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1 # An assay for quantitative analysis of polysialic acid expression in cancer cells

2 Xiaoxiao Guo,[a] Sara M. Elkashef,[b] Anjana Patel, Goreti Ribeiro Morais, Steven D. Shnyder, Paul M.
3 Loadman, Laurence H. Patterson, and Robert A. Falconer*

4 Institute of Cancer Therapeutics, Faculty of Life Sciences, University of Bradford, Bradford BD7 1DP, United
5 Kingdom

6 [a]Current address: Discovery Sciences, R&D, AstraZeneca, Cambridge CB4 0WG, United Kingdom

7 [b]Current address: University Health Network, Princess Margaret Cancer Research Center, Toronto ON, M5G
8 2M9, Canada

9 *Corresponding author. Tel +44 (0)1274 235842. E-mail address: r.a.falconer1@bradford.ac.uk

10 ## Abstract

11 Polysialic acid (polySia) is a linear polysaccharide comprised of *N*-acetylneuraminic acid residues and its
12 over-expression in cancer cells has been correlated with poor clinical prognosis. An assay has been
13 developed for quantitative analysis of cellular polySia expression. This was achieved by extracting and
14 purifying released polySia from glycoproteins by mild acid hydrolysis and optimised organic extraction. The
15 polySia was further hydrolysed into Sia monomers, followed by fluorescent labelling and quantitative
16 analysis. The assay was qualified utilising endoneuraminidase-NF to remove polySia from the surface of C6-
17 ST8SiaII cancer cells (EC_{50}=2.13 ng/ml). The result was comparable to that obtained in a polySia-specific
18 cellular ELISA assay. Furthermore, the assay proved suitable for evaluation of changes in polySia
19 expression following treatment with a small molecule inhibitor of polysialylation. Given the importance of
20 polySia in multiple disease states, notably cancer, this is a potentially vital tool with applications in the fields
21 of drug discovery and glycobiology.

22

23 **Keywords:** polysialic acid, sialic acid, quantitative analysis, cell-based method, inhibitor screening

24
25

1. Introduction

Polysialic acid (polySia, **Fig. 1**) is an unusual linear polysaccharide, comprised of negatively-charged *N*-acetylneuraminic acid residues (Sia, Neu5Ac), linked specifically *via* α-2,8- and α-2,9-glycosidic bonds (Finne, 1982; Angata et al., 1997). The polymer length, or degree of polymerisation (DP), typically varies from 8 to up to 400 sialic acid monomer units (Finne, 1982). As an important post-translational modification of several important proteins, notably the neural cell adhesion molecule (NCAM), polySia plays significant roles in cellular and physiological processes, including cell-cell and cell-matrix adhesion, cell migration, synapse formation and functional plasticity of the developing nervous system (Bruses & Rutishauser, 2001). Due to the bulky and polyanionic nature of polySia, cell adhesion molecules that are heavily-polysialylated display anti-adhesive properties, which are important in regulating cell-cell interactions and cell migration (Ulm et al., 2013).

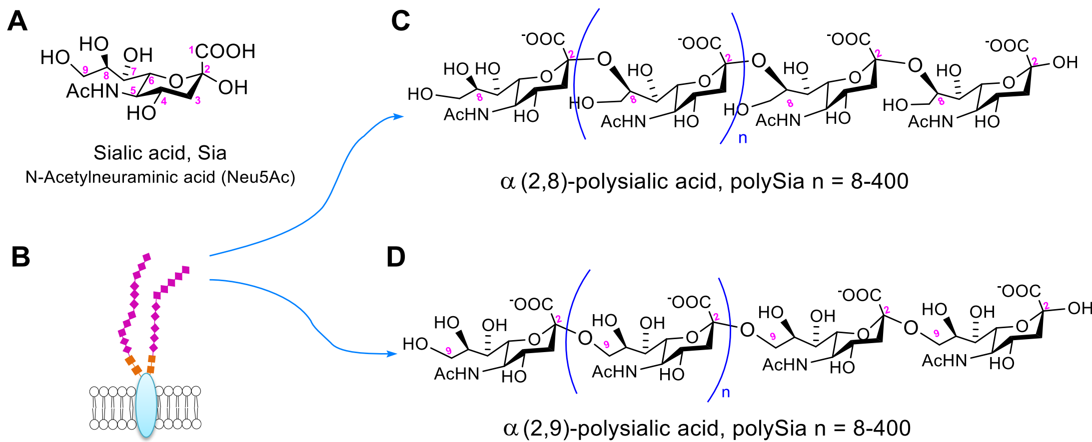

Figure 1. Structure of sialic acid (Neu5Ac, Sia) (A) and polysialic acid (C & D). PolySia found in glycoproteins (B) can be linked by α-(2,8) glycosidic bonds (C) or α-(2,9) glycosidic bonds (D).

PolySia expression is abundant in human embryos, but is relatively limited in adulthood, where it is only detectable at low levels in discrete areas of the adult brain, blood, milk, semen, and immune cells (Finne, 1982; Angata et al., 1997; Yabe, Sato, Matsuda, & Kitajima, 2003; Simon et al., 2013; Ulm et al., 2013; Zlatina et al., 2018). Interestingly, polySia expression is recapitulated in a large number of malignant human tumours, notably neuroblastoma and small cell lung cancer, where it is closely correlated with cancer progression and poor patient prognosis in the clinic (Tanaka et al., 2000; Suzuki et al., 2005; Valentiner, Muhlenhoff, Lehmann, Hildebrandt, & Schumacher, 2011; Falconer, Errington, Shnyder, Smith, & Patterson, 2012), driving tumour migration, invasion and metastasis (Falconer et al., 2012). The two enzymes responsible for polySia biosynthesis in cells, i.e. polysialyltransferases ST8SiaII and ST8SiaIV (polySTs), are thus potential therapeutic targets, and efforts are currently underway to design inhibitors as potential anti-metastatic agents (Falconer et al., 2012; Al-Saraireh et al., 2013). The ability to analyse and quantify the levels of endogenous polySia in biological samples, and in cellular systems in particular, is therefore of great importance.

During recent decades, chemical methods based on anion-exchange high performance liquid chromatography (AE-HPLC) separation have been developed to structurally characterise polySia from mammalian brain tissues and cells. Generally speaking, polySia liberated from glycoproteins by mild acid hydrolysis is labelled with a fluorescent tag (i.e. 1,2-diamino-4,5-methylenedioxybenzene, DMB), followed by separation using anion-exchange chromatography according to the DP (C. Sato, Inoue, Matsuda, & Kitajima, 1998; Inoue, Lin, Lee, & Inoue, 2001). These methodologies allow for quantitative determination of chain length, but suffer from low sensitivity, particularly in the case of long-chain polySia present in low abundance, as is usually the case in biological samples (Inoue et al., 2001; Guo, Elkashef, Loadman, Patterson, & Falconer, 2019). On the other hand, multiple antibodies have been developed to recognise specific oligosialic acids, polySia, and polysialylated proteins in biological samples (Chihiro Sato & Kitajima, 2008; Galuska, 2013; Guo et al., 2019). Compared to chemical methods, antibody-based methodologies offer

rapid, specific and even high-throughput analysis. That said, antibody-based analysis relies simply on the specificity of the antibody in question, and does not allow polySia polymers of various lengths to be distinguished (Nishimura et al., 2014).

To accurately and reproducibly quantify the effects of polyST inhibitors on cellular polySia levels in cancer cells, a sensitive chemical assay is required. Since mild hydrolysis-based HPLC assay methodology is not sufficiently sensitive for quantitative analysis of long-chain polySia, a sensitive assay for indirect measurement was developed here. We hypothesised that chemically cleaving polySia selectively from the cell surface, followed by solvent extraction from the crude cellular protein mixture, would allow for quantitative cellular polySia analysis. In this case, the assay involves evaluation of Sia monomers derived from cellular polySia, which were quantified to allow determination of changes in total cellular polySia after inhibitor treatment. This was achieved by extraction and purification of cellular polySia after mild acid hydrolysis, followed by further hydrolysis into Sia monomers prior to DMB fluorescence labelling. Furthermore, in order to minimise the cellular background of Sia derived from non-polysialylated glycoproteins following hydrolysis, of which there are many (Rodrigues & Macauley, 2018), polySia purification was optimised. To do so, colominic acid, a commercially-available polySia source with a wide range of polymer chain lengths, was spiked into the lysate of wild-type cells that do not naturally express polySia. The recovery of this 'spiked' polySia from the lysates of cells using precipitation by various organic solvents was then evaluated. The assay was qualified by analysis of isogenic cancer cells as a model system: C6-ST8SiaII (transfected to express ST8SiaII and thus polySia) and C6-WT (which do not express polySia, as control) utilising endoneuraminidase NF (endo-NF), an enzyme that specifically recognises and hydrolyses polySia (Gerardy-Schahn et al., 1995). The assay was subsequently applied to the evaluation of polyST inhibitors.

2. Materials and Methods

2.1 Chemicals & reagents

All general chemicals, media and media supplements were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. The water used throughout the study was obtained from a Milli-Q Reagent Water System (Triple Red Ltd., UK). HPLC-grade acetonitrile and methanol were purchased from Merck. The reverse-phase chromatography analytical column (RPB, 2.1 x 250 mm, 5 μm particle size) was purchased from Hichrom Ltd. UK, and the anion exchange chromatography DNA-Pac™ PA100 Oligonucleotide column (250 x 4 mm, 13 μm particle size) was purchased from Dionex, Ltd. UK. ICT3176 is an analogue of *N*-acetylmannosamine, and was synthesised as previously described (Elkashef, Allison, et al., 2016).

2.2 Cell culture

Cell lines - The rat glioma C6-ST8SiaII cell line and C6-WT cell line were kind gifts from the Minoru Fukuda lab (SBP Medical Discovery Institute, CA, USA), as described previously (Suzuki et al., 2005; Elkashef, Allison, et al., 2016). Briefly, both cell lines were cultured in Alpha MEM Eagle medium (Lonza Ltd., UK) supplemented with 10% (v/v) foetal bovine serum (Sigma, UK). Cells were incubated in a humidified incubator with 5% CO_2/ 95% air atmosphere at 37 °C.

Endo-NF activity analysis - One million C6-ST8SiaII cells were treated with Endo-NF (a kind gift from the Rita Gerardy-Schahn lab) at various concentrations in 100 μl of 100 mM Tris-HCl (pH 6.8) at 37 °C for 1 h on a heating block. Subsequently, cells were lysed and the cellular polySia was further extracted, as described in section 2.3, hydrolysed and analysed.

Effect of polyST inhibitor ICT3176 on cells - Cells were seeded on a 6-well plate at a density of 2 x 10^5 per well in complete medium at 37 °C in the presence of 5% CO_2. After overnight culture, cells were treated with known ST8SiaII enzyme inhibitor ICT3176 (initially dissolved in dimethyl sulfoxide (DMSO), and then diluted to various concentrations in complete medium to give a final concentration of 0.1% DMSO), for 48 h at 37 °C. Vehicle controls were cells treated with 0.1 % DMSO in parallel, while negative controls were cells treated with 0.5 μg/ml Endo-NF in the last 3 h of the compound treatment in parallel. After ICT3176

1 treatment, cells were rinsed with phosphate-buffered saline (PBS) and harvested using Accutase Cell
2 Detachment solution (Sigma, UK). Cell pellets were washed by PBS prior to cell lysis and further analysed.

3 **2.3 Extraction of polySia from cells**

4 Cell pellets were lysed and de-lipidated as previously described (Inoue et al., 2001), with minor
5 modifications. Briefly, pellets were re-suspended in lysis buffer (100 µl) consisting of Tris-HCl (100 mM, pH
6 6.8), 0.5% CA-630 and protease inhibitors cocktail (Sigma, UK). The cell suspension was then gently
7 sonicated in ice and allowed to stand in ice for 30 min to allow complete lysis. Subsequently, the cell lysate
8 was mixed with 400 µl of a chloroform/methanol solution (1:2, *v:v*) for 10 min at room temperature, followed
9 by centrifugation at 20,000 g for 20 min at 4 °C. Supernatant containing lipids was discarded, and the protein
10 pellets were further washed twice with 400 µl of 80% ethanol and subsequently dried in a Speedvac
11 (Genevac Ltd., UK).

12 Cell Lysate protein pellets were first re-dissolved in a 2% CA-630 aqueous solution, with gentle vortex
13 mixing. An equal volume of buffer containing trifluoroacetic acid (TFA, 40 mM), beta-mercaptoethanol (1 M)
14 and $Na_2S_2O_4$ (18 mM) was then added to the mixture, followed by incubation at 50 °C for 1 h, to release
15 polySia from protein carriers. The released polySia was further isolated from the protein suspension by 90%
16 acetone precipitation, followed with a 60% methanol wash to recover any precipitated polySia from the pellet.
17 The combined supernatant was then dried in a Speedvac, followed by re-dissolving in H_2O (22 µl) and NaOH
18 (1 M, 7.5 µl) to adjust the solution to pH 9.0, and incubated at room temperature for 30 min to reverse the
19 lactonisation of polySia. The de-lactonised polySia was further incubated with 90% ethanol in NaCl (final
20 concentration 100 mM) at -20 °C for 30 min, prior to centrifugation at 20,000 g for 20 min. The supernatant
21 was subsequently discarded, and the pellet washed with 80% ethanol twice, and dried in a Speedvac. The
22 purified polySia was then hydrolysed into Sia monomers with TFA (0.1 M, 100 µl) for 3 h at 80 °C. After
23 hydrolysis, samples were dried in a Speedvac, prior to DMB labelling.

24 **2.4 Analytical detection of polySia and Sia monomers**

25 ***DMB labelling of colominic acid and Sia monomers***. Sialic acid (Neu5Ac) and colominic acids were
26 labelled with the fluorophore 1,2-diamino-4,5-methylenedioxybenzene (DMB). Briefly, DMB (40 mM) was
27 dissolved in labelling buffer containing TFA (40 mM), sodium hydrosulfite (18 mM) and β-mercaptoethanol (1
28 M). Then, samples were mixed with an equal volume of DMB (40 mM, 1:1, *v*:*v*) and incubated at 50 °C for 1
29 h. The reaction was terminated by adding NaOH (1 M) to the reaction mixture (final concentration 100 mM)
30 and allowing it to stand at room temperature for 30 min to neutralise the pH of reaction mixture. Samples
31 were then dried in a Speedvac to remove the TFA prior to analytical analysis.

32 ***Reverse-phase chromatographic analysis of DMB-labelled Sia.*** Samples for analysis were subjected to
33 separation with a reverse-phase chromatographic column (2.1 x 250 mm, 5 µm, RPB-Hichrom, Ltd., UK),
34 utilising eluent A (aqueous 5 mM ammonium formate), and eluent B (90% acetonitrile in 5 mM ammonium
35 formate). Samples were analysed using a 25 min linear gradient of 5%-12% of eluent B, followed by a 5 min
36 linear gradient to 100% B, at a flow rate of 450 µl/min at room temperature. Each chromatographic
37 separation was conducted over 50 min. The DMB-labelled Sia was detected on a Waters Alliance system
38 equipped with a RF-10A XL fluorescence detector (Shimadzu, UK) at an excitation wavelength of 373 nm
39 and an emission wavelength of 448 nm (Elkashef, Sutherland, Patterson, Loadman, & Falconer, 2016). The
40 acquired chromatograms were analysed by Masslynx 4.1 and Quanlynx software (Waters, UK).

41 ***Anion-exchange chromatographic analysis of DMB-labelled PolySia.*** Samples for analysis were
42 subjected to separation with a DNAPac™ PA100 chromatographic column (250 x 4 mm, 13 µm, Dionex, Ltd.,
43 UK). Elution was performed at a flow rate of 1.0 ml/min as follows: i) isocratic 100% eluent A (HPLC grade
44 water) for 5 min; ii) linear gradients of eluent B (1 M ammonium formate, pH 8.0, in HPLC grade water) to 20,
45 25, 32.5, 38.5, and 40% at 15, 20, 35, 51, and 55 min, respectively, followed by a column wash with 100%
46 eluent B for 10 min. Each chromatographic separation was conducted over 80 min at room temperature. The
47 DMB-labelled polySia was visualised as described above. The acquired chromatograms were analysed by
48 Masslynx 4.1.
49

1 ## 2.5 In-Cell ELISA

2 Cells were seeded in 96-well plates at a pre-determined density (5,000 and 10,000 cells per well) overnight
3 for attachment, followed by treatment with ICT3176 at various concentrations, in complete medium for 48 h
4 at 37 °C. After compound treatment, cells were fixed and permeabilised by adding an equal volume of 2x
5 fixation solution (8% paraformaldehyde and 1% Triton X-100 in PBS) into each well for 15 min at room
6 temperature. After fixation and a PBS wash, BSA (3% in PBS, 100 μl) was added to block the wells for 30
7 min at room temperature, followed by incubation with mouse anti-polySia monoclonal antibody (mAb 735, 15
8 μg/ml) overnight at 4 °C. After the PBS wash, cells were further incubated with HRP-conjugated anti-mouse
9 antibody (1:5000 in 3% BSA/PBS, 100 μl) for 1 h at room temperature. Cells were carefully washed with PBS
10 to remove unbound HRP-conjugated antibody prior to the addition of substrate solution (Ultra TMB-ELISA
11 substrate solution, Sigma, UK) into each well. Substrate reactions were then quenched by addition of H_2SO_4
12 (2 M, 100 μl) into each well. Absorbance was measured at 450 nm. Data were analysed by using Graphpad
13 Prism 8.0.

14 ## 2.6 Data analysis

15 Inhibition of cellular polysialylation was calculated as a percentage by measuring the reduction of fluorescent
16 signal peak areas (measured as Relative Fluorescence Units, RFU) of DMB-labelled Sia monomers
17 hydrolysed from polySia from both treated and untreated cells, according to the equation 1:

18 % **Inhibition** = $[1- (RFU_{(i)} - RFU_{(Endo\text{-}NF)}) / (RFU_{(Control)} - RFU_{(Endo\text{-}NF)})] \times 100\%$ (equation 1)

19 The RFU of Sia derived from polySia in the Endo-NF treated cells was used as a Sia background control.

20 All data points presented are representative of 3 independent experiments, each with a minimum of 2
21 technical replicates. Analyses were conducted using Graphpad Prism software.

22

23 ## 2.7 Overview of assay methodology

24 A schematic representation of the methodology developed for cellular polySia isolation, purification and
25 analysis using the developed assay is shown in **Fig. 2**.

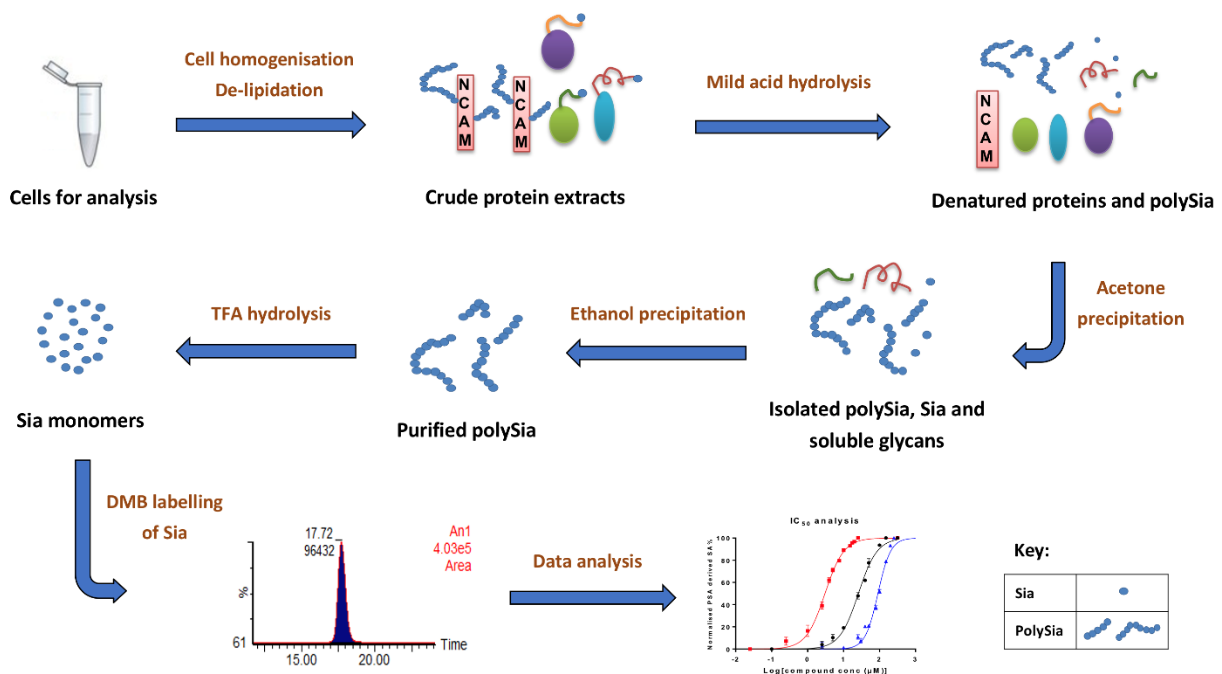

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Figure 2: Schematic representation of the assay methodology for cellular polySia isolation, purification, and analysis. PolySia can be quantitated in cultured cells, followed by cell harvesting, lysis and de-lipidation. The polySia is then released from cell glycoproteins by mild acid hydrolysis. PolySia is subsequently isolated and purified from protein and Sia monomers from non-polySia sources by organic solvent precipitation. The isolated polySia is then hydrolysed to Sia monomers which are fluorescently labelled with DMB and quantitated using fluorescence-based RP-HPLC.

3. Results & Discussion

3.1 Extraction of polySia from protein mixture by organic precipitation

A prerequisite for sensitive detection of polySia (a polymer of sialic acid) in biological systems is to minimise the background due to mono-sialic acids found in other glycoproteins. Sialic acid is commonly found as the terminal sugar of cellular glycoproteins, and this presents a significant challenge. In this newly-designed assay, lipid-containing gangliosides were first removed from the cell lysate using an organic solvent (chloroform/methanol) extraction, as described previously (Inoue & Inoue, 2001; Inoue et al., 2001). After delipidation, polySia was then cleaved from its carrier protein (NCAM) using mild acid hydrolysis (Inoue & Inoue, 2003). In order to improve the assay sensitivity, hydrolysis of polySia into Sia monomers was employed. To reduce the forementioned background Sia, polySia was further purified from both the cell protein mixture and background Sia monomers by a series of organic solvent precipitation steps (**Fig. 2**).

Colominic acid is a commercially available source of polySia, containing a range of polymer chain lengths, and was a convenient tool for method development. Colominic acid was shown to be highly soluble in aqueous (90% and 95%) acetone solutions following centrifugation (**Fig. 3A**), while cell protein homogenates were almost entirely precipitated (**Fig. 3B**). We noted that colominic acid could be co-precipitated with acetone when added to delipidated cell lysate, however (**Fig. 3C**). The anionic nature of polySia is likely to facilitate its co-precipitation with cationic protein fragments and salts in the lysis buffer.

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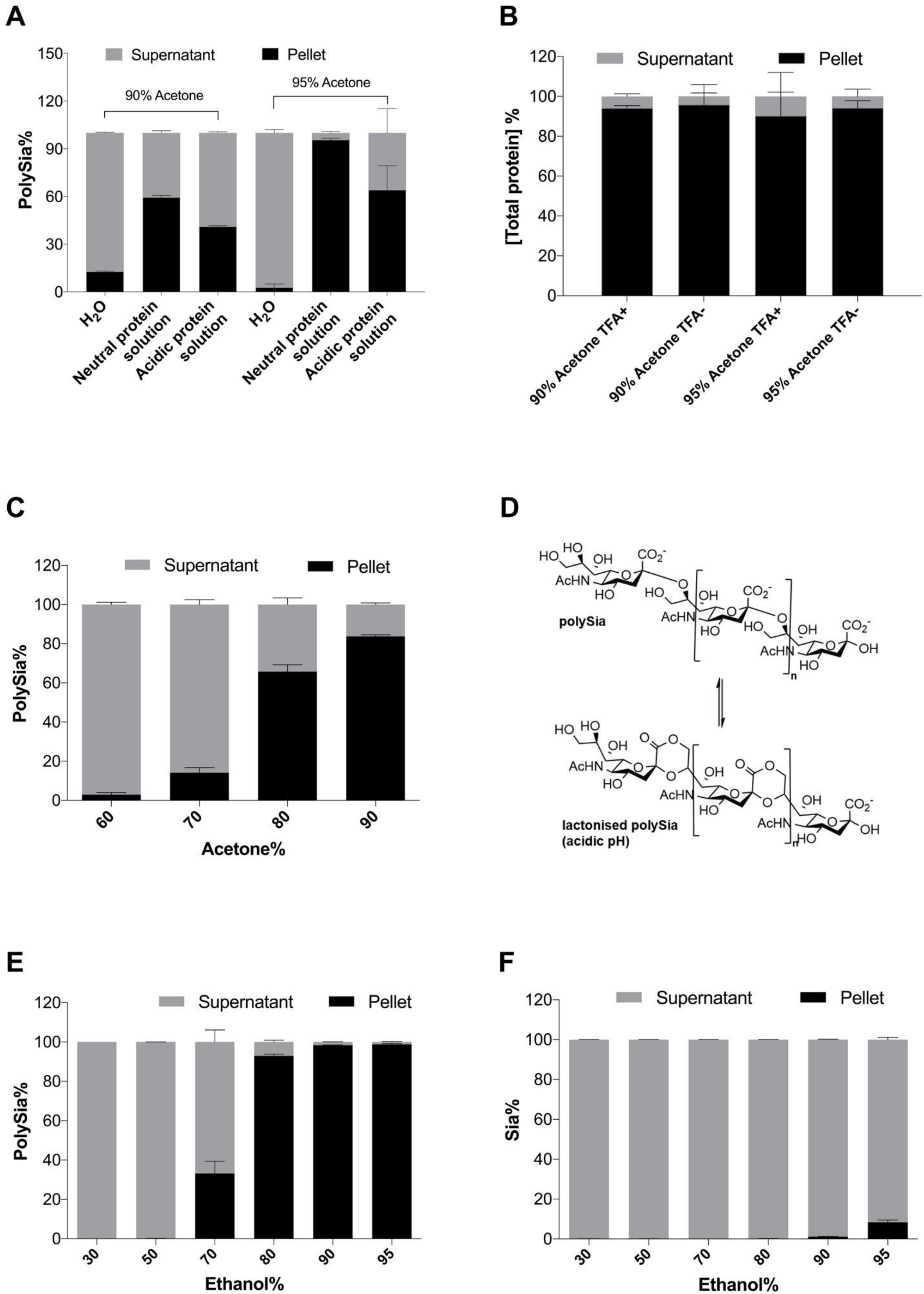

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3 **Figure 3: organic solvent extraction of polySia.** Effects of acetone on precipitating polySia (5 µg
4 colominic acid) added to water, neutral or acidic protein **(A)**; Effects of precipitating cell lysate proteins

suspended in acidic and neutral solutions by acetone **(B)**; Acetone recovery of polySia from pelleted cell lysate protein **(C)**; Structures of polySia and lactonised polySia (**D**); Effect of ethanol concentration on precipitation of non-lactonised polySia **(E)** and Sia monomers **(F)** in the presence of sodium salts (100 mM). Data presented are the mean ± SD (n = 3) from 2 independent experiments

Since polySia is known to lactonise readily in acidic conditions (Galuska, 2013) (**Fig. 3D**), we further analysed the effect of acetone in precipitating a colominic acid/protein mixture under the mild acid conditions employed. The results show that colominic acid precipitation was less in the acetone (90 or 95%) extracted acidic aqueous solution than from the pH neutral solution (**Fig. 3A**). The decreased recovery of colominic acid in the lysate pellet/increased recovery in aqueous phase could be due to lactonisation of Sia residues in the colominic acid. In this case, the increase in lactone hydrophobicity and decreased number of negative charges would diminish its interaction with salts and proteins required to facilitate its precipitation (**Fig. 3D**). Since the aim of the acetone solvent treatment is to harvest the polySia in the solvent phase whilst precipitating lysate polySia and proteins, acetone (90%) precipitation was performed immediately after mild acid hydrolysis, i.e. without neutralising the pH in aqueous phase. The co-precipitated polySia was carefully recovered from the re-suspended lysate protein pellet by using 60% acetone, a concentration which produced minimal precipitation of polySia (**Fig. 3C**).

After the removal of cell lysate proteins, the next step was to isolate the polySia from the Sia monomers (terminal Sia hydrolysed from glycans from other glycoproteins) in the supernatant solution. Since natural polySia contains a large number of negative charges (one per Sia monomer), polySia and Sia monomers could be separated by ethanol precipitation in the presence of a high concentration of salts. As shown in **Fig. 3E, 3F,** the majority of colominic acid was precipitated by a high percentage of ethanol (90-95%), in the presence of sodium salts (**Fig. 3E**), while most Sia monomers extracted from the cell lysate remained in the supernatant at 95% ethanol (**Fig. 3F**).

While ethanol precipitation can effectively separate natural polySia from Sia monomers in the presence of sodium salts, little is known about this process when polySia is in its lactonised form. Ethanol precipitation was therefore investigated using spiked colominic acid (10 μg) in the WT cell lysate (which does not contain polySia) in the presence of sodium salt. Following mild acid hydrolysis and acetone extraction (Section 2.3), colominic acid samples with or without lactonisation were analysed (polySia would be lactonised in the acidic conditions of the hydrolysis, while non-lactonised in alkaline solution). These were precipitated using 90% ethanol in the presence of sodium salts. Both the supernatant and pellet samples were subsequently subjected to anion exchange chromatography analysis after fluorescent labelling with DMB.

Anion exchange chromatography has previously been successfully employed to elegantly separate DMB-derivatised polySia of differing chain lengths (Galuska, 2013), a good example of which is shown in **Fig. 4A**. Each individual DP is separated, with longer chain lengths exhibiting increased retention times due to the increased negative charge. Sia monomers are eluted as the first peak (before 5 min), as shown in **Fig. 4A-D**. For non-lactionised colominic acid, a relatively high concentration of DMB-polySia was measured in the ethanol precipitation pellets (**Fig. 4A**) over the supernatant (**Fig. 4B**, which indicates only Sia monomers and small Sia oligomers). Conversely, the majority of lactonised colominic acid was present in the supernatant (**Fig. 4D**), with only a small amount precipitated after 90% ethanol treatment in the presence of sodium salts (**Fig. 4C**). In order to maximise the recovery of polySia from Sia monomers mainly present in the supernatant following ethanol precipitation, reversal of polySia lactonisation was necessary, ensuring that the majority of polySia could be collected in the precipitation pellets. This was achieved by adjusting the pH of the sample to pH 8 prior to the ethanol precipitation. Using this approach, ethanol (90%) precipitation resulted in > 96% colominic acid isolation, while Sia monomers present was determined to be < 2% **(Fig. 3E, 3F)**.

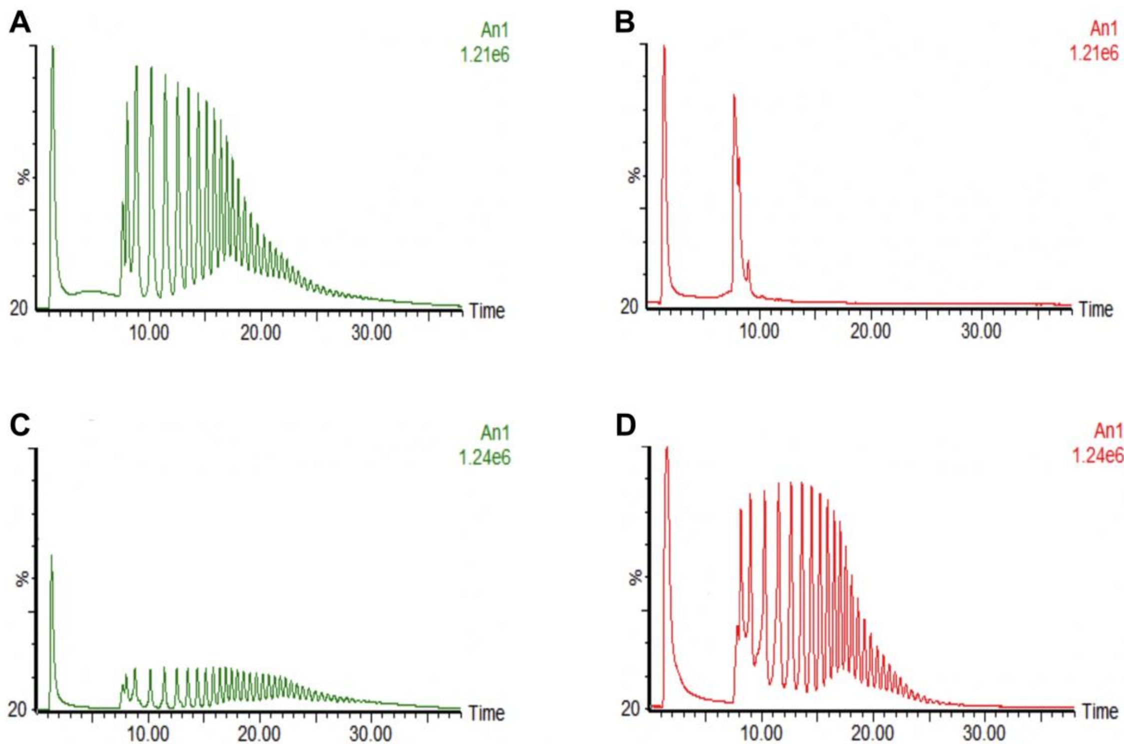

Figure 4: Effects of polySia lactonisation on ethanol precipitation of colominic acid. Anion-exchange chromatography of polySia remaining in **(A)** the cell lysate pellet, and **(B)** the cell lysate supernatant, and lactonised polySia remaining in **(C)** the cell lysate pellet, and **(D)** the cell lysate supernatant.

3.2 Assay Qualification

Qualification of the anion-exchange chromatography assay for polySia was determined by measuring the levels of total DMB-labelled Sia monomers hydrolysed from known concentrations of polySia (colominic acid). From the calibration curve prepared, the limit of detection of polySia hydrolysate was 0.005 ng, while the limit of quantification was 0.05 ng (**Fig. 5A**). Assay specificity was investigated by analysing the polySia-derived Sia concentration in C6-ST8SiaII and C6-WT cell lysates (each 10^6 cells) respectively (**Fig. 5B**). Moreover, to avoid the potential variation of expression of Sia background from non-polySia sources in C6-ST8SiaII and C6-WT cells, a polySia-negative control was generated by Endo-NF treatment of a C6-ST8SiaII 10^6 cell lysate to exhaustively hydrolyse polySia as described previously (Valentiner et al., 2011). As shown in **Fig. 5B**, a strong polySia signal was detected in the C6-ST8SiaII cell lysate. The C6-ST8SiaII tumour (rat glioma) cells are genetically-engineered to express a polysialyltransferase and hence polySia. The C6-WT (wild-type) tumour cells, which are devoid of a polysialyltransferase and thus polySia, revealed a 25-30% Sia background level. Similarly, Endo-NF-treated C6-ST8SiaII cells which effectively had their polySia enzymically removed, as previously described (Al-Saraireh et al., 2013), demonstrated a similar Sia fraction. This indicates that there was 25-30% Sia monomer signal not derived from cellular polySia. The concentration-dependent hydrolysis of Endo-NF on cellular polySia in C6-ST8SiaII lysates was determined using this cell-based assay, and the EC_{50} for Endo-NF was 2.13 ng/ml (**Fig. 5C**). A cell-based polySia-detecting ELISA assay produced a similar low ng/ml detection level (**Fig. 5D**). These results demonstrate the feasibility of the developed assay for measuring cellular polySia levels.

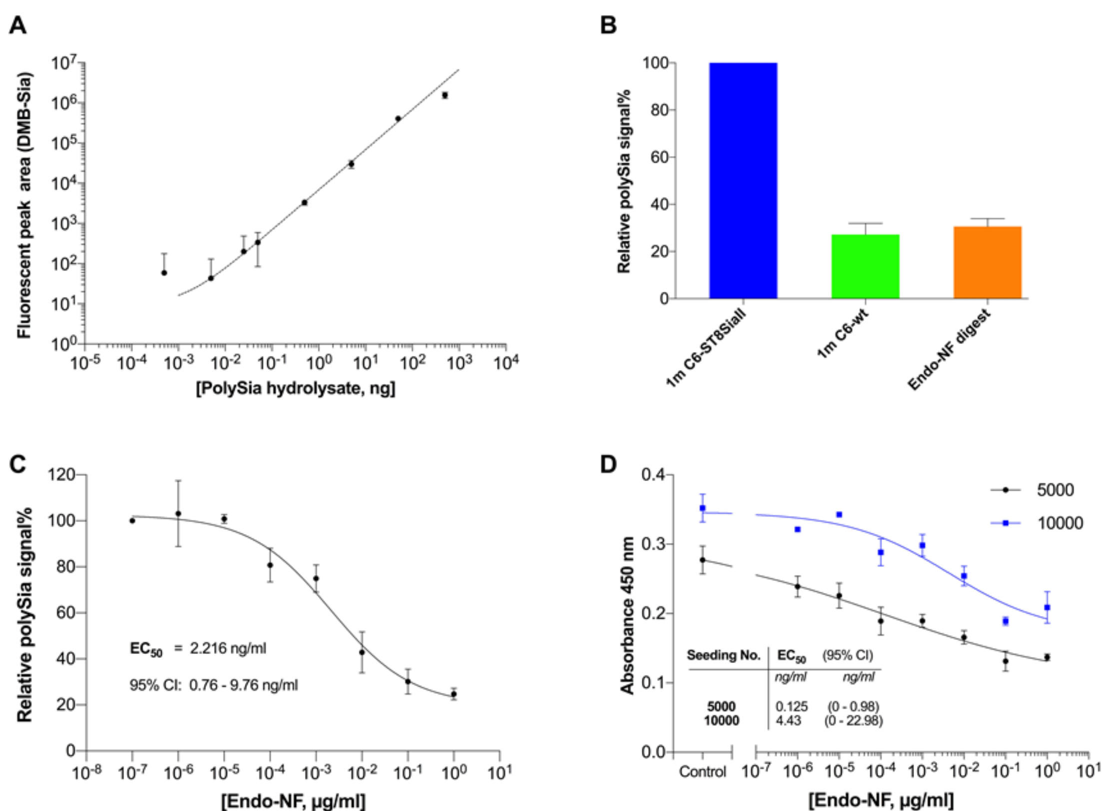

Figure 5: Assay qualification. Linearity range of assay sensitivity for polySia hydrolysate using colominic acid (**A**); Analysis of Sia background using a polySia-negative cell line (C6-WT) and Endo-NF enzyme treatment of the polySia-positive cell (C6-ST8SiaII) lysate, respectively (**B**); Enzymatic activity of Endo-NF on cleaving cellular polySia from C6-ST8SiaII cells, determined by RPLC-fluorescence detection (**C**); Cellular ELISA assay for polySia (**D**). Data are the mean values ± SD from 3 independent experiments.

3.3 Analysis of the effect of an ST8SiaII inhibitor on polySia expression in C6-ST8SiaII

To investigate the suitability of the assay for evaluating inhibition of polysialylation in cellular systems, ICT3176, compound previously shown to inhibit polysialylation (Elkashef, Allison, et al., 2016) was investigated using the assay developed. Cells were treated with ICT3176 for 48 h, after which time the cellular polySia levels were determined using anion exchange chromatography of polySia cell extracts, and a cellular polySia ELISA assay was also conducted for comparison (in the absence of an assay for direct comparison). In the RPLC-fluorescence detection assay, ICT3176 had an IC_{50} of 250 µM (**Fig. 6A**), which compared favourably to the ELISA-based assay (IC_{50} 146 µM with 5 x 10^3 cells, and 174 µM with 5 x 10^4 cells) (**Fig. 6B**). The limitation of the cellular polySia ELISA assay is its reliance on antibody recognition mainly reflecting changes in polySia quantity, i.e. the number of polySia chains, following compound treatment, without providing information as to any change in the polymer chain length. In contrast, the RPLC-fluorescence detection assay provides quantification of potential chain length changes by measuring the total Sia residues derived from cellular polySia before and after the compound treatment, irrespective of the changes in polySia chain numbers.

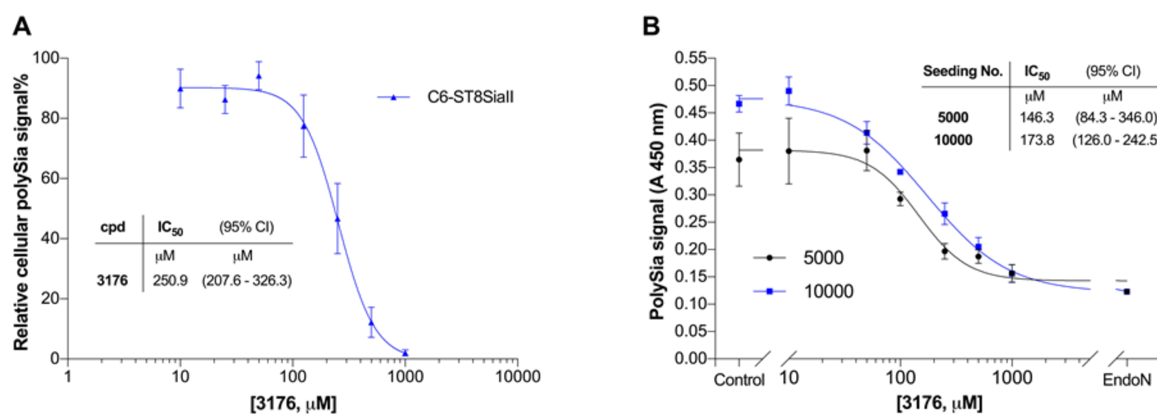

Figure 6: Effect of polysialylation inhibitor ICT3176 on polySia expression using (**A**) the newly-developed RPLC-fluorescence detection assay, and (**B**) a cellular polySia ELISA assay.

Conclusion

In summary, this study describes the development of a cell-based method for quantitative evaluation of polySia expression in cellular systems. We have proven our hypothesis, having demonstrated that the assay enables characterisation of changes in Sia content of polySia, through extraction and purification of the polymer from the complex cellular environment, and sensitive analysis using RPLC-fluorescence detection. The assay offers the opportunity for accurate evaluation of polyST inhibition in cellular systems. Moreover, this assay is complementary to a cell-free polyST enzyme assay (Guo et al., 2020), and offers important quantitative methodology to evaluate the functional expression of polySia in cellular systems. Given the importance of polySia in multiple disease states, notably cancer, this is a potentially vital tool with applications in drug discovery and in understanding the complex biological roles associated with this unique carbohydrate polymer.

Acknowledgements

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Author Contributions

Conceived and designed the experiments: XG, SME & RAF. Performed the experiments: XG, SME, AP & GRM. Data analysis: XG, SME, AP & GRM. Supervisory team: SDS, PML, LHP, RAF. Manuscript composition and editing: XG, LHP & RAF. Final proof-reading: All.

Conflict of interest statement

There are no conflicts of interest to declare.

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