8 mUnits/ μ L neuraminidase specific for 2-3 sialic acid linkage, only the 2-3 sialyllactose was converted to lactose (Figure 5 trace B). Triplicate runs with and without the fixed enzyme zone generated peaks that had a relative standard deviation in area of 4% and in time of 1%. These studies were performed using fluorescence detection. Data found in Figure S-5 in the Supporting Information expanded on these analyses by incorporating a fixed enzyme zone of 0.6 mUnits/ μ L α 2-3 neuraminidase to confirm 3'-sialyllactose was converted to lactose and not just shifted in time.

A similar strategy to identify the sialic acid linkage was applied to the mixture of trisialylated triantennary complex N-glycan shown in Figure 6. The N-glycan determinations were accomplished using the fixed nanogel enzyme zone embedded in capillary filled with an enzyme-free nanogel. A nanogel

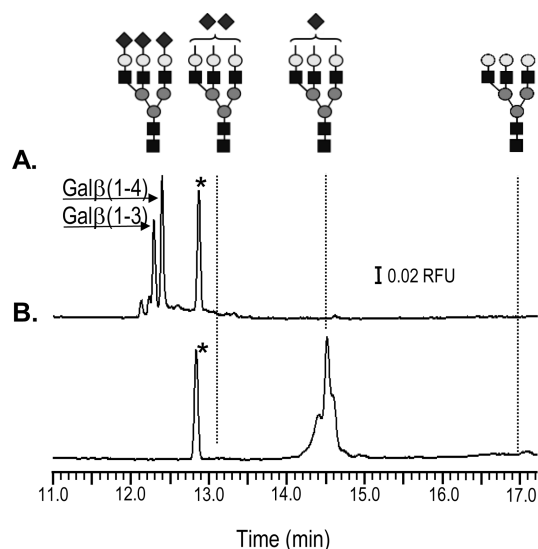


Figure 6. Electropherograms of 0.15 nM trisialylated triantennary complex N-glycan incubated in α 2-3 neuraminidase to determine linkage position. Trace A was obtained without enzyme, while trace B was obtained using 80 mUnits/ μ L α 2-3 neuraminidase suspended in phospholipid nanogel. The fixed zone of α 2-3 neuraminidase (loaded at 69 kPa for 35 s) generated an electropherogram of N-glycan devoid of all 2-3 linked sialic acid. The peak marked with the asterisk was present in the reaction blank. Experimental conditions were the same as those used in Figure 5.

separation (Figure 6 trace A) of the N-glycan in the absence of enzyme revealed that the N-glycan was fully sialylated and contained both Gal β (1-3)GlcNAc and Gal β (1-4)GlcNAc. These isomers were resolved electrophoretically when the capillary was filled with nanogel.³⁰ The separation in trace B was obtained with a fixed zone of 80 mUnits/ μ L α 2-3 neuraminidase, resulting in complete cleavage of the 2-3 linked sialic acid. A higher concentration and larger fixed enzyme zone increased the relative standard deviation in area to 11% and in time to 2% ($n = 3$), but was required to desialylate the trisialylated N-glycan. No product peaks were generated that contain two sialic acids. A product peak associated with asialo N-glycan (Figure 6 trace A) comprised <1% of the area of the N-glycan peaks. The peaks obtained in these traces indicated that two-thirds of the sialic acid linkages were 2-3, while one-third were 2-6.

To demonstrate that different catalytic rates of general neuraminidase for 2-3 vs 2-6 sialic acid linkages could be harnessed to distinguish linkage position, the experiment was repeated using general neuraminidase. The separation in trace A of Figure 7 was obtained without enzyme, while the separation in trace B was obtained with a fixed enzyme zone of 4 μ Units/ μ L general neuraminidase. This resulted in complete cleavage of the 2-3 linked sialic acid. Triplicate runs with the fixed zone of general enzyme generated peaks that had a relative standard deviation in area of 10% and in time of 2%. No product peaks were generated that contained two sialic acids. The separation in trace C of Figure 7 was obtained with a fixed zone of 2.4 mUnits/ μ L general neuraminidase, resulting in complete cleavage of sialic acid. The same sialic acid linkage composition obtained with specific enzyme was obtained using general enzyme by controlling the concentration of the general neuraminidase.

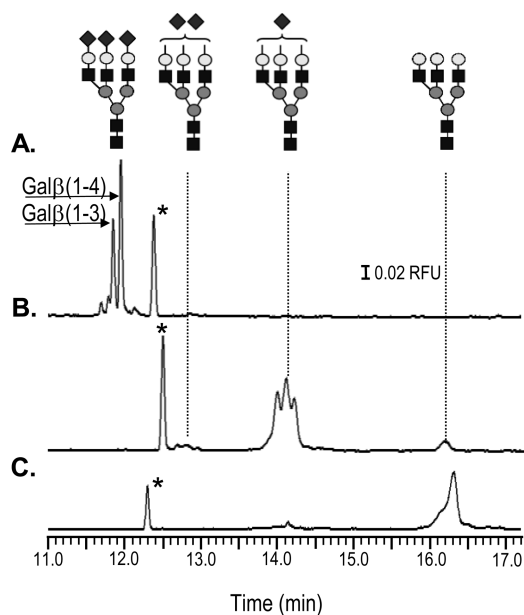


Figure 7. Electropherograms of 0.15 nM trisialylated triantennary complex N-glycan incubated in 2-3',6',8',9' neuraminidase to determine the 3' versus 6' sialic acid composition. The separation in trace A was obtained in the absence of enzyme. The separation in trace B was obtained using 4 $\mu\text{Units}/\mu\text{L}$ α 2-3,6,8,9 neuraminidase suspended in phospholipid nanogel (loaded at 69 kPa for 21 s) and generated an electropherogram of N-glycan devoid of all 2-3 linked sialic acid. The fixed zone of 2.4 mUnits/ μL α 2-3,6,8,9 neuraminidase used in trace C (loaded at 69 kPa for 21 s) generated an electropherogram of N-glycan devoid of all sialic acid. The experimental conditions are the same as those used in Figure 5.

CONCLUSIONS AND FUTURE DIRECTIONS

Nanogels are a biocompatible separation additive. At a concentration of 150 $\mu\text{Units}/\mu\text{L}$, enzyme reconstituted in aqueous electrolyte has a rate that is approximately half of what is obtained when it is reconstituted in nanogel made in the same aqueous solution. Furthermore, when the preparation does not contain nanogel, the activity decreases dramatically at an enzyme concentration of 150 $\mu\text{Units}/\mu\text{L}$ such that product was not detectable on the second day of measurement. In contrast, neuraminidase reconstituted in nanogel maintained a rate of 500 ± 40 μM lactose/min, as seen in the generation of product measured throughout a 30-day period. A single enzyme stock in nanogel is a cost-effective means to deliver the subnanoliter volumes required for the capillary method.

Nanogel preparations are inexpensive, costing \$0.09 for 5 μL .^{60,61} The nanogel enzyme analyses provide an inexpensive, rapid, and simple means to analyze and quantify the linkage composition of oligosaccharides and are an alternative technology to current methods that rely on derivatization or benchtop digestion. Although the specific enzyme provides greater confidence in the glycan linkage composition, the same information is achievable with the general enzyme that has preferential catalytic specificity for one substrate linkage over another, such as the α 2-3,6,8,9 neuraminidase. This is particularly useful in cases where a specific enzyme is not readily available (e.g., α 2-8 or α 2-9 specific sialidases) or is prohibitively expensive. The method will be harnessed to determine the linkage composition in mixtures of complex N-glycans. Future efforts will also center on adapting the method for different enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b04074.

Additional experimental methods; position of the fixed enzyme zone in-capillary; Ohm's Law plot; Michaelis–Menten data for 6'-sialyllactose with α 2-3,6,8,9 neuraminidase; Michaelis–Menten data for 3'-sialyllactose with α 2-3 neuraminidase; summary of Michaelis–Menten parameters; electropherograms that distinguish linkages in sialyllactose (PDF)

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Notes

The authors declare no competing financial interest.

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