Limited hydrolysis of polysialic acid by human neuraminidase enzymes

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ABSTRACT

Regulation of sialic acids by human neuraminidase (hNEU) enzymes is important to many biological processes. Defining hNEU substrate tolerance can help to elucidate the roles of these enzymes in regulating sialosides in human health and disease. Polysialic acid (polySia) is a polyanion of α(2→8) linked sialic acids with roles in nervous, reproductive, and immune systems and is dysregulated in some malignancies and mental disorders. The unique chemical properties of this polymer, which include an enhanced susceptibility to acid-catalyzed hydrolysis, have hampered its study. Herein we describe the first systematic study of hNEU isoenzyme activity towards polysialic acid *in vitro.* The experimental design allowed us to study the impact of several factors that may influence polysialic acid degradation including pH, polymer size, and the relative ionic strength of the surrounding media. We report that short chains of polysialic acid (degree of polymerization, DP 3-8) were substrates of NEU3 and NEU4 at acidic pH, but not at neutral pH. No hNEU-catalyzed hydrolysis of longer polymers (DP 10-20) was detected. These findings suggest a neuraminidase-independent mechanism for polysialic acid turnover such as internalization and degradation in endosomes and lysosomes.

INTRODUCTION

Hydrolysis of the glycosidic bonds of sialic acid by neuraminidase (also known as sialidase) enzymes modulates cell signaling by removing neuraminic acid (sialic acid) epitopes or by revealing epitopes masked by this residue. The regulation of neuraminic acid by enzymes plays important roles in many biological processes including cell-cell interaction, host-pathogen interactions, and tumor malignancy.[1, 2] To date, four human neuraminidase (hNEU) isoenzymes have been identified which differ in subcellular localization and substrate tolerance.[1] Small molecule inhibitors[3] and unnatural neuraminic acid substrates[4-6] have been valuable tools to further understanding of hNEU structure and substrate tolerance; however, many natural sialosides are unstable and difficult to isolate which has impeded their study.[7, 8]

Diversity in sialic acid presentation is generated at three levels: modification of the monosaccharide (e.g. hydroxyl groups or the *C*-5 amido group), the glycosidic bond (typically α(2→3, 6, or 8)), or the reducing end glycans or aglycone.[9] Previous work has indicated that all three levels of sialic acid diversity influence hNEU catalyzed hydrolysis, both independently and in synergy.[4, 10-12] These results suggest that in order to understand the interaction of uncommon sialic acids with hNEU it is important to study a range of well-defined substrates.

Polymers of α(2→8) linked sialic acids (polysialic acid, polySia) are sialylglycoconjugates with unique chemical properties. In human systems polySia has been predominantly studied on NCAM, which can account for as much as 80% of total polySia,[13] and where the degree of polymerization (DP) can be up to 90 residues.[14] Polysialic acid has also been found on synCAM,[15] neuropilin,[16] E-selectin,[17] CD36 in

human milk,[18] the α-subunit of the voltage-sensitive sodium channel,[19] and on polysialyltransferases ST8SiaII and ST8SiaIV.[20] The biological role of polySia is often ascribed to that of a non-specific anti-adhesive or pro-migratory molecule for cells.[21, 22] In recent years specific binders to polySia have emerged, including neurotrophins like BDNF,[23] and FGF2.[24] Polysialyltransferase activity regulates the activation of CD4+ T-cells[25] and knock-out of the polysialyltransferase ST8SiaIV resulted in T-cell defects in mice.[26] PolySia has been found on other immune cells,[16, 26, 27] and is more abundant in the serum of males compared to females, suggestiong the possibility that polySia may contribute to sex differences in immune response.[28] In some cancers dysregulation of polySia promoted malignancy.[29-32] Polysialic acid regulates neuronal development and plasticity,[33] and it has recently been gaining traction as a molecule associated with mental disorders.[34] In the brain polySia levels are increased in bipolar disorder,[35] but decreased in depression,[35] schizophrenia,[36] and acute stress.[37] Understanding the mechanisms of polySia regulation are critical to elucidation of specific roles for this polyanion in human health and disease.

The unique chemical properties of polySia can largely be attributed to the C1 carboxylate of sialic acid monomers, making the polymer polyanionic at neutral pH. As a result, polySia is known to have a large hydration volume,[38] which is influenced by charge screening from ions in the surrounding solution.[39] The pKa of polySia increases with increasing chain length (**Figure S1A**), and can range from 2.9-5.5.[40] The carboxylate groups influence stability of the polymer. An intramolecular self-cleavage mechanism, where a protonated C1 carboxylic acid acts as a proton donor to the glycosidic O2, is proposed to explain the susceptibility of polySia to acid-catalyzed

hydrolysis (**Figure S1B**).[41] Notably, polysialic acid is known to hydrolyze even under the mild acidic conditions present in cellular compartments such as endosomes and lysosomes.[41] Protonation of the C1 carboxylate can result in formation of lactones between the C1 and C9 of the neighboring reducing-end residue.[42, 43] The C1 carboxylate contributes to the formation of polySia tertiary structure – which is generally thought to be an extended helix.[44]

The chemical properties of polySia make it challenging to study. Polysialic acid studies *in vitro* have relied heavily on reducing-end reactivity in a thiobarbituric acid assay,[45, 46] or 1,2-phenylenediamines for analysis of chain length using anion-exchange HPLC.[46] Both reducing-end chemistries require acidic conditions which would degrade the polymer, making accurate analysis of polySia difficult.[47, 48] *In cellulo* analysis of polySia has relied almost exclusively on anti-polySia antibodies that recognize epitopes that are not always well defined.[48] A polySia-specific probe has been developed from an inactive mutant of endosialidase that binds, but does not cleave, polySia;[49-51] however, it has not yet been widely adopted.

Literature reports of hNEU hydrolysis of polySia have been limited and sometimes contradictory. *In vitro* and *in cellulo* studies have reported that NEU4 is the only isoenzyme to cleave polySia,[46, 52] while a separate report suggests only NEU1 regulates polySia.[13] An *in vivo* study in rats detected increased NEU1 in brain tissues upon LPS stimulation corresponded to a decrease in polySia.[53] Knockdown of NEU1 led to an increase in polySia on hippocampal granule cells.[54] Although these results implicate NEU1 in the regulation of polySia, they do not provide direct evidence for NEU1 hydrolysis of polySia. We considered that a systematic study of polySia hydrolysis by

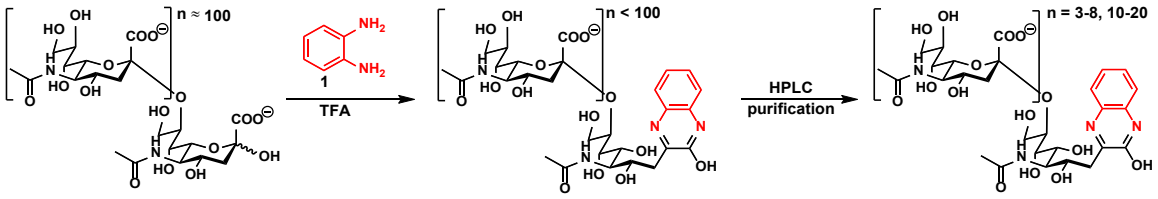
hNEU isoenzymes would provide important insight into this problem. Here, we describe in vitro hydrolysis assays of polySia of varying lengths with each of the hNEU isoenzymes. We find that only NEU3 and NEU4 show significant amounts of hydrolysis for polySia under acidic conditions; and that the hNEU isoenzymes do not act on polySia substrates of high DP (10-20).

RESULTS AND DISCUSSION

Preparation of substrates for degradation assays.

To study polysialic acid degradation we required defined polySia substrates and a reliable method to detect polySia chain length. Reducing-end labeling of sialic acids with 1,2-phenylenediamines followed by HPLC separation is a well-established method for the analysis of sialic acid monosaccharides[55, 56] and polymers.[57] Sialic acid labeling with 1,2-phenylenediamines requires acidic conditions which in turn promote the intramolecular self-cleavage of polySia.[41] The labeling reaction has been optimized to minimize, but not completely eliminate, polySia hydrolysis.[47, 57] Further, under acidic conditions polySia of higher DP hydrolyzed faster than that of lower DP, so background hydrolysis from 1,2-phenyldiamine derivatization may be inconsistent across samples.[41, 47] The drawbacks to determining polySia chain length through labeling with 1,2-phenyldiamines can be circumvented by labeling the polySia substrates prior to submission to test conditions. This early labeling not only eliminated background hydrolysis due to labeling from the polySia degradation assay, but also allowed us to separate and isolate pools of polysialic acid of known DP using preparative HPLC to provide defined substrates.[47]

The most common diamine used for sialic acid labeling is 1,2-diamino-4,5-methylenedioxyenzene dihydrochloride (DMB) which enables fluorescent detection. However, DMB is unstable to both light and oxygen while being relatively expensive.[58] A more stable and inexpensive alternative,[59] *o*-phenylenediamine (OPD, **1**) (**Scheme 1**), provided sufficient sensitivity for our purposes. Starting from commercially available and inexpensive colominic acid (average DP ≈ 100), shorter fragments of labeled polySia were produced during reducing-end labeling with OPD over 2 hours at 50 °C to promote hydrolysis to smaller chain lengths (**Scheme 1**).[57] After labeling, anion exchange chromatography with preparative HPLC was used to isolate polymers of defined length. For our degradation assays we generated pools of short chain (DP 3-8) and long chain (DP 10-20) sialic acids, which we henceforth refer to as oligo- and poly-sialic acid, respectively.[48] These lengths were selected based on conformational studies that suggested polysialic acid adopts extended helical structures where one helical turn is 8-9 sialic acid residues.[44, 60, 61] This model indicates that oligoSia (DP < 8) may have a different tertiary structure relative to longer chain polySia (DP 10+), which we reasoned could influence its availability for hNEU-catalyzed hydrolysis.



Scheme 1: Preparation of sialic acid polymers for degradation assays

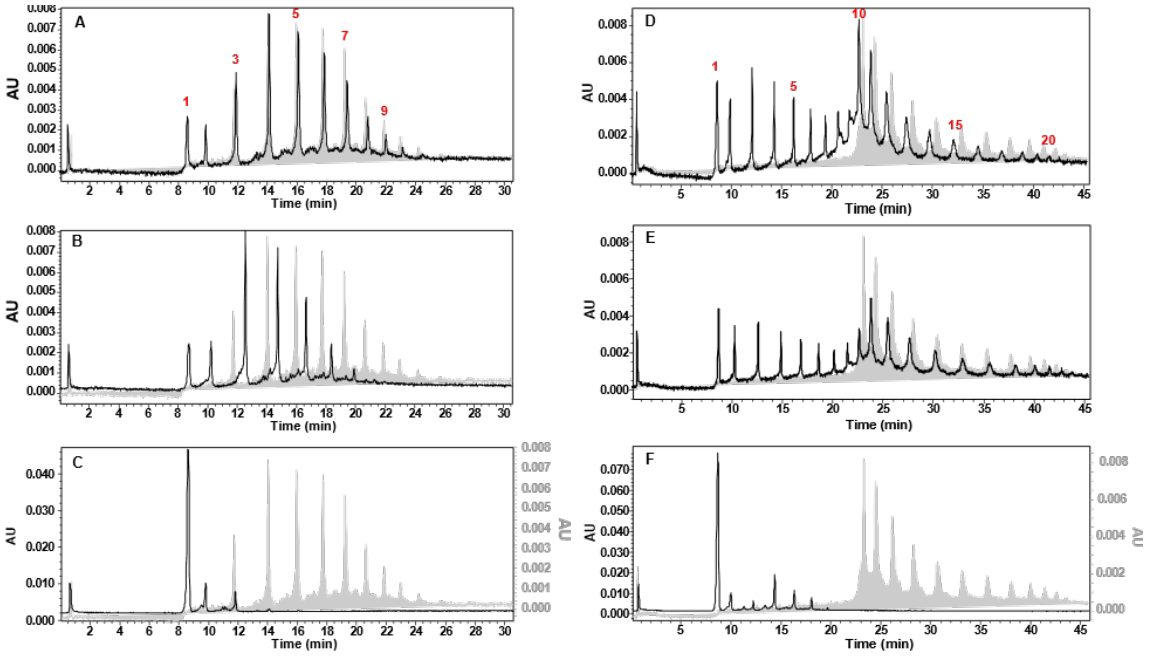


Figure 1: Representative runs of oligosia (A-C) and polysia (D-F) degradation assays. Samples were measured after 5 hrs at 37 ℃ where the grey chromatogram indicates time = 0, and the black chromatogram indicates treatment with A, D) pH 4.5 control B, E) 1 mU NEU3 C, F) 65 µg/mL Endo-N.

Degradation assays.

To study the degradation of oligo- and polySia, we implemented an endpoint assay where the labeled substrates were incubated with neuraminidase for 5 hrs at 37 ℃ before quenching. The samples were analyzed by HPLC using anion exchange chromatography (DNAPac PA-100) to separate polymers of different DP. For analysis, we calculated a weighted average of the peak areas from the raw data (**Figure 1**) to produce an average DP. Because polymers of sialic acid undergo intramolecular self-cleavage at the pH optimum of hNEU enzymes (pH 4.5 for NEU1, NEU3, and NEU4 and 5.5 for NEU2); enzyme-free pH controls were implemented in addition to a time = 0 control (**Figure 2**). The enzyme-free controls used hNEU storage buffer to ensure consistency in pH and salt

concentration. Pooled oligoSia (DP 3-8) and polySia (DP 10-20) were stable at pH 7 over the 5 h experiment, in agreement with previous results,[62] and only underwent minor hydrolysis at pH 5.5. At pH 4.5 oligoSia had a decrease in average DP of 0.82 ± 0.03 over 5 h (a decrease in molecular weight of 50.7 g mol^{-1} hr^{-1}) (**Figure 1A**), and hydrolysis occurred more rapidly for polySia (**Figure 1D, Figure 2**) (decrease in DP of 4.15 ± 0.1, -247.6 g mol^{-1} hr^{-1}), consistent with previous reports.[41, 47] Taken together, these data support the postulated intramolecular self-cleavage (**Figure S1**).[41]

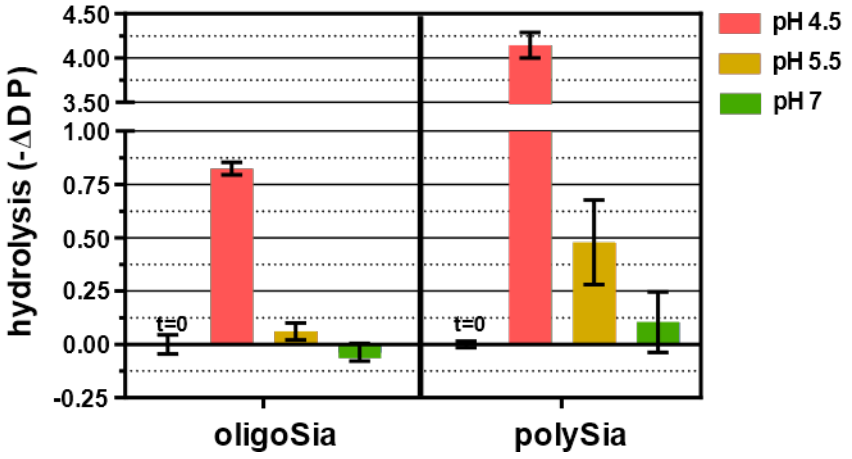


Figure 2: Enzyme-free hydrolysis of oligosia and polysia over 5 hours at pH 4.5, 5.5 and 7. Hydrolysis is presented as -ΔDP (over 5 hrs), the change in the average degree of polymerization of the sample normalized to time = 0 (at left).

Bearing in mind that pH has a strong influence on enzyme-independent hydrolysis of oligoSia and polySia, we tested oligoSia degradation by neuraminidases both at the enzyme optimum and at pH 7. Endoneuraminidase-N (Endo-N, from *E. coli* K1 bacteriophage) was used as a positive control for endosialidase activity. EndoN treatment

(65 μg/mL)[63] of oligoSia converted most of the sample to monosaccharide (**Figure 1C**) at both pH 4.5 and pH 7 (**Figure 3A**), confirming that the reducing-end labeling conditions did not block oligoSia cleavage. We were surprised to observe that neuraminidase from *A. ureafaciens* (siaAU), reported to be a relatively indiscriminate NEU,[45, 64] did not show increased hydrolysis of oligoSia or polySia when compared to pH controls (**Figure 3B**).

The hNEU isoenzymes exhibited pH-dependent activity towards oligoSia. The enzyme activity for all neuraminidase enzymes was normalized to 0.5-1 mU for the indicated pH. At pH 4.5, NEU3 hydrolyzed oligoSia fastest with a decrease in average DP of 2.00 ± 0.04 compared to 0.82 ± 0.03 for the pH 4.5 control (**Figure 3E**). The NEU4 isoenzyme had significant activity on oligoSia (decrease in DP of 1.53 ± 0.01, **Figure 3F**). At its optimum pH, NEU2 did not show detectable hydrolysis of oligoSia relative to the control (**Figure 3D**), nor did it have activity at more acidic pH (4.5, data not shown). These results agree with reports that NEU2 has a strong preference for α(2→3) over α(2→6) or α(2→8) linked sialic acids and that it does not to cleave colominic acid.[65] Previous studies suggested that the NEU2 active site interactions with the glycerol chain are constrained, limiting its substrate tolerance.[11, 66, 67] We observed moderate, but not significant, enzyme-catalyzed hydrolysis of oligoSia by NEU1 at pH 4.5 (**Figure 3C**). Our NEU1 enzyme preparation was not active at pH 7, preventing measurement under this condition. In general, hNEU isoenzymes showed activity at acidic enzyme optimum pH (excepting NEU2); however, none of the hNEU cleaved oligoSia at pH 7. This observation suggests that oligoSia may only be degraded by hNEU in acidic compartments *in vivo*.

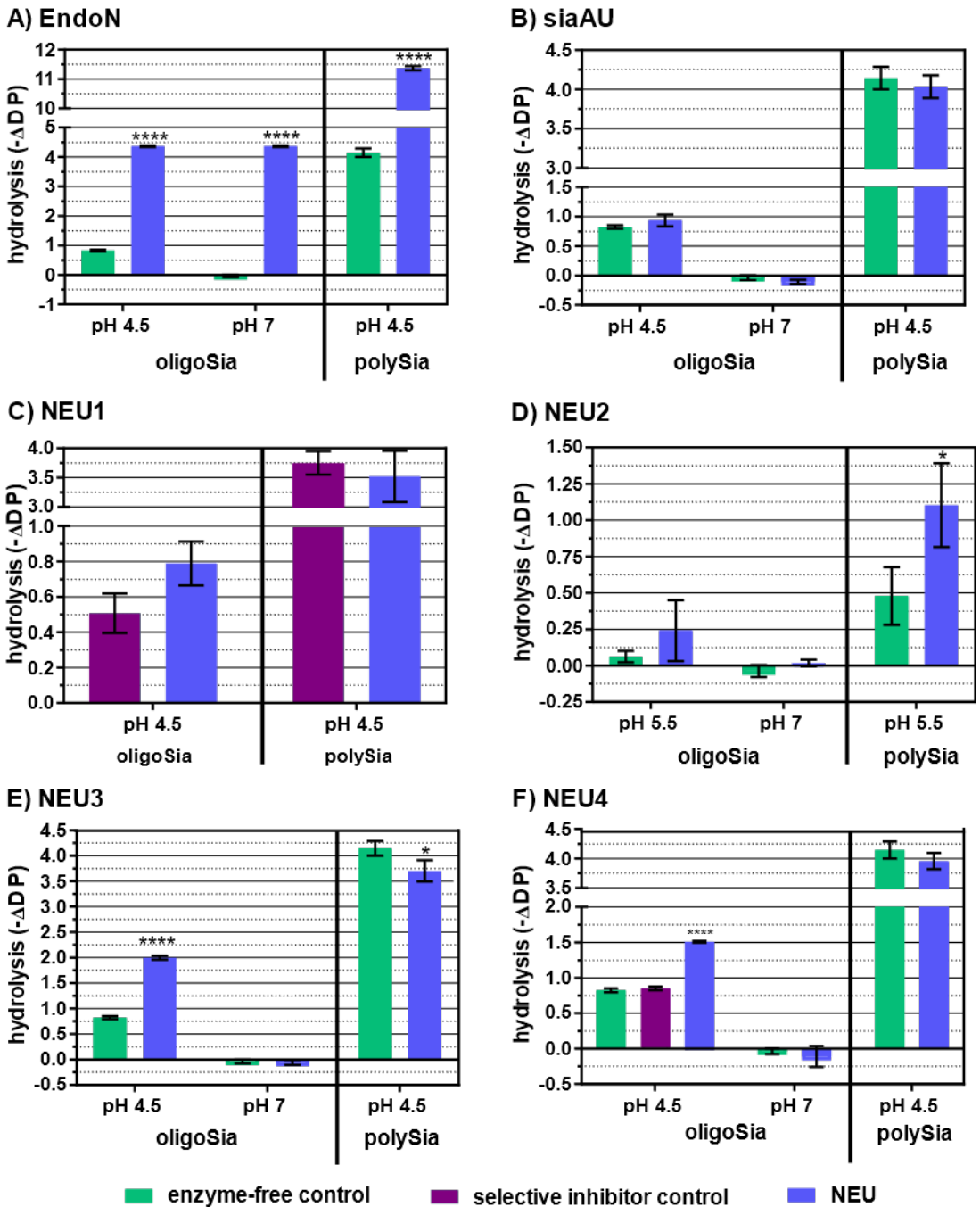


Figure 3: Neuraminidase-catalyzed degradation assays. Enzyme activity was normalized to 1 mU enzyme activity apart from Endo-N, for which enzyme activity could not be normalized and so was used at 65 µg/mL, and NEU1 which was normalized to 0.5 mU. For NEU3 at pH 7, 0.75 mU of enzyme activity was used due to its low activity away

from the enzyme optimum pH. Unpaired t-tests with Welch's correction were performed comparing the enzymatic conditions to their respective pH controls, where * = $p < 0.05$ and **** = $p<0.0001$.

We proceeded to test hNEU activity on polySia (DP 10-20) (**Figure 3**). Our results showed that, with the exception the Endo-N positive control, none of the neuraminidases tested had appreciable activity on polySia. Among these, NEU2 was the only isoenzyme to show increased hydrolysis compared to the pH 5.5 control ($p < 0.05$). NEU3 showed a small decrease in hydrolysis ($p < 0.05$). Taken together, we ascribe these small differences in activity to variability in the assay rather than authentic differences in enzyme activity. Previous reports found that NEU2 did not cleave colominic acid,[65] and we conclude that all four hNEU isoenzymes have minimal or no activity towards polySia.

Inhibition of oligoSia degradation by selective hNEU inhibitors.

Our group and others have developed isoenzyme-specific inhibitors of hNEU.[67-71] Inhibitors of NEU3[68] and NEU4[69] can block glycolipid processing *in vitro*. To confirm that oligoSia degradation under hNEU treatment conditions was a result of neuraminidase activity, we used a NEU4 selective inhibitor (**Figure 4, CY16600**) to block enzymatic hydrolysis of oligoSia.[69] Addition of the inhibitor to the assay mixture at 1.6 μM (10x the IC_{50})[69] gave a ΔDP identical to the pH control (**Figure 3F**), indicating that NEU4-catalyzed hydrolysis of oligoSia was completely blocked by the NEU4-selective inhibitor. Our NEU1 enzyme is produced by overexpression in HEK293 cells, and should not contain other NEU isoenzyme activity.[11] Due to the sensitivity of oligoSia/polySia to small changes in pH and salt concentration (*vide infra*) we could not use the enzyme-free

conditions as controls for NEU1. Thus, we used a selective NEU1 inhibitor, **CG33300,**[67] (10x the $IC_{50,}$ **Figure 3C**) to confirmed that the differences observed could be attributed to NEU1 enzyme activity on oligoSia. As with the other isoenzymes, we did not observe NEU1 activity towards polySia compared to control.

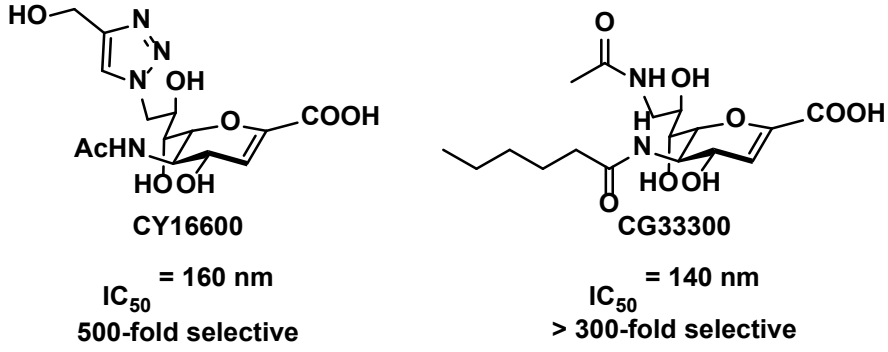


Figure 4: Structures of selective hNEU inhibitors. NEU4 inhibitor **CY16600** is 500-fold selective for NEU4 and NEU1 inhibitor **CG33300** is 300-fold selective for NEU1.

Influence of salt concentration on oligoSia degradation.

Our systematic investigation of hNEU activity on oligoSia/polySia provides evidence that hNEU may only cleave short polymers of sialic acid and only at acidic pH. We hypothesize that these results could be explained through charge repulsion of polySia substrate to hNEU, or by different conformations of the substrates. These two factors are difficult to isolate experimentally.[44, 60] To test whether the relative ionic strength of the polymer influenced its accessibility to hNEU, we monitored oligoSia hydrolysis over a range of salt concentrations (260 mM to 80 mM; **Figure 5**). Increased buffer salt concentration had a small protective effect against hNEU-independent hydrolysis.[41] Varying the buffer salt concentration did not change the ΔDP for EndoN treatment.

Conversely, decreasing the salt concentration correlated with a decrease in NEU4-catalyzed hydrolysis of oligoSia. These data are consistent with charge screening of polysialic acid,[39] and suggest that the relative ionic strength of oligoSia/polySia may influence the association kinetics of the polymer to the enzyme. We note that the α(2→8) SiaT, ST8SiaIII, has a large electropositive groove to accommodate its polyanionic substrate not found in other SiaTs.[72] These experiments do not exclude a role for conformational differences between oligoSia and polySia in hNEU catalysis.

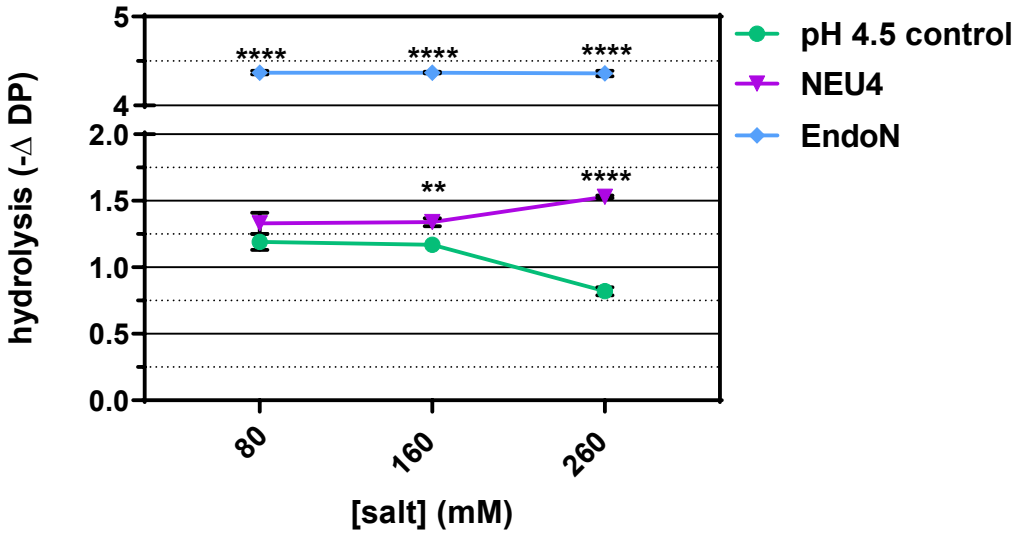


Figure 5: Effect of buffer salt concentrations on oligosia hydrolysis. Each point represents a mean of triplicate experiments with error bars denoting one standard deviation. Enzyme activity was normalized to 1 mU with the exception of Endo-N, which was used at 65 µg/mL. Unpaired t-tests with Welch's correction were performed comparing the enzymatic conditions to the pH control at each buffer concentration, where ** = $p < 0.01$ and **** = $p < 0.0001$.

The ongoing challenge of studying polySia.

The sensitivity of our degradation assays to different salt concentrations highlights the challenge of studying polysialic acid. The unique chemistry of polySia makes it a challenging target. Factors which complicate the study of polysialic acid include sample consistency (DP), pH, salt concentration, and method of detection. This challenge becomes apparent when reviewing the literature for examples of neuraminidase activity on polySia substrates. The siaAU enzyme is reported to cleave α(2→3,6,8,9) linkages[45, 64] and has been used to degrade polySia to smaller DP. Enzyme activity has been measured using a thiobarbituric acid assay, requiring strong acid and boiling.[45, 73] Many reports using siaAU employed high concentration of enzyme or were performed at acidic pH.[14, 42, 74-76] In this context, our observation that siaAU did not cleave oligoSia/polySia indicates that much, if not all, of reported activity may be attributed to acid-catalyzed hydrolysis.

Previous reports have identified NEU1, NEU2, and NEU4 isoenzymes as having activity on polysialic acid substrates. Murine NEU2 and NEU4 exhibited similar activity towards oligoSia while NEU1 was approximately twice as active.[75] Conversely, a study of murine sialidases determined that only NEU4 cleaved oligoSia (DP 2 – 6) using the thiobarbituric acid assay.[46] Antibodies to polySia supported that NEU4b could cleave polysialic acid *in vitro* and *in cellulo*; however, we note that the antibodies used (12E3 and 12F8) recognize epitopes of DP > 5 and an undefined epitope, respectively.[48] Murine NEU4b-catalyzed removal of polySia from NCAM *in vitro* is reported at near-neutral pH.[46] Thus, there is some disagreement in the literature on the specific NEU isoenzymes

that hydrolyze polysialylated glycoproteins. Candidates also include murine NEU4a (polysialylated NCAM)[52] and human NEU1.[13, 53]

CONCLUSIONS

This work suggests that degradation of polysialic acid substrates, especially larger polymers (DP > 8), may be largely independent of neuraminidase-catalyzed hydrolysis. *In vitro* studies showing enzyme-free polysialic acid hydrolysis under mild acidic conditions also found in biological systems has already prompted speculation that enzyme-free hydrolysis of polySia may occur in acidic environments of endosomes and lysosomes.[41, 77, 78] Desialylation of polysialylated NCAM was found to be dependent on endocytosis.[79] More recently, an antibody specific for polysialic acid (DP > 3) was internalized by cells and co-localized with endosomal and lysosomal markers.[80] Further investigation of polySia trafficking and degradation, as well as follow-up studies of hNEU isoenzyme activity on polySia, will be essential to confirming mechanisms of polySia regulation. Our assay design has enabled us to directly compare several factors that influence polySia degradation kinetics, including substrate DP, pH, and relative ionic strength of the media. Our results suggest that hNEU can only hydrolyze polymers of sialic acid with low DP at acidic pH.

METHODS

General methods.

All reagents were purchased from commercial sources and used without further purification unless otherwise noted. HPLC was performed with a Waters Delta 600 pump, and a Waters 600 controller with Empower 2 software. Eluted peaks were detected with a Waters 2996 photodiode array (PDA) detector (Waters Ltd.). Neuraminidase from *A. ureafaciens* was purchased from Millipore Sigma. Endo-N was a generous gift from Dr. Lisa Willis. Human neuraminidase enzymes NEU2-NEU4 were expressed as fusion proteins with maltose binding protein and were purified as described.[4, 81, 82] Human neuraminidase enzyme NEU1 was produced from HEK293E cells and was used as a crude cell lysate.[11] Specific activity of the exosialidase enzymes was determined in comparison to a standard curve of neuraminidase from *Clostridium perfringens* (NanI) against 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NANA). Neuraminidase activity was normalized to 1 mU, with exceptions. Our NEU1 expression system has lower activity[11] than the other isoenzymes, and 0.5 mU NEU1 was used at pH 4.5. At pH 7, NEU1 had no detectable activity and NEU3 had reduced activity, so was used at 0.75 mU. Endo-N could not be normalized using the exosialidase assay so was used at 65 μg/mL.

Oligo- and polysialic acid sample preparation.

Colominic acid (average MW 30 000 kDa, 45 mg) was dissolved in 4.5 mL of 0.25 M β-mercaptoethanol, 9 mM $Na_2S_2O_4$, and 20 mM trifluoroacetic acid. The 1,2-phenylenediamine (8 mg, 0.07 mol) was added and the mixture was heated at 50 °C for 2

hrs. NH_4OH (10 µL of 28%) was added and the mixture was incubated at 37 °C for 2 h.[55, 57]

Labelled polysialic acid was separated on a DNA Pac PA-100 anion exchange column (9 x 250 mm) with a gradient of 2 M NH_4OAc (pH 8) in water (3 mL/min flow rate: 0%, 0%, 20%, 25%, 32.5%, 100%, 100%, at 0, 5, 20, 35, 45, 48, 55 minutes, respectively.)[46, 47] Peaks were detected at 350 nm. Fractions were collected and pooled to obtain oligosialic acid (DP 3-8) and polysialic acid (DP 10-20). For oligosialic acid, solvent was removed under vacuum and the remaining salt was removed by repeated washes through a centrifugal filter unit (3 kDa MWCO) before lyophilization. For polysialic acid, solvent was concentrated under vacuum and salt was removed by dialysis (3 kDa MWCO) followed by repeated washes through a centrifugal filter unit (3kDa MWCO) before lyophilization.

Oligo- and polysialic acid hydrolysis assays.

Assay buffer (20 µL) was added to aliquots of oligo- or polysialic acid (0.1 mg of oligo- or 0.8 mg of poly-). Unless otherwise stated, the assay buffer was 0.3 M sodium acetate HEPES at pH 4.5, 5.5, or 7. For controls, 20 µL of neuraminidase buffer (0.2 M NaCl, 20 mM MOPS, 10 mM maltose and 10% glycerol, pH 7.2) was added, and for enzymatic assays 20 µL of 1 mU of enzyme in neuraminidase buffer was added (for NEU1, 0.5 mU of enzyme in 1X RIPA buffer was used). Mixtures were incubated at 37 °C for 5 h. Ethanol (10 µL) and NH_4OAc (30 µL, 0.2 M, pH 8, with 1 mM benzoic acid) were added. The mixture was washed through a centrifugal filter unit (30 kDa MWCO at 14 000 rpm for 30 min) and the filtrate was analyzed after separation on a DNA Pac PA-100 anion exchange column (4 x 150 mm), monitored at 350 nm. The gradient was 2 M

NH_4OAc (pH 8) in water 2 mL/min: 0%, 0%, 20%, 25%, 100%, 100%, at 0, 5, 20, 35, 38, 43 minutes, respectively (oligosia) or: 0%, 0%, 20%, 25%, 32.5%, 100%, 100%, at 0, 5, 20, 35, 45, 48, 55 minutes, respectively (polysia).

For inhibitor assays NEU1 or NEU4 was incubated with their respective inhibitors for 10 min at room temperature prior to addition to oligosialic acid. Final concentrations of each inhibitor was 10x the reported IC_{50} values. For the NEU1 inhibitor (C5-hexanamido-C9-acetamido-DANA, CG33300) the concentration was 1.4 μM,[67] and for the NEU4 inhibitor (9-[4-hydroxymethyl-[1,2,3]triazol-1-yl]-2,3-didehydro-*N*-acetylneuraminic acid, C9-4HMT-DANA, CY16600) the concentration was 1.6 μM.[69]

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SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI:

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