Video Article

Metabolic Glycoengineering of Sialic Acid Using *N*-acyl-modified Mannosamines

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Abstract

Sialic acid (Sia) is a highly important constituent of glycoconjugates, such as *N*- and *O*-glycans or glycolipids. Due to its position at the non-reducing termini of oligo- and polysaccharides, as well as its unique chemical characteristics, sialic acid is involved in a multitude of different receptor-ligand interactions. By modifying the expression of sialic acid on the cell surface, sialic acid-dependent interactions will consequently be influenced. This can be helpful to investigate sialic acid-dependent interactions and has the potential to influence certain diseases in a beneficial way. Via metabolic glycoengineering (MGE), the expression of sialic acid on the cell surface can be modulated. Herein, cells, tissues, or even entire animals are treated with C2-modified derivatives of *N*-acetylmannosamine (ManNAc). These amino sugars act as sialic acid precursor molecules and therefore are metabolized to the corresponding sialic acid species and expressed on glycoconjugates. Applying this method produces intriguing effects on various biological processes. For example, it can drastically reduce the expression of polysialic acid (polySia) in treated neuronal cells and thus affects neuronal growth and differentiation. Here, we show the chemical synthesis of two of the most common C2-modified *N*-acylmannosamine derivatives, *N*-propionylmannosamine (ManNProp) as well as *N*-butanoylmannosamine (ManNBut), and further show how these non-natural amino sugars can be applied in cell culture experiments. The expression of modified sialic acid species is quantified by high performance liquid chromatography (HPLC) and further analyzed via mass spectrometry. The effects on polysialic acid expression are elucidated via Western blot using a commercially available polysialic acid antibody.

Video Link

The video component of this article can be found at https://www.jove.com/video/55746/

Introduction

Sialic acid is a monosaccharide that can typically be found at the non-reducing termini of glycoconjugates, such as *N*- and *O*-glycans or glycolipids. Among all monosaccharides, sialic acid has some unique chemical characteristics. It has a 9 C-atom backbone, a carboxylic group in the C-1 position, that is deprotonated and thereby negatively charged under physiological conditions, and an amino function in the C-5 position. Although over 50 naturally occurring variants of sialic acid have been characterized to date[1], the predominant form of sialic acid found in humans is *N*-acetylneuraminic acid (Neu5Ac). Other mammals also express higher amounts of *N*-glycolylneuraminic acid (Neu5Gc)[2,3].

Due to its exposed position in glycoconjugates, sialic acid is involved in a plethora of receptor-ligand interactions, *e.g.,* the hemagglutinin dependent binding of the influenza virus to host cells[4]. A sialic acid epitope with important biological functions, especially during embryogenesis and in the nervous system, is polysialic acid. Polysialic acid is a polymer of up to 200 alpha 2,8-linked sialic acids. The major protein carrier of polysialic acid is the neural cell adhesion molecule (NCAM). Polysialic acid expression modulates the adhesive property of NCAM in that polysialic acid expression decreases the adhesion and increases plasticity with the nervous system[5].

Changes in the expression of (poly)sialic acid will ultimately affect a multitude of different biological interactions. This can be used to study known sialic acid dependent processes on a molecular level, to uncover novel glycoconjugate interactions, or explore possible therapeutic approaches. There are different methods available by which the expression of sialic acid on the cell surface can be modulated, for example treatment with sialic acid specific glycosidases (sialidases), inhibition of enzymes involved in the sialic acid biosynthesis[6,7,8], or knocking down or changing the expression of the key enzyme of sialic acid biosynthesis[9].

Another versatile method to modulate sialic acid expression is MGE (also known as metabolic oligosaccharide engineering, MOE). Herein, cells, tissues, or even animals are treated with non-natural derivatives of ManNAc that bear C2-amino modifications. Being precursor molecules for sialic acid, after cellular uptake, these ManNAc analogs are unidirectional metabolized to non-natural sialic acids and can be expressed on sialylated glycoconjugates. Cells treated with ManNAc derivatives carrying aliphatic C2-modifications, such as ManNProp or ManNBut, do incorporate *N*-propionylneuraminic acid (Neu5Prop) or *N*-butanoylneuraminic acid (Neu5But) in their glycoconjugates[10,11]. By using functional

groups introduced to the C2-position of ManNAc, the occurring non-natural sialic acids can be coupled, *e.g.,* via the Staudinger ligation or the azide alkine cycloaddition, with fluorescent dyes and therefore visualized on the cell surface[12].

The expression of these non-natural sialic acids has intriguing effects on many biological processes, including pathogen infections, the adhesion and migration of tumor cells, general cell adhesion, as well as vascularization and differentiation (for review see: Wratil *et al.*[13]). Interestingly, MGE with *N*-acyl modified mannosamines can also be used to interfere with the expression of polysialic acid. Polysialic acid is generated by two different polysialyltransferases (ST8SiaII and ST8SiaIV). It has been demonstrated, that polysialyltransferase ST8SiaII is inhibited by unnatural sialic acid precursors, such as ManNProp or ManNBut[14,15]. In addition, it has been demonstrated in human neuroblastoma cells that ManNProp or ManNBut application also reduces sialylation in total[15].

MGE with *N*-acyl modified mannosamines is an easy to apply method that has been successfully used, not only in mammalian and bacteria cell culture but also in entire animals of different species, such as *Caenorhabditis elegans*[16], zebrafish[17], or mice[18,19,20,21]. Especially ManNAc derivatives bearing aliphatic modifications, including ManNProp and ManNBut, are negligibly cytotoxic, even at millimolar concentrations in cell culture medium or blood plasma. Furthermore, they are relatively easy to synthesize.

Here, we provide details on how to use MGE with *N*-acetyl modified mannosamines. First, the chemical synthesis of two of the most widely used ManNAc derivatives in this field, ManNProp and ManNBut, is explained. Next, we show how MGE can be applied in an *in vivo* experiment. As an example, the neuroblastoma cell line Kelly was chosen to demonstrate decreased expression of the polysialic epitope by Western blot after treatment with the ManNAc derivatives. The non-natural sialic acids on the cell surface were quantified by HPLC and further analyzed via mass spectrometry.

Protocol

1. Preparation of Buffers and Reagents

1. **Preparation of 3 mM sodium methoxide solution**
   1. Dissolve 8.1 mg sodium methoxide in 50 mL methanol (3 mM) in a 100 mL glass bottle with a stir bar. Store at room temperature (RT) for several weeks.

2. **Preparation of Tris-HCl buffer**
   1. Combine 8.766 g NaCl, 157 mg Tris-HCl, and 146 mg EDTA in a 100 mL glass bottle with a stir bar and dissolve in 80 mL water.
   2. Add sodium hydroxide (1 M, in water) or HCl (20%, in water) to the stirring solution, while observing the pH with a pH-meter, and adjust the pH to 8.0.
   3. Add the volume of water needed to achieve a final volume of 100 mL. Filter the solution using a 0.22 µm sterile filter system.
      NOTE: 100 mL Tris-HCl buffer will contain 150 mM NaCl, 10 mM Tris-HCl, and 5 mM EDTA (pH 8.0). The solution can be stored at 4 °C for several weeks.

3. **Preparation of aprotinin solution**
   1. Dissolve 10 mg aprotinin in 1 mL water (1.54 mM). Aliquot the aprotinin solution into 10 x 1.5 mL-plastic tubes (100 µL each). Store at -20 °C for several weeks.

4. **Preparation of leupeptin solution**
   1. Dissolve 10 mg leupeptin in 2 mL water (10 mM). Aliquot the leupeptin solution 10 x 1.5 mL-plastic tubes. Store at -20 °C for several weeks.

5. **Preparation of phenylmethylsulfonyl fluoride (PMSF) solution**
   1. Dissolve 34.8 mg PMSF in 2 mL ethanol (100 mM). Aliquot the PMSF solution in 10 x 1.5 mL-plastic tubes. Store at -20 °C for several weeks.

6. **Preparation of 1,2-diamino-4,5-methyldioxybenzol-dihydrochloride (DMB) solution**
   1. Combine 15.62 mg DMB, 352 µL β-mercaptoethanol, and 48 µL sodium bisulfite-solution (39%), and add 9.6 mL water (6.9 mM DMB, 500 mM β-mercaptoethanol, and 0.19% sodium bisulfite). Aliquot the DMB solution in 40 x 1.5 mL-plastic tubes (250 µL each). Store protected from light at -20 °C for several weeks.

7. **Preparation of 1 M trifluoroacetic acid (TFA) solution**
   1. Add 770.3 µL of 100% TFA to 9.23 mL water in a 15 mL plastic tube (1 M). Store at 4 °C for several weeks.

8. **Preparation of 120 mM TFA solution**
   1. Add 92.4 µL TFA (100%) to 9.91 mL water (120 mM) in a 15 mL plastic tube. Store at 4 °C for several weeks.

9. **Preparation of 400 mM sodium hydroxide (NaOH) solution**
   1. Dissolve 160 mg NaOH in 10 mL water (400 mM) in a 15 mL plastic tube. Store at 4 °C for several weeks.

10. **Preparation of Western blot lysis buffer**
    1. Dissolve 5.84 g NaCl, 0.1 g Tris(hydroxymethyl)-aminomethan (Tris), 0.1 g $CaCl_2$, 0.95 g $MgCl_2$, and 1 g Triton-X100 in 100 mL water and adjust the pH to 7.8. Store at 4 °C. Add 100 µL of each protease inhibitor (aprotinin, leupeptin, and PMSF) before using the lysis buffer.

11. **Preparation of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer**

1. Dissolve 0.3 g Tris, 1.5 g glycine, and 0.1 g SDS in 100 mL water and adjust the pH to 7.3. Store at RT.

12. **Preparation of Western blot buffer**
    1. Dissolve 0.3 g Tris, 1.1 g glycine in 90 mL water and add 10 mL ethanol. Store at RT.

13. **Preparation of blocking solution**
    1. Dissolve 1 g of fat free milk power in 25 mL phosphate buffered saline (PBS). Always prepare fresh.

2. Synthesis of ManNProp and Related *N*-acyl-modified Mannosamines

1. Dissolve 431.2 mg mannosamine hydrochloride in 10 mL 3 mM sodium methoxide solution in a 50 mL glass bottle with a stir bar.
2. Cool the mixture on ice to 0 °C. To the stirring solution, slowly add dropwise 210 µL propionyl chloride (2.4 mmol), or 248 µL butanoyl chloride (2.4 mmol) to synthesize ManNProp or ManNBut, respectively. Incubate the stirring mixture at 0 °C for 4 h.
3. Transfer the solution into a 50 mL plastic tube. Poke 4 - 8 holes into the lid of the plastic tube with a thin needle. Rapidly freeze the solution using liquid nitrogen and subsequently lyophilize (freeze dry) it for 48 h or until it has completely dried.
4. Mix 350 g silica gel 60 with 750 mL ethyl-acetate/methanol/water (15:2:1) (this is a suspension). Fill a glass column (35 mm diameter and 70 cm length) with the silica gel 60 suspension and wash the prepared column with an additional 100 mL ethyl-acetate/methanol/water (15:2:1) before use.
5. Dissolve the dried products from step 2.3 (5 g dried product) in 10 mL ethyl-acetate/methanol/water (15:2:1) and load them using a pipette onto the silica gel column. Collect 4 mL fractions after 500 mL (for ManNProp). NOTE: ManNBut will elute from the column approximately after 700 mL.
6. Transfer the eluted fractions into 15 mL plastic tubes. Poke 3 - 6 holes into the lid of the plastic tube with a thin needle. Rapid freeze the solution using liquid nitrogen and subsequently lyophilize it until it has completely dried.
   NOTE: The lyophilized products can be stored for several months at RT.
7. Verify the purity of the products by mass spectrometry (see section 9).
   NOTE: Alternatively, purity can also be verified by ^{1}H Nuclear Magnetic Resonance (NMR).

3. Cell Culture

1. Culture Kelly neuroblastoma cells in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, 100 U penicillin, 100 mg streptomycin, 2 mM L-glutamine containing 5 mM ManNAc, 5 mM ManNProp, or 5 mM ManNBut, at 37 °C and 5% CO_2.
   NOTE: Cells cultured in medium without ManNAc or its analogues are used as a control.
2. **Prior to application of the mannosamines, detach the Kelly neuroblastoma cells by incubating them for 10 min with trypsin/EDTA (0.25%, 0.02%, in PBS) and count using a Neubauer-chamber or cell counter. Seed the cells at defined numbers based on the size of the plate/dish.**
    1. To measure sialic acid monosaccharides, seed 1 x 10^5 cells in 500 µL medium into a 48-well tissue culture plate. For the analysis of polysialic acids, seed 1 x 10^6 cells in 3 mL medium onto a 6 cm diameter cell culture dish. Incubate at 37 °C and 5% CO_2. Replace the medium every 24 h.
       NOTE: The effect of MGE increases with the total time of treatment. For high metabolic efficiency treat the cells for 5 - 7 days.

3. **After treatment, decant the medium. Wash the plates/dishes with PBS. Detach the cells by incubating them for 10 min with trypsin/EDTA (0.25%, 0.02%, in PBS). Neutralize the trypsin by adding the same volume of cell culture medium to the detached cells. Transfer the detached cells into 15 mL plastic tubes. Centrifuge the samples for 3 min at 500 x g and discard the supernatant.**
    1. Add 5 mL PBS and centrifuge cells (see step 3.3). Repeat the washing procedure two more times.
       NOTE: The washed cell pellet without supernatant can be stored at -20 °C for several days. If the expression of polysialic acid is to be elucidated continue to section 10.

4. Cell Lysis for HPLC Analysis

1. **Preparation of HPLC lysis buffer**
    1. Combine 5 mL Tris-HCl buffer, 5 µL aprotinin solution, 20 µL leupeptin solution, and 50 µL PMSF solution.
       NOTE: 5 mL HPLC Lysis buffer will contain 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1.54 µM aprotinin, 40 µM Leupeptin, and 1 mM PMSF (pH 8.0). This buffer should always be prepared fresh prior to cell lysis.

2. Re-suspend the collected cell pellets (step 3.3) each in 500 µL ice-cold HPLC lysis-buffer, and ultra-sonicate the samples with an ultra-sonic processor needle three times for a period of 30 s at medium-high amplitudes. Cool the samples on ice for at least 1 min in between the cycles.

5. Separation of the Membrane Fraction

1. Centrifuge the lysed cell samples for 2 h at 20,000 x g and 4 °C.Separate the supernatant (approximately 480 µL), representing cytosolic proteins, in 1.5 mL plastic tubes. Determine the protein concentration of these fractions using, *e.g.*, the Bradford or the bicinchoninic acid (BCA) assay.
   NOTE: The protein concentration (approximately 1 mg/mL) of the cytosolic fractions can later be related to the measured amounts of the respected sialic acid species. The pellet represents the membrane fraction, which is used in step 6.1.

6. Acidic Hydrolysis

NOTE: Oligo- and polysaccharides in the cell membranes are hydrolyzed to monosaccharides. Further, possible *O*-modifications in the monosaccharides are hydrolyzed, as well. This is necessary for quantitative HPLC analysis, because the overwhelming majority of sialic acids in the membrane fractions are naturally incorporated in glycoconjugates and might possibly bear *O*-acetyl or *O*-lactolyl modifications.

1. Add 150 µL 1 M TFA-solution to the separated membrane fractions (pellet from section 5). Incubate the samples for 4 h at 80 °C with shaking at 200 - 600 rpm.
2. Centrifuge the samples for approximately 30 min at 20,000 x g and 21 °C using 0.5 mL filter tubes with 3 kDa exclusion membranes, until the upper phase volume is less than 20 µL.
   NOTE: This step is important to dispose higher molecular debris.
3. Transfer the lower phase containing smaller molecules, including the sialic acid species, into 2 mL plastic tubes. Poke 2 - 4 holes into the caps of the plastic tubes with a thin needle. Rapid freeze the samples using liquid nitrogen and then lyophilize them overnight.
   NOTE: The dried samples can be stored for several days at RT. Replace a cap without holes for longer storage.

7. Fluorescent Labeling

1. Re-suspend the dried samples in 10 µL 120 mM TFA solution and add 50 µL DMB solution. Transfer samples into dark 1.5 mL tubes to protect them from ultraviolet light.
2. For standards, dissolve 1 µL Neu5Ac (60 ng/mL, in water) or 1 µL Neu5Gc (60 ng/mL, in water) in 10 µL 120 mM TFA solution in dark 1.5 mL tubes. Add 50 µL DMB solution.
3. Incubate the cell membrane samples and the standards for 1.5 h at 56 °C protected from light. After incubation, add 4 µL 400 mM NaOH solution to each sample to stop the labeling reaction.

8. HPLC Analysis

NOTE: Labeled samples are analyzed on a HPLC system equipped with a C18 RP column (110 Å, 3 µm particle size, 4.6 x 150 mm), a fluorescence detector, and a fraction collector. The solvents used are methanol, acetonitrile, and water. Make sure to degas all solvents prior to the measurements, if the HPLC system lacks an internal degasser.

1. Set the temperature of the column to 40 °C and configure the fluorescence detector with 373 nm for excitation and 448 nm for emission, respectively. Inject 10 µL sample volume and separate the probes for 50 min at 0.5 mL/min flow rate with methanol/acetonitrile/water (6:8:86) as the eluent. For mass spectrometry analysis, collect the peaks of interest.
   NOTE: Neu5Gc is expected to eluate after 6-8 min, Neu5Ac after 9 - 12 min, Neu5Prop after 17 - 23 min, and Neu5But after 38 - 44 min. The fractionated samples can be stored for several h at 4 °C protected from light.
2. After measuring each sample, wash the column for 7.5 min at 1.0 mL/min flow rate (≈ 1.5 column volumes) with methanol/acetonitrile/water (6:25:69) and re-equilibrate the system for 7.5 min at 1.0 mL/min flow rate with methanol/acetonitrile/water (6:8:86).
3. For quantitative analysis, calculate the area under the curve of the sialic acid peaks of interest using the operating software of the respective HPLC-system[22].
   NOTE: Obtained data can be related to the Neu5Ac or the Neu5Gc standard and to the measured protein concentrations in the cytosolic fractions (see section 5.1).

9. Mass Spectrometry Analysis of Sialic Acid Monosaccharides

NOTE: HPLC retention peaks of interest can be further analyzed by liquid chromatography (LC) electrospray-ionization mass spectrometry (ESI-MS), in order to verify the unnatural sialic acid species.

1. For mass spectrometry analysis, inject 20 µL of collected sample into a LC/Mass Selective Detector (MSD) system with 79.9% methanol, 20% isopropyl alcohol, and 0.1% formic acid as eluent, 0.5 mL/min flow rate, 4 kV capillary voltage, and 350 °C capillary temperature[22,23].
2. In the evaluation software of the respective LC/MSD system, select the peak of interest in the total ion chromatogram. View the mass spectrum of the resolved peak. Display the positive mass/charge ratio between 300 and 700.
   NOTE: DMB labeling of sialic acids leads to an increase in the molecular mass of 116.2 g/mol. The DMB-labeled sialic acid species have the following molecular masses: DMB-Neu5Ac = 424.2 g/mol, DMB-Neu5Gc = 441.8 g/mol, DMB-Neu5Prop = 436.2 g/mol, DMB-Neu5But = 453.2 g/mol. Common adducts are acetonitrile, sodium, or isopropyl alcohol.

10. Western Blot Analysis of Polysialic Acid in Kelly Neuroblastoma Cells

1. **Preparation of cell lysates**
   1. Add 1 mL Western blot lysis buffer to the cell pellets from step 3.3 and vortex. Incubate for 30 min on ice. Vortex every 5 min.Centrifuge the samples for 1.5 h at 20,000 x g and 4 °C. Collect the supernatant containing the proteins from the lysed cells.
   2. Determine the protein concentration of the protein fractions, *e.g.*, the Bradford or the BCA assay. Dilute the samples to a protein concentration of 1.5 µg/µL in lysis buffer.
   3. Prepare the samples for the SDS-PAGE by adding 10 µL Laemmli sample buffer to 90 µL protein fraction (1.5 µg/µL) and boil the sample for 5 min[24].

2. **Immunoblotting**
   1. Use 8% SDS-acrylamide gels with 20 - 30 µL pockets and 0.75 or 1 mm thickness. Run the gel at 25 mA for 2 h at RT.

2. Carefully remove the glass cover from the gel. Cut out and discard the upper part of the gel containing the loading pockets. Place the gel on a nitrocellulose membrane (0.2 µm), previously soaked in Western blot buffer. Transfer the proteins to nitrocellulose according to the recommendation of the system (*e.g.,* 250 mA for 1 h at 4 °C).
3. Remove the nitrocellulose membrane from the blotting system. Stain the blot with Ponceau red to visualize the proteins. Transfer the nitrocellulose membrane into a plastic chamber and add 10 mL blocking solution. Incubate for 1 h at RT or overnight at 4 °C.
4. Decant the blocking solution and add monoclonal anti-polySia 735 antibody (1 µg/mL) in PBS. Incubate the membrane for 1 h at RT or overnight at 4 °C. After incubation, wash the membrane at least three times for 5 min with PBS.
5. Add polyclonal anti-mouse IgG secondary antibody (1 µg/mL) coupled to horseradish peroxidase (HRP) or a fluorescent label in PBS. Incubate the membrane for 1 h at RT. After incubation, wash the membrane at least three times for 5 min with PBS.
6. Transfer the membrane onto a plate of an appropriate imager. Detect the signal according to the manufacturer's instructions.

Representative Results

HPLC chromatograms of the fluorescent labeled Neu5Ac and the Neu5Gc standards are depicted in **Figure 2**. Using the herein described method, DMB-labeled Neu5Gc typically elutes between 7 - 9 min elution time, and DMB-Neu5Ac between 10 - 12 min. Several smaller peaks in the chromatogram usually appear between 2 - 6 min. These peaks represent unreacted DMB and reaction intermediates[25].

Figure 3 shows representative chromatograms of cell lysates. Compared to the chromatograms of the DMB-labeled standards (**Figure 2**), in these chromatograms several undefined peaks are visible. This is most likely due to the internal impurity of cell lysates and to the fact that DMB not only reacts with sialic acid species, but also with other α-keto acids present in cell lysates, including pyruvate, succinate, and α-ketoglutarate[26]. The presence of small amounts of sialic acids bearing *O*-acetyl modifications that have not been cleaved by acetic hydrolysis might also be discussed as a reason for these undefined peaks[23]. Chromatograms from lysed untreated cells are compared with chromatograms from cells that have previously been treated with ManNAc or its analogs. As shown here, treatment with ManNAc often leads to an almost entire depletion of Neu5Gc on the cell surface. In chromatograms of lysed cells treated with Neu5Prop or Neu5But, peaks are visible that do not appear in chromatograms of untreated cells. These peaks (for ManNProp treated cells at approximately 19 min elution time and for ManNBut treated cells at 42 min) indicate the appearance of the corresponding non-natural sialic acids, Neu5Prop and Neu5Gc. As described in section 8.3 of the protocol, the HPLC chromatograms can be analyzed in order to quantify the amounts of the different sialic acid species in the cell lysates.

The fact that distinct HPLC retention peaks correspond to certain sialic acid species was verified via mass spectrometry. Representative ESI-MS data from collected HPLC peaks of interest are depicted in **Figure 4**: the spectrum of the collected DMB-Neu5Ac retention peak in the upper left panel, DMB-Neu5Gc in the upper right panel, and the two non-natural sialic acid species, DMB-Neu5Prop and DMB-Neu5But in the lower left and right panels. The reaction with DMB leads to an increase in the molecular mass of 116.2 Da, compared to the sialic acid species that are not labeled with this dye. In the mass spectra, when displaying the positive mass/charge ratio of the injected samples between 300 and 700, several peaks are visible. This is due to adduct formation of the respected DMB-labeled probe. Besides the protonated ion $[M+H]^+$, the sodium adducts $[M+Na]^+$ and $[M+2Na-H]^+$ appear. As acetonitrile (ACN) is present during the LC analysis, it may be found as an adduct in the mass spectra (shown for DMB-Neu5Gc, **Figure 4**: upper right panel). Due to fragmentation caused by collisional decompensation activations generated in the electronspray transport region between the capillary and the first skimmer, dehydrated sialic acid species $[M+H]^+$-H_2O can also be observed (shown for DMB-Neu5Ac, **Figure 4**: upper left panel)[23,27]. In the LC setup, DMB-Neu5Gc elutes shortly after the unreacted or partly reacted DMB-species. Therefore, the collected DMB-Neu5Gc probe may contain impurities caused by these reaction intermediates. This serves as an explanation for the appearance of undefined peaks in the mass spectrum of DMB-Neu5Gc (**Figure 4**: upper right panel).

Treatment of Kelly neuroblastoma cells with ManNProp or ManNBut leads to reduced expression of polysialic acid on NCAM, as shown by Western blot analysis of the cell membrane from lysed cells (**Figure 5**). Polysialic acid normally appears as a smear do to its heterogeneity in size and charge of (≈ 200 kDa). Treatment with ManNAc analogs leads to the reduction of polysialic acid on the cells surface: the longer the aliphatic *N*-acyl side chain, the stronger the reduction[28].

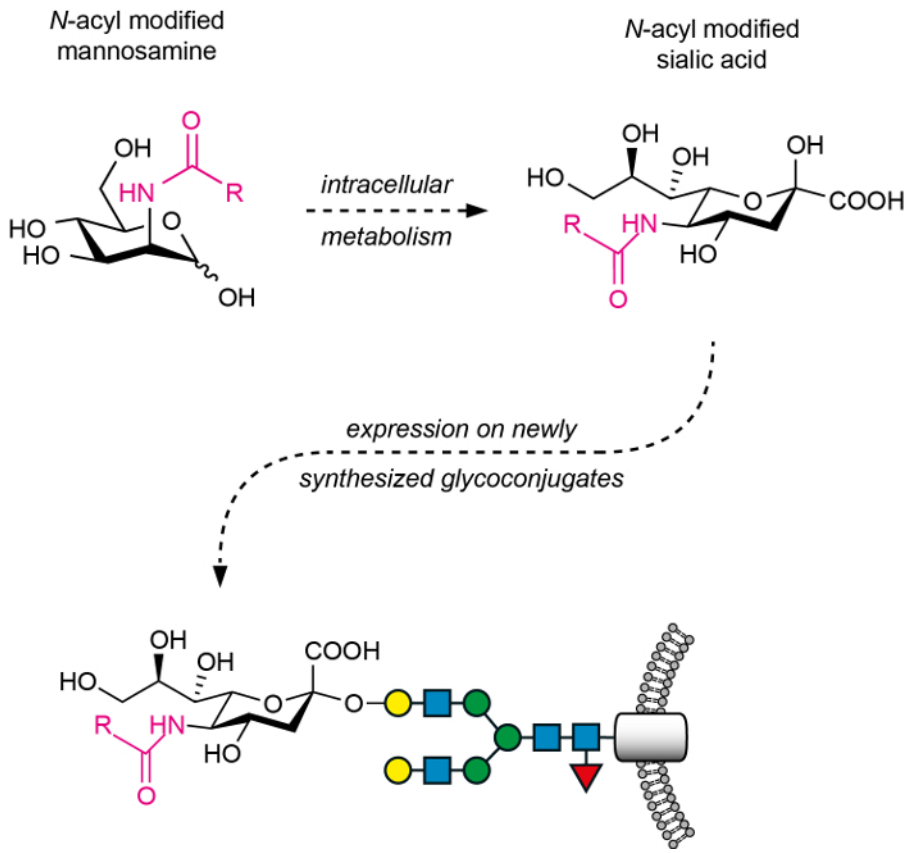

Figure 1: The general principle of MGE with *N*-acyl modified mannosamines. After treatment with the *N*-acyl modified mannosamines, these ManNAc analogs are unidirectionally metabolized (intracellular metabolism) to the corresponding non-natural sialic acids. These are transported to the Golgi, transferred to the respective oligosaccharide acceptor by sialyltransferases, and expressed on the cell surface as sialosides (here, a glycoprotein). Please click here to view a larger version of this figure.

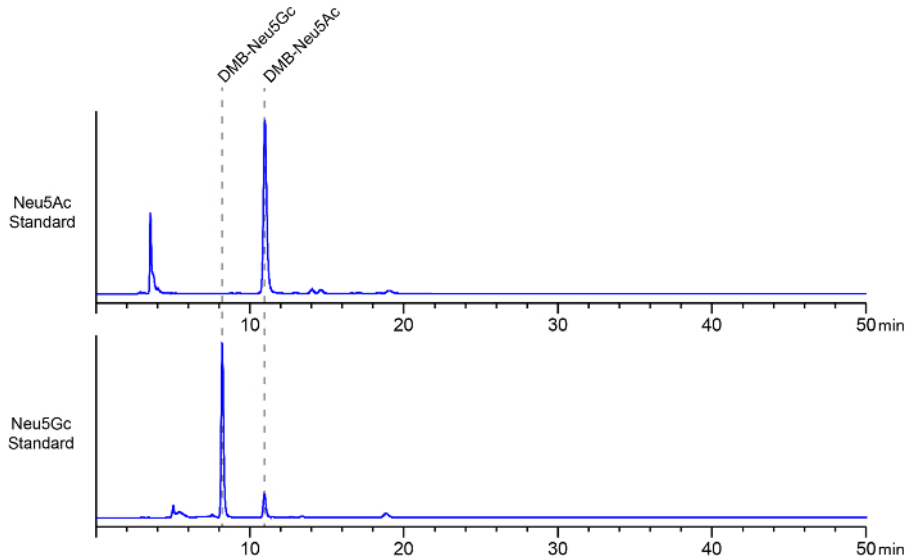

Figure 2: Representative chromatograms of DMB-labeled sialic acid standards. DMB-labeled Neu5Ac (upper panel) and Neu5Gc (lower panel) were analyzed by HPLC. Retention times (dashed lines) and peak areas of both sialic acids were determined after injecting 10 ng of the respected DMB-labeled species into the HPLC system. In the herein shown representative chromatograms, DMB-Neu5Gc eluted after approximately an 8 min retention time and DMB-Neu5Ac at 11 min, respectively. Several smaller peaks in the chromatogram usually appear between 2 - 6 min, representing unreacted DMB and reaction intermediates. A small fraction of DMB-labeled Neu5Ac appears as a minor impurity in the DMB-Neu5Gc standard (lower panel). This figure is modified from reference[22]. Please click here to view a larger version of this figure.

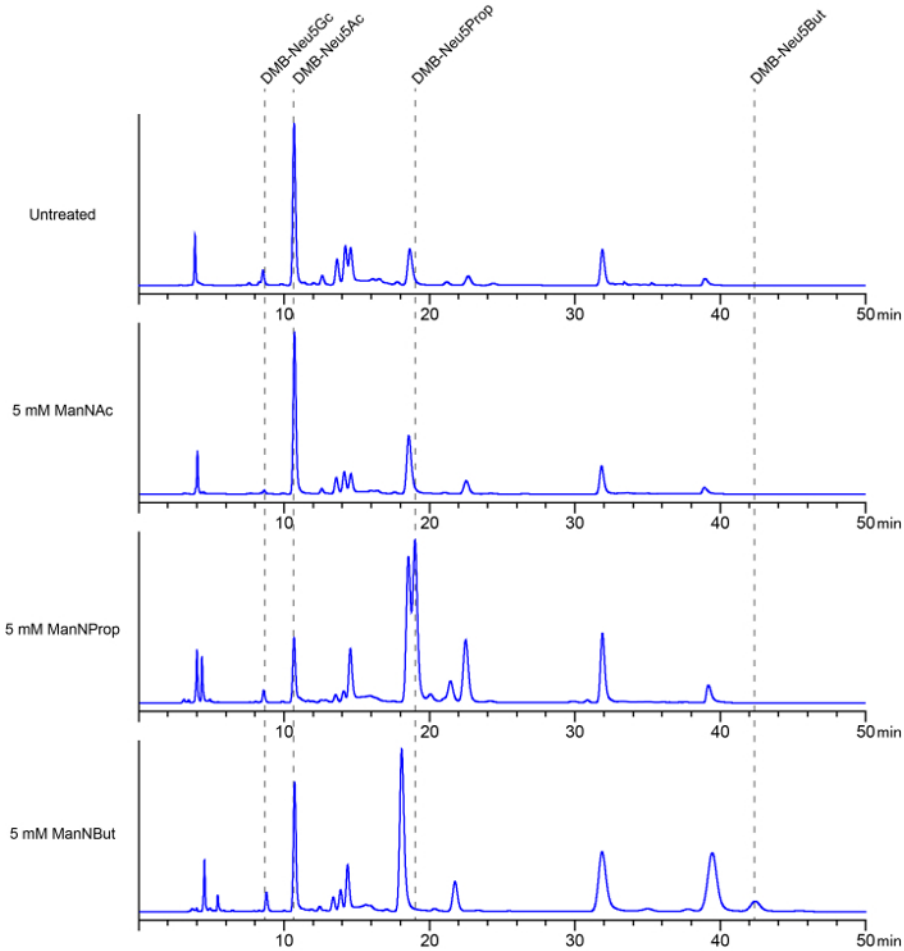

Figure 3: Characterization of membrane-bound sialic acids by DMB-HPLC. Cells were cultured in the absence (untreated, upper panel) or presence with 5 mM ManNAc (second panel from top), ManNProp (third panel from top), or ManNBut (lower panel), respectively for 7 days. The medium was changed every 24 h. Cells were harvested and membrane fractions were prepared, labeled with DMB, and analyzed by HPLC. By comparison with the chromatograms of DMB-labeled sialic acid standards (**Figure 2**), peaks at the retention times between 8-9 min were identified as DMB-Neu5Gc, and peaks between 10 - 11 min as DMB-Neu5Ac. Compared to the peaks obtained from injecting DMB-labeled sialic acid standards, chromatograms from cell lysates show additional, undefined peaks. This is due to the internal impurity of the probes, as well as to the fact that DMB can react not only with sialic acid present in the cell lysates, but also with other α-keto acids present in cell lysates, including pyruvate, succinate, and α-ketoglutarate. Peaks that only appear in the chromatograms of samples from cells cultured in the presence of *N*-acyl modified mannosamine analogs indicate the corresponding DMB-labeled non-natural sialic acids. This figure is modified from reference[22]. Please click here to view a larger version of this figure.

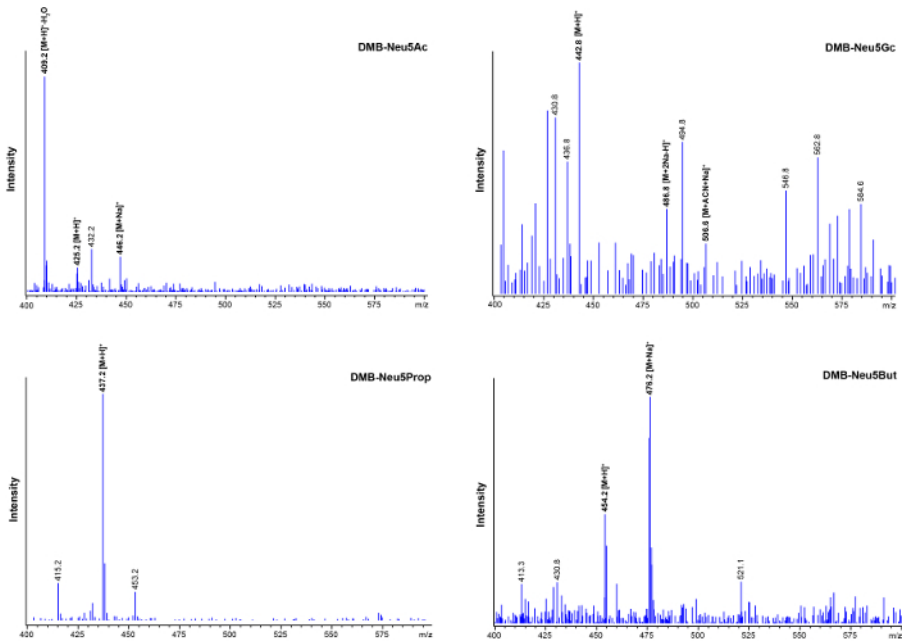

Figure 4: Mass spectra of DMB-labeled sialic acid species found in cell lysates. HPLC retention peaks of interest (see **Figure 3**) were collected and subsequently analyzed by ESI-MS. 20 µL of the collected retentions peaks were injected into the LC-MSD system. Reaction with DMB leads to an increase in the molecular mass of 116.2 Da compared to the corresponding unreacted sialic acid species. The positive mass/charge ratio spectrograms obtained from different sialic acid species are depicted (DMB-Neu5Ac: upper left panel, DMB-Neu5Gc: upper right panel, DMB-Neu5Prop: lower left panel, DMB-Neu5But: lower right panel). Due to adduct formation several peaks are visible. Besides the protonated ion $[M+H]^+$, sodium adducts $[M+Na]^+$ and $[M+2Na-H]^+$ appear. Acetonitrile, which is present during the LC analysis, can also be found as an adduct (upper right panel). Dehydrated DMB-Neu5Ac, which is generated by fragmentation in the instrument $[M+H]^+$-H_2O can be observed (upper left panel). The collected DMB-Neu5Gc probe may contain impurities caused by unreacted DMB, as well as reaction intermediates seen as additional undefined peaks (upper right panel). ACN, acetonitrile; H, hydrogen; Na, sodium. This figure is modified from reference[22]. Please click here to view a larger version of this figure.

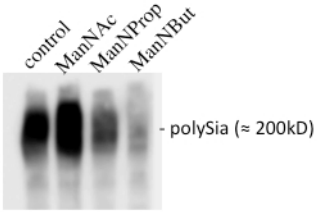

Figure 5: Analysis of the polysialylation of NCAM. Kelly neuroblastoma cells were cultured in the absence or presence of 5 mM ManNAc, ManNProp, or ManNBut for 7 days. Cells were harvested and proteins were separated on 8% SDS-acrylamide gels and analyzed by Western blot using monoclonal anti-polySia antibody 735. Note that polySia appears as a smear due to its heterogeneity in number of Sia, resulting in different sizes and charges of polySia.

Discussion

If the chemically synthesized ManNAc derivatives, ManNProp and ManNBut are analyzed via mass spectrometry, only the correct mass peak for both specimens should be identified. Therefore, the products can be assumed to have a purity of over 99%. Small amounts of Neu5Gc, which is normally not found in human cells[29], are detected in the membrane fractions of the lysed cells. This most likely occurs through a salvage pathway that recruits Neu5Gc from fetal bovine serum sialoglycoconjugates in the media[30]. Treatment with the natural precursor of sialic acid biosynthesis, ManNAc, drastically lowers the expression of the Neu5Gc epitope on the cell surface.

Application of modified mannosamines is negligibly toxic to all cells investigated so far. Cells accept increased millimolar sugar concentrations within the medium, which is necessary, since there are no specific transporters for *N*-acylmannosamines. It has been demonstrated that signal transduction pathways are activated upon application of *N*-acylmannosamines, which could affect cell growth or differentiation[31,32]. Whether this is due to a changed sialylation pattern or reflects direct effects on the modified sialylation is not known so far. Growth of cells in the presence of the C2-modified ManNAc analogs, ManNProp or ManNBut results in the expression of non-natural sialic acid species carrying the corresponding *N*-acyl substitution. The metabolic efficiency of the two tested *N*-acyl modified mannosamines varies. Treatment with ManNProp usually leads to expression of the corresponding Neu5Prop on the cell surface and to a decreased expression of Neu5Ac as well as Neu5Gc. Other *N*-acyl modified mannosamine analogs, like *N*-cyclopropylcarbamyl mannosamine, tend to have an even higher metabolic efficiency[22].

Previous studies revealed that sialic precursors interfere with polysialylation. Polysialic acids are nearly exclusively expressed on the NCAM. Interestingly, NCAM can be polysialylated by two distinct polysialyltransferases expressed in the Golgi (ST8SiaII and ST8SiaIV). It turned out that ManNProp, ManNBut, and other *N*-acyl-modified mannosamines interfere with polysialylation and that this is predominantly due to interaction with ST8SiaII[14]. This sialyltransferase is expressed during development and is important for the development of the brain. Interestingly also, many tumors from neuronal origin re-express ST8SiaII increasing their malignancy and further making them undetectable from the immune system[33]. In contrast, the application of ManNAc leads to an increase of polySia, since it leads to an increase of CMP-Neu5Ac, which is the natural substrate of both polysialyltransferases.

MGE is a unique method to introduce novel sialic acids on proteins of interest (*e.g.,* erythropoietin) and to modulate thereby its function. Furthermore, it can be used to modulate cell surface glycosylation, which might be of interest in many diseases, such as cancer, since many cancer cells try to escape the immune system by expressing high levels of sialic acid. The method presented here allows: first, to perform glycoengineering using cell cultures, and second, to analyze engineered glycans to prove successful glycoengineering. The method is very robust and up to now, no pitfalls are reported; in contrast, the method has been expanded to introduce chemical active groups to perform click-chemistry on cells[13].

Here we present two ManNAc analogs with aliphatic *N*-acyl side chain modifications that are comparably simple to synthesize, even by laboratories with less equipment and less experience in chemical synthesis. Groups that are more familiar to organic chemistry could synthesize other ManNAc analogs with more complex structures and use these for MGE, including the so-called 'bioorthogonal' analogs, which can be utilized, for example, to visualize sialylated glycoconjugates. There are review articles giving an overview of the *N*-acetyl-mannosamine derivatives that have been synthesized to date[13,34]. *N*-azidoacetyl mannosamine (ManNAz), a substance that is frequently used, to visualize sialylated glycans, is commercially available. Because of the low membrane permeability of ManNAc analogs, it can be advantageous to use derivatives of these sugars with protected hydroxyl-groups, *e.g.,* peracetylated ManNAc analogs. After entering the cytoplasm, the protecting groups are cleaved by cytoplasmic esterases, thereby releasing the active monosaccharides[35,36,37]. However, peracetylated ManNAc analogs expose higher cytotoxicity compared to the corresponding unprotected derivatives.

In this manuscript, we chose immortalized neuroblastoma cells for our experiments with MGE. Generally, MGE (especially with ManNProp and ManNBut) can be applied in a wide variety of cell lines, including immortalized cell lines (see Wratil *et al.*[13] for examples) and primary cells[38,39]. Furthermore, MGE was successfully utilized to alter sialylation *in vivo* in *C. elegans*[40], zebrafish[17,41], mouse[19,20,42], and rat[43,44]. The treatment time and the concentration of the ManNAc analogs in MGE experiments can be varied to affect the metabolic efficiency of this method. From our experience, longer treatment and higher concentrations correspond with better replacement of the naturally occurring sialic acid species in glycoconjugates by modified Neu5R. If very high concentrations of ManNAc derivatives are to be used, the cytotoxicity of the treatment should be evaluated, *e.g.,* by the MTT assay or the resazurin reduction assay.

In this manuscript, we suggest using ultra-sonication for cell homogenization. Other cell lysis methods were tested in our laboratory, including douncing on ice (approximately 250 strokes) and french press disruption (10,000 psi, 4 passages), with comparable outcomes regarding the amount of sialic acid detected in the samples. The advantage of ultra-sound homogenization is that it needs little time and does not require the addition of DNAase to the samples prior to lysis.

We focused our analysis on the membrane fractions of cell lysates, because we wanted to show alterations in the sialylation of membrane bound glycoconjugates. Sialic acid derivatives and their concentrations can also be measured in the cytosol of cells, after the membrane fraction has been separated by high-speed centrifugation, using the same method as described above. However, the concentrations of sialic acid in the cytosol are up to 100x lower compared to sialic acid expression on the cell membrane[7]. Thus, larger amounts of cells are needed to generate reliable results. The pool of cytosolic, activated CMP-sialic acid can be measured indirectly by pre-treating the cytosolic fractions of cell lysates with sodium borohydride prior to hydrolysis[7,45]. In order to hydrolyze sialic acid in glycoconjugates and sialic acid with hydroxyl modifications, the cells were incubated with 1 M TFA. Other acidic solutions can be used for hydrolysis, as well, *e.g.,* 2 M propionic acid, 2 M acetic acid, or 1 M HCl.

A possible encountered issue during HPLC analysis is that peaks of interest are overlapping with undefined peaks. This is especially problematic when the amount of sialic acid in a sample is to be calculated by the area under the curve of a certain peak. To separate a peak of interest from an undefined peak, the acetonitrile concentration in the HPLC solvent might be adjusted (± 3%). We suggest using steady solvent concentrations for HPLC analysis, giving the advantage of preparing the solvent mixture before chromatography. Therefore, our method requires only one HPLC pump and is feasible for a wide range of different HPLC systems. If more than one pump is available in the HPLC system, an isocratic gradient with increasing concentrations of acetonitrile can be applied, *e.g.,* 4-9% in 35 min[45]. By using this technique, the retention time of certain HPLC peaks will be altered considerably. This strategy can also help to achieve better separation of overlapping peaks. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was successfully established to verify the expression of modified sialic acid species on the cell surface[45], and thus serves as an equivalent to the ESI-MS method presented here.

Due to the hydrophilicity of ManNAc analogs and the lack of specific transporters for the uptake of these derivates[46], high concentrations are needed in MGE experiments with these compounds. Therefore, in up-scaled experiments and, especially in *in vivo* trials, large amounts of the respective ManNAc analogs are necessary showing a possible limitation of this technique. On the other hand, depending on the cell lines used, a certain minimal number of treated cells is necessary to obtain reliable results in the HPLC analysis. From our experience, a minimum of about 100,000 adherent cells or 150,000 suspension cells (1-2 wells of a 96-well plate with confluent cells) is sufficient. This can become a bottleneck, if the analysis is to be downsized, for example in screening approaches.

With the methods presented in this manuscript, sialic acid and its derivatives can be detected in cell lysates. However, the structure and composition of glycoconjugates in cells treated with ManNAc derivatives cannot be assessed with the herein described technique. Resolving the structure of glycoconjugates, *e.g.,N*- and *O*-glycans, generally requires greater experience and a glycomics facility[47]. To our knowledge, so far, no data are available on the distinct structure of glycoconjugates from cells that have been treated with ManNAc analogs.

Western blot analysis of polysialic acid is a very rapid and easy method. However, data obtained by this method are not quantitative, since the detection uses antibodies and chemiluminescence. Therefore, the use of HPLC methods is an advantage over Western blotting. Nevertheless, we suggest using the immunoblotting method, since it is easy to apply and known to work very reliably.

The expression of sialic acid on the cell surface can be altered also by methods other than MGE. As described above, cells can be treated with sialidase, an enzyme that cleaves sialic acid residues from glycoconjugates in a relatively unspecific manner. Sialidase treatment consequently induces hyposialylation in treated cells. Unfortunately, sialidase treatment is a rather limited technique, as it is cytotoxic, not feasible for long time treatment, and not applicable in *in vivo* experiments. Another technique to induce hyposialylation in cells is by using inhibitors of sialic acid biosynthesis. A variety of inhibitors is available that either target the key enzyme of sialic acid biosynthesis, the UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine-kinase (GNE/MNK), or the group of sialyltransferases[6,7,8,48]. One of these compounds, a C3-fluorinated sialic acid analog, was shown to lower the expression of sialic acid in living mice[49]. Animals treated with this substance, however, showed liver impairment, irreversible kidney dysfunction, and failure to thrive[49]. These results confirm the crucial role of Neu5Ac in liver and kidney function and further indicate the limits of sialylation inhibitors for *in vivo* experiments. A third method to generate cells with altered cell surface sialylation is by interfering with the expression of enzymes that are crucial for sialic acid biosynthesis. A knock-down of the GNE/MNK in immortalized cells, for example, was shown to render these cells hyposialylated [9]. The possibility to relatively easily knock-in and -out genes *in cellular* and *in vivo* using the novel and well recognized CRISPR-Cas9 technique might lead to novel discoveries regarding sialic acid biosynthesis and cell surface sialylation.

The advantage of MGE compared to the techniques described here, is that it is easy to apply and adaptable to a myriad of different experiments, from *in vitro* trials to cell based assays and *in vivo* experiments. An alternative method to measuring the expression and concentrations of different sialic acid species in cell samples is by high-performance anion-exchange chromatography (HPAEC) with pulsed electrochemical detection[50]. By using this method, the expression of sialic acid is measured directly and not after labeling with a fluorescent dye. The advantage of this method is that it can be utilized also to analyze monosaccharides other than sialic acid and its derivatives in cell- and protein-samples. Unfortunately, the sensitivity of HPAEC is lower compared to fluorometric detection of DMB-sialic acid. Thus, HPAEC is limited to experiments with the availability of large sample amounts[51].

The herein described methods can be used to reveal unknown characteristics of glycoconjugates. A multitude of examples for the use of MGE to investigate sialic acid dependent interactions already exists[13], showing the feasibility of this technique in context of a wide range of experimental setups.

Nowadays, MGE with *N*-acetylmannosamines is mainly used by researchers in the field of glycobiology. We hope that this technique will be recognized by a broader audience as a versatile tool to modify glycoconjugates. Other fields, including immunology, virology, neurology, or oncology will benefit by adapting this technique for their own purposes. Such cross-over research might enable the discovery of yet uncharted areas, and therefore give a better understanding of health and disease. In early studies, for example, this technique was used to produce glycoproteins with modified glycosylation, which later serve for *in vivo* vaccination. In certain cases, treatment with such glycoengineered vaccines lead to production of antibodies that exposed advanced avidity and toxicity to the respective targets[52,53]. Others successfully used MGE to develop a model for targeted tumor therapy in mice[54]. Another study showed that systemic *in vivo* injection of ManNProp promoted nerve regeneration[55]. In the case of polySia, it was shown that diminishing the expression of this epitope in neuroblastoma cells by the methods presented in this manuscript, increases the sensitivity of these tumor cells to radiation or treatment with anticancer drugs[56].

The amounts of sialic acid measured within samples might differ depending on the method used to detach the treated cells. In this manuscript, incubation with trypsin was used to detach cells. If other techniques are to be used, such as scraping the cells or long-time treatment with PBS + EDTA, the amounts of sialic acid measured in the samples might differ. Even adapting the incubation time with trypsin can have an impact on the sialic acid concentrations measured later on. From our experience, the impact of the cell detachment method on the amounts of sialic acid measured is below 15%, but if results are to be normalized and compared, this might become an important bottleneck. Additionally, the method for lysis of the cells can affect the outcome of the HPLC analysis. Thus, we recommend the reader to standardize cell detachment and lysis in their experiments.

To achieve separation of the membrane fraction in cell lysates, we used centrifugation for 2 h at 20,000 x g and 4 °C (section 5.1). Other centrifugation protocols might affect the separation and therefore the amounts of sialic acid detected in the samples. A critical compound within this protocol is DMB, because it is very sensitive to light and degrades over time. Here we recommended to aliquot small portions of the DMB-solution and store it as stocks at - 20 °C, protected from light (section 1.6.1). This way the DMB-solution can be stored for several weeks. If the results from DMB-HPLC analysis show decreasing signals for sialic acid species or increasing signals within the first 6 min of the HPLC measurement, representing DMB and its reaction products, new DMB-solution should be prepared. Do not re-freeze and re-use DMB-solution after thawing it. After DMB labeling, all samples should be analyzed immediately. If larger numbers of samples (> 10) are to be analyzed, these samples should be divided into smaller badges and gradually labeled with DMB. Both the labeling reaction as well as the DMB-labeled probes should be protected from light at all times. After elution, the DMB-labeled sialic acid species should be analyzed immediately by ESI-MS. Direct coupling of the HPLC with the ESI-MS system (in a LC-ESI-MS setup) is not necessary, but can be advantageous to achieve higher quality of the mass spectrometry analysis.

Disclosures

The authors have nothing to disclose.

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