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An optimised assay for quantitative, high-throughput analysis of polysialyltransferase activity

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The polysialyltransferases are biologically important glycosyltransferase enzymes responsible for the biosynthesis of polysialic acid, a carbohydrate polymer that plays a critical role in the progression of several diseases, notably cancer. Having improved the chemical synthesis and purification of the fluorescently-labelled DMB-DP3 acceptor, we report optimisation and validation of a highly sensitive cell-free high-throughput HPLC-based assay for assessment of human polysialyltransferase activity.

Introduction

Polysialic acid (polySia) is a homopolymer of N-acetyllactosaminic acid (sialic acid, Neu5Ac), which is linked specifically by α-2,8-glycosidic bonds, and post-translationally modifies the neural cell adhesion molecule (NCAM). PolySia is abundantly expressed throughout embryonic development, but is subsequently downregulated during maturation and differentiation. PolySia has received considerable interest due to its potential role in many biological processes, including inflammatory diseases, neurodegenerative diseases, schizophrenia, psychotic and mood disorders, autism and other related psychiatric disorders and most notably, cancer.

As an onco-developmental antigen, polySia is re-expressed during the progression of a number of malignant human tumours such as neuroblastoma, Wilms’ tumour, medulloblastoma, pheochromocytoma, medullary thyroid carcinoma, lung cancer, pituitary adenomas and breast cancer. In these tumours, NCAM polysialylation correlates with metastatic potential and poor prognosis and plays a key role in tumour cell migration, invasion and metastasis. Furthermore, a trimer (degree of polymerisation, DP=3) of polySia biosynthesis, abolishes tumour cell growth and dissemination.

Despite its key role in cancer, polySia has to-date only been efficiently modulated by genetic manipulation, enzymatic digestion or by the use of inhibitors that lack drug-like properties for in vivo use (inhibitors of the sialic acid biosynthesis pathway, or CMP analogues). A major factor that has hindered progress in the development of novel polyST inhibitors is the lack of an efficient technique for high throughput, sensitive and quantitative assessment of human polyST enzyme activity.

Studying polySTs presents particular challenges since they are less catalytically active when compared to the majority of the wider sialyltransferase (ST) family. Furthermore, the catalytic mechanisms underpinning polyST function are still poorly understood. PolySTs are one of the few STs which predominantly glycosylate a specific protein. A pressing need remains for a new strategy to assay polyST activity.

Several methods have been reported for the assessment of the activity of different mono-ST enzymes, each with significant shortcomings when applied more specifically to polyST activity measurement. These methods can be broadly divided into four groups: radioactive assays, non-radioactive assays, phosphatase-coupled assays and chemical conjugation methods.

In summary, none of these approaches is satisfactory for polyST analysis, requiring huge quantities of enzyme and acceptor proteins, at considerable cost. These assays additionally lack the ability to show the characteristics of the reaction product(s) in terms of polySia chain length (or molecular weight) in order to enable characterisation of enzyme activity, do not allow for high throughput analysis of enzyme inhibitors (primarily due to a lack of sensitivity) and rapid analysis of results and/or are not suitable for efficient analysis of the human polyST enzymes. Mammalian and bacterial polySTs share no significant sequence identity. Although they both catalyse the same reaction, different cellular environments and acceptor substrates are involved. Subsequently, high throughput analysis techniques specific for the human enzymes must be optimised.

Here we have utilised a non-ganglioside fluorescent acceptor to assess polyST activity. A trimer (degree of polymerisation, DP=3) of α-2,8-linked sialic acid (DP3) directly conjugated to a 1,2-diamino-4,5-methylenedioxybenzene (DMB) label, thereby forming DMB-DP3, has been previously reported by the Gerardy-Schahn group as a useful acceptor for bacterial and murine ST8SiaI enzymes. We have previously utilised DMB-DP3 to determine the activity of human polyST following inhibition by CMP (Figure 1).

In this article, we describe the optimisation of a cell-free chromatographic assay that enables simple, quantitative and high-throughput analysis of human polyST. This assay enables the characterisation of reaction products in terms of molecular weight, does not depend on radioactive material nor antibodies for product detection and overcomes the issues of low sensitivity experienced with the other methodologies described above. Furthermore, it is cost-effective, using only minimal quantities of enzyme.

![Figure 1](image-url)

**Figure 1.** The main steps of the optimised assay for the analysis of human polyST.
Experimental section

Chemicals and reagents
General chemicals and reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise specified. Human recombinant ST8SiaII (EC 2.4.99; accession number Q92186, UniProtKB) was synthesised as described previously.28

DMB-DP3 labelling reaction and purification
DMB labelling was performed as previously described.44 Briefly, DP3 (10 mg/ml) was dissolved in a labelling solution (1 ml) consisting of DMB (20 mM), sodium hydrosulphite (40 mM) and β-mercaptoethanol (1 M). The solution was then mixed with an equal volume of ice-cold trifluoroacetic acid (40 mM). The reaction mixture was subsequently incubated for 24 hours at 4°C. The derivatisation reaction was then stopped by the addition of one fifth reaction volume of sodium hydroxide (200 µM).

DMB-DP3 purification by reversed phase chromatography
A Waters 2695 alliance HPLC system operated by Masslynx software was used to analyse samples. Separation was achieved by reversed phase chromatography on a Hichrom RP8 C18 column (25 cm x 4.6 mm, 250 Å, Hichrom, UK). Chromatography was performed at 1.2 ml/min with ammonium formate (5 mM, pH 6) (mobile phase A) and 55% methanol in distilled water (mobile phase B). Elution of DMB-DP3 was achieved with an isocratic method of 90% mobile phase B over 70 minutes. The separation was monitored by mass spectrometry (Waters ZMD, Micromass, United Kingdom), photodiode array detector (373, 303 and 250 nm) (Waters 2996) and fluorescence detector (RF-10A), (excitation 373, emission 448 nm) all connected in series. A single fraction containing the DMB-DP3 was collected (confirmed by detecting the molecular weight and comparing the retention time with a DMB-DP3 standard obtained from the Gerardy-Schahm group). Fractions from several injections were pooled and freeze-dried. The purified material was weighed and dissolved in water to create a 200 µM DMB-DP3 solution and stored at -20°C.

Mass spectrometric analysis
Liquid chromatography-mass spectrometry analysis was carried out using a Waters ZMD (Micromass, Manchester, United Kingdom) single quadrupole mass spectrometer connected in series to a Water Alliance 2695 system. The mass spectrometer was operated in negative ion electrospray mode. A solvent flow of 1.2 ml/min (split 1:10). The cone voltage was set at 30 V. The mass spectra were continuously scanned from m/z 100 to m/z 1500 throughout the entire separation. Masslynx software was used to process the mass spectral data and produce both total ion chromatograms and single ion recording chromatograms for the key masses of interest.

DMB-DP3 purification by anion exchange chromatography
Separation was achieved using a DNA Pac PA-100 analytical anion exchange column (25 cm x 4 mm, packed with 0.1 µM microbeads, Dionex, UK). DMB fluorescence was monitored with a fluorescence detector (RF-10A) as described above. The mobile phases used were: distilled water (mobile phase A) and ammonium acetate buffer (5 M, pH 7.4, mobile phase B) for 30 minutes and 1 ml/min flow rate, with the gradient outlined in Supplementary Data, Table 1.

Measurement of polyST enzyme activity
Recombinant human polyST (ST8SiaII) enzyme (250 ng/µl final concentration) was incubated at 25°C overnight in a solution of DMB-DP3 (10 to 100 µM), MgCl2 (5 mM), CMP-Neu5Ac (0.5 mM) and sodium cacodylate buffer (0.1 M, pH 6.7). Aliquots were taken after 1, 2, 4, 6 and 24 hours and the reaction was stopped by 10-fold dilution with Tris-HCl (100 mM, pH 8.0), EDTA (5 mM), followed by centrifugation at 10,000 g for 10 minutes at 4°C.

Analysis of polyST enzyme activity using anion exchange HPLC system
Separation was achieved using a DNA Pac PA-100 analytical anion exchange column as described previously. The mobile phases used were: distilled water (mobile phase A) and ammonium acetate (0.5 M, pH 7.4; mobile phase B) with flow rate of 1.2 (ml/min) and the solvent gradient outlined in Supplementary Data, Table 2.

Analysis of polyST enzyme activity using reversed phase HPLC
Reversed phase chromatography was performed at 1 ml/min with ammonium formate (5 mM, pH 6, mobile phase A) and 100% methanol (mobile phase B). The elution of DMB-DP3 was performed with an isocratic method of 20 % mobile phase B over 6 minutes. The separation was monitored by both photodiode array (Waters 2996) and fluorescence detector (RF-10A) as described above.

Western blotting for PolySia-NCAM
Samples were prepared by incubating NCAM (2.5 ng/µL) with human polyST (ST8SiaII) enzyme (12.5 ng/µl) suspended in manganese chloride (10 mM) and sodium cacodylate buffer (10 mM, pH 6.7) for five minutes at 37°C. The reaction then was started by adding CMP-N-acetylneuraminic acid (CMP-Neu5Ac, 10 mM) and incubated at 37°C for 30 minutes. A negative control was prepared without polyST enzyme.

Samples were resolved on 6% polyacrylamide gels and blotted onto PVDF membranes (Amersham). Non-specific antibody binding was blocked via incubation with skimmed milk (0.05 g/ml) and the blot was probed with anti-polySia antibody mAb 735 (obtained as a kind gift from the Gerardy-Schahm group; 1:3000 dilution) overnight at 4°C. Antibody reactivity was detected by horseradish peroxidase (HRP)-conjugated antibody and chemiluminescence using ECL-Plus (Amersham).

Results and discussion

Optimisation of the DMB labelling conditions for DP3
DP3 labelling was performed as described previously.44 Three different incubation conditions for DP3 labelling with DMB have been previously published28, 45 but have never been compared. In order to investigate the optimum conditions, reaction mixtures were each incubated for 30 minutes at 50°C, 24 minutes at 4°C and 48 minutes at 4°C. It was found that opting for labelling conditions of 30 minutes at 50°C instead of 48 or 24 hours at 4°C not only reduced the time required for DMB-DP3 labelling, but also improved efficiency, delivering a final yield of approximately 85 ± 6% by mass after purification. This follows reversal of lactonisation (MW 988.3) into the unlactonised form (MW 1006.3), as described below. This represents a significant improvement over previously published studies that reported yields of only 38%.44
Optimisation of the DMB-DP3 purification methodology

Two different HPLC methods were used to obtain DMB-DP3 of high purity: RP-HPLC and anion exchange-HPLC. These methodologies were chosen for the purification of DMB-DP3 after evaluation of the impurities was performed using LC-MS (Figure 2).

It was found that the impurities were mainly: sialic acid monomer (DP1, molecular weight 309.2), DMB-DP1 (molecular weight 424.3) and DMB-DP3 [H2O] (molecular weight 988.3, DMB-DP3 [H2O] is the lactonised form of DMB-DP3 reported previously). These impurities possess different polarity and charge, which allows both RP- and anion exchange-HPLC system to separate them effectively.

RP-HPLC purification

Three methods were utilised in order to determine the optimum conditions for DMB-DP3 purification using RP-HPLC (Supplementary Data, Table 3). The purification of DMB-DP3 dissolved in distilled water (1 µl DMB-DP3: 1000 µl water) was achieved first by RP-HPLC where the mobile phases used were: 1% methanol, 0.01% formic acid (pH 3.5) (mobile phase A) and 55% methanol, 0.01% formic acid (mobile phase B). The peaks were analysed by mass spectrometry. Both lactonised and non-lactonised forms of DMB-DP3 were detected (see Figure 2B).

Lactonisation of sialic acids and oligomers has been previously reported in more than one study and has been attributed to acidic conditions (Figure 2A). In order to prevent this lactonisation with DMB-DP3, the pH of the mobile phase was increased: mobile phase A was modified from 1% methanol, 0.01% formic acid (pH 3.5) to ammonium formate buffer (pH 6). However, observed lactonisation of DMB-DP3 was not sufficiently improved using these conditions (Figure 2C).

Increasing the pH further by changing mobile phase B from 55% methanol, 0.01% formic acid (pH 5.8), to 55% methanol only (pH 7.2) and changing the method from 15% B to 10% B for better chromatographic separation proved successful. These conditions successfully minimised DMB-DP3 lactonisation (as calculated by peak area of lactonised form compared to the total peak area). While lactonisation was not completely suppressed, it did result in a more efficient separation (Figure 2D). A single fraction containing the DMB-DP3 product was collected between 11 and 15 minutes. Fractions from several injections were pooled and freeze-dried. The purified DMB-DP3 was dissolved in water to create a 200 µM solution and stored at -20°C. Analysis of the material using MS-HPLC and FD-HPLC revealed compound purity of ≥99%, representing a significant improvement.

Anion exchange-based HPLC purification

DMB-DP3 purification using anion exchange-HPLC was initially performed using the methodology outlined in Supplementary Data, Table 4. Three peaks were detected by fluorescence (emission λ, 448 nm). DMP-DP3 was determined to be a peak with a retention time of 9.42 min (data not shown), as confirmed by comparison with a standard. As the mobile phase used in the anion exchange HPLC methodology was not MS-compatible (5 M ammonium acetate), DMB-DP3 identity was not confirmed directly by mass spectrometry.

Although the product peak appeared well resolved when using fluorescence detection and photodiode array detection at 373 nm (Figure 3A), when examined by the photodiode array detector at 303 nm (Figure 3B), it emerged that the peak representing DMB-DP3 was in fact two merged peaks.

In order to resolve these peaks, the methodology was modified as described in the experimental section (Supplementary Data, Table 1), which proved successful. The product from the peak representing DMB-DP3 (13.8 min, Figure 3D) was collected from several injections and freeze-dried. The solid material was analysed, confirmed as pure (Figure 3E), weighed and dissolved in distilled water to create a 200 µM solution and stored at -20 °C.

Two different methodologies were explored in order to determine the optimum method of purification. Although RP-HPLC purification resulted in ≥ 99% pure compound, a 45 minute method was required. Utilising anion exchange HPLC enabled use of a method requiring only 30 minutes per injection and was thus ultimately preferable.
As mentioned previously, it was observed that under all conditions, samples gave a higher yield of DMB-DP4 after 24 hours of incubation with polyST. This could be explained by minimisation and/or reversal of the lactonisation of DMB-DP3, which leads to a product not accepted by polyST, after incubation with sodium cacodylate buffer (pH 6.7).

In an attempt to improve the yield of DMB-DP4 without the need for a lengthy 24 hour incubation, purified DMB-DP3 was pre-incubated for 1 hour at 37°C in cacodylate buffer (pH 6.7) in order to ensure the equilibrium was shifted towards formation of the desired form of DMB-DP3 prior to starting the reaction with polyST. This protocol showed considerably higher efficiency than that previously observed (Figure 4C). To confirm that the improvement in polysialylation efficiency was indeed caused by pre-incubation of DMB-DP3 (and thus minimisation of lactonisation of DMB-DP3), the polysialylation of DMB-DP3 purified by RP-HPLC, anion-exchange HPLC or purified by both systems was similarly pre-incubated and the results were compared to that without a pre-incubation step (Figure 4C). It was found that equilibrium is crucial.

The reduction in polysialylation reaction efficiency associated with DMB-DP3 lactonisation could be explained by the relative inability of polyST to incorporate new sialic acid monomers within the lactonised structure. Human polyST catalyses the reaction between an incoming sialic acid residue and DMB-DP3. The 2-position of the incoming sugar (in the form of CMP-sialic acid) is glycosylated to the 8 position of the non-reducing terminal sugar of DMB-DP3. In lactonised DMB-DP3 these positions are involved in lactone ring formation (Figure 2A).

**Optimisation of the polyST enzyme reaction with DMB-DP3**

To determine enzyme activity, human polyST (ST8SiaII) was incubated with DMB-DP3 and the product was detected by anion-exchange HPLC, as described in the experimental section. The results were compared with a negative control (i.e. without enzyme).

Following polysialylation of DMB-DP3 in the presence of polyST, a range of polymer chain lengths (DP) of polySia could be expected. The reaction conditions were adjusted and optimised in order to maximise production of DMP-DP4, i.e. only one sialic acid monomer unit extension. This was desirable to allow for easier detection and data analysis, and to ensure the reliability and reproducibility of the assay. It was found that incubating polyST with DMB-DP3 for 24 hours, showed significant increase in the yield of DMB-DP4 product over 2 hours incubation yield (Figure 4A).

To determine the optimum temperature for enzyme activity, human polyST (ST8SiaII) was incubated with DMB-DP3 at 15, 25, 37 and 50°C. Interestingly, incubating the reaction at 25°C resulted in a higher production of DMP-DP4 (Figure 4B) than at 37°C (i.e. normal body temperature). This was noted previously with bacterial and murine polyST enzymes. Clearly the conditions and buffers used to perform the reaction in vitro are different to the situation in vivo.

Further optimisation was performed by incubating polyST with different concentrations of DMB-DP3 (10 -100 µM) and detection of the product DMB-DP4 after 2 and 24 hours. There was no significant advantage of increasing the DMB-DP3 above 20 µM (Figure 4A).

![Figure 3. Purification of DMB-DP3 using anion exchange HPLC. The peak initially identified as DMB-DP3 by fluorescence detection, as analysed by photodiode array at 373 nm (A) and 303 nm (B), revealing two peaks in the latter. Modifying the FD-HPLC method that resulted in merged peaks (C) to the gradient described in Supplementary data, Table 4, enabled complete separation of the two merged peaks (D). (E) Purified DMB-DP3 as observed by anion exchange FD-HPLC.](image-url)

**Optimisation of the assay: DMB-DP4 product quantification**

In order to accurately quantify the DMB-DP4 produced by the reaction of DMB-DP3 and polyST, DMB-DP4 was synthesised and purified utilising methodology similar to that employed for DMB-DP3. A calibration graph was constructed (quantity DMB-DP4 produced vs peak area, figure 4D).

**Analysis of polysialylation reaction kinetics, assay sensitivity and product stability**

PolyST activity was determined by incubating the enzyme with the substrate (CMP-Neu5Ac) and varying amounts of DMB-DP3 acceptor. The assay kinetic parameters (K<sub>in</sub>, V<sub>max</sub>) were then calculated. The K<sub>in</sub> value of the DMB-DP3/STX reaction was calculated from a Lineweaver-Burk plot (Figure 5A). The experiment was repeated three times and the average K<sub>in</sub> value was calculated as 29.5 ± 3.0 µM. The V<sub>max</sub> value of the reaction (14.8 ± 2.7 µmol/min/mg) was also calculated using the same plot. These values are consistent with those observed previously and serve to validate the new assay.

In order to determine assay sensitivity and suitability for determination of polyST inhibition, enzyme activity, as measured by DMB-DP4 product formation, was assessed in the presence of varying concentrations of the polyST inhibitor, CMP (Sigma-Aldrich, UK). The quantity of DMB-DP3 acceptor was maintained at a constant level. It was found that a direct relationship between inhibitor concentration and the degree of DMB-DP3 polysialylation could be established, and that even small changes in inhibitor concentration proved detectable by analysis of product formation (Figure 5B). In order to determine the inhibition constant (K<sub>i</sub>) value for CMP, three concentrations of CMP were used (20, 50 and 150 µM) and a control (no CMP). Each concentration was assessed in the presence of different concentrations (250, 425 and 500 µM) of CMP-Neu5Ac (substrate).

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**Figure 3. Purification of DMB-DP3 using anion exchange HPLC. The peak initially identified as DMB-DP3 by fluorescence detection, as analysed by photodiode array at 373 nm (A) and 303 nm (B), revealing two peaks in the latter. Modifying the FD-HPLC method that resulted in merged peaks (C) to the gradient described in Supplementary data, Table 4, enabled complete separation of the two merged peaks (D). (E) Purified DMB-DP3 as observed by anion exchange FD-HPLC.**
The $K_i$ value was determined by constructing a Dixon plot, which additionally confirmed competitive inhibition, as indicated by the location of the intercept. The experiment was repeated three times; the average $K_i$ value was measured as 12 $\mu$M (Figure 5B). Once more, this value is consistent with the results published previously and serves to validate the new optimised assay.

**Optimisation of chromatography for high-throughput analysis**

For ultimate utility as a compound screen for identification of novel polyST inhibitors, high-throughput analysis is an essential requirement. In order to achieve this, further optimisation was performed by utilising RP-HPLC, as described in the experimental section.

Using these conditions, DMB-DP4 eluted at 3.9 minutes while DMB-DP3 eluted at 5.9 minutes. The methodology was repeated six times independently to ensure its reproducibility (Figure 5C), which was confirmed.

RP-HPLC allowed for rapid, sensitive and reproducible analysis, due to the fact that the column needs no time for equilibration between runs: the method is isocratic, rather than the gradient elution used with anion exchange chromatography. The short retention times and excellent resolution allow for rapid analysis (less than 10 minutes) per sample. Another advantage of using RP-HPLC is the ability to conjugate it to mass spectrometry, which allows very specific product characterisation and molecular weight information.

**Analysis of DMB-DP4 product stability**

High-throughput analysis of large numbers of polyST inhibitors will potentially mean large numbers of HPLC samples for analysis, which could thus potentially remain in an HPLC machine for prolonged periods. In this event, the stability of the DMB-DP3 acceptor and DMB-DP4 product over this lengthy period are critically important. Stability of DMB-DP3 and DMB-DP4 were evaluated over a 48 hour period at room temperature. It was determined that both DMB-DP3 and DMB-DP4 are stable under these conditions in the context of the assay (Figure 4D, 4E).

**Analysis of the assay limit of detectability**

In order to determine the limit of detection of the product DMB-DP4 (smallest peak area that is distinguishable peak from baseline) produced in the DP-DP3 polysialylation reaction, different concentrations of standard DMB-DP4 were injected (1 – 50 nM). The peak area corresponding to each concentration was recorded and plotted against the DMB-DP4 concentration. It was found that using the previously described optimised conditions, a concentration of 1 nM of DMB-DP4 could be detected (which is 1/10,000 of the concentration of DMB-DP3 added) (Figure 5F).

**Analysis of assay reproducibility**

In order to assess assay reproducibility and reliability, seven independent control samples using the same concentration of DMB-DP3, polyST enzyme and CMP-Sia substrate were analysed. The percentage of the product DMB-DP4 was calculated (relative to the total peak area) and the standard deviation and coefficient of variation were calculated.

It was found that the results were reproducible with an average of 7.8% conversion of DMB-DP3 into DMB-DP4 and standard deviation of 0.58 and coefficient of variation of 0.07, meaning the results were reproducible.

**Validation of the assay**

CMP was utilised in order to validate the developed high throughput cell-free assay by evaluating its effect on polySia synthesis using both the developed assay and previously described western blot analysis.

The average product (DMB-DP4) formation relative to control sample after treatment with different concentrations of CMP was calculated using the developed assay and compared with the results of western blot analysis. According to the results obtained from this assay, it was found that 250 $\mu$M CMP resulted in 70% inhibition of polySia synthesis as measured by the developed assay and 74% as measured by western blot (using imageJ software for western blot quantification, Figure 6). This demonstrates concordance between the two assays.

![Figure 4](image)

Figure 4. Optimisation of the DMB-DP3 reaction with human polyST enzyme. (A) Optimisation of the DMB-DP3 concentration and duration of incubation with polyST shows no significant difference between the 20 and 100 $\mu$M DMB-DP3, but incubating the enzyme for 24 hours instead of 2 hours showed significant increase in DMB-DP4 yield; (B) Incubation of DMB-DP3 with human polyST showed that 25 °C incubation temperature is optimum; (C) incubation of human polyST with DMB-DP3 purified by RP-HPLC, anion exchange HPLC or both techniques combined with incubation of the purified DMB-DP3 for 1 hour at 37°C in sodium cacodylate buffer (pH 6.7) compared to incubation of human polyST with DMB-DP3 purified by RP-HPLC without incubation in sodium cacodylate buffer. The results show that the efficiency of polysialylation of DMB-DP3 depends on the reversal of lactonisation of the DMB-DP3, achieved by a pre-incubation step rather than purification technique. (D) Calibration graph for DMB-DP4 quantification.
Figure 5. Analysis of assay kinetics and optimisation of the chromatography to allow high throughput-HPLC analysis. Lineweaver-Burk plot (A) and Dixon plot (B). $K_m$ of DMB-DP3 for ST8SiaII (derived from x axis intercept, Lineweaver-Burk plot) was calculated as $29.5 \pm 3.0 \mu M$ and $V_{max}$ value (calculated from y axis intercept) was calculated as $14.8 \pm 2.7 \mu mol/min/mg$. The $K_i$ value of CMP was calculated from the Dixon plot. Graph lines converge above the x axis; where they intersect is $-K_i$. Data points are from a single determination representative of three independent experiments. (C) The reproducibility of the results when running 6 samples of standard DMB-DP4 and DMB-DP4 consecutively. The first peak at 3.9 minutes represents DMB-DP4 while the second peak at 5.9 minutes represents DMB-DP3, the last chromatogram shows DMB-DP3 only. (D) Stability of DMB-DP4 and (E) DMB-DP3. The experiment was carried out by incubating DMB-DP3 and DMB-DP4 at room temperature for 48 hours. 10 µl of the sample at T0 was analysed using HPLC, then similar volumes of sample were analysed at regular intervals over 24h, then again at 48h. Peak area was measured and percent peak area compared to T0 was calculated and plotted. The experiment was repeated three times and the standard deviation was determined. (F) Limit of detectability of DMB-DP4 standard.

Conclusion

This study describes the development of the first reliable, sensitive, reproducible, cost-effective and high-throughput technique for quantitative analysis of human polyST activity. Furthermore, the protocol for synthesis and purification of DMB-DP3 has been closely optimised to allow large scale production of DMB-DP3. This assay offers significant advantages over previously reported techniques. It is the first study that allows analysis of human polyST enzymes using RP-HPLC combined with mass spectrometry allowing specific detection of polysialylation products. In addition, the assay will allow further opportunity to study these important polyST enzymes and will enable development of chemical probes to better understand biological processes associated with polyST activity and may ultimately pave the way to the development of novel therapeutics.

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Figure 6. The effect of polyST inhibitor CMP on polySia biosynthesis as measured by both the cell-free chromatographic assay and western blot analysis. (A) Represents polyST inhibition using 0.25 M CMP, (B) represents polyST inhibition using 0.5 M CMP. Lower chromatogram represents the control (no CMP) while upper chromatogram represents reaction in the presence of CMP the inhibitor (C) Lane 1 represents the negative control (without polyST enzyme), lane 2 represents the positive control (without CMP), lanes 3 - 7 represent reactions with 0.1, 0.25, 0.5, 0.8 and 1 mM CMP respectively. The blot is representative of three independent experiments.

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