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Quantifying the Efficiency of *N*-Phenyl-D-mannosamine to Metabolically Engineer Sialic Acid on Cancer Cell Surface

Zhifang Zhou, Guochao Liao, Sergejs Stepanovs, and Zhongwu Guo

Department of Chemistry, Wayne State University, Detroit, Michigan 48202, USA

Abstract

A convenient method was developed for the quantification of sialic acids expressed by cells and used to analyze the efficiency of *N*-phenylacetyl-D-mannosamine (ManNPhAc) to metabolically glycoengineer SKMEL-28 cancer cell. For this purpose, ManNPhAc-cultured cells were treated with 2M acetic acid to release sialic acids, and the products were treated with 1,2-diamino-4,5-methylenedioxybenzene to form the corresponding derivatives that had strong UV absorptions. The reaction mixture was then applied to HPLC-UV analysis to determine the amounts and the ratios of natural sialic acid and its unnatural analog. It was confirmed that after incubation with ManNPhAc SKMEL-28 cell was effectively glycoengineered to express a significant amount of unnatural sialic acid.

Keywords

mannosamine; metabolic engineering; sialic acid; analysis; cancer; 1,2-diamino-4,5-methylenedioxybenzene

Introduction

Sialic acids are a family of nine-carbon keto carbohydrates, and the most common member is *N*-acetylneuraminic acid (Neu5Ac, Figure 1). Neu5Ac is usually present at the non-reducing end of glycans and plays important roles in various biological processes, such as specific recognition, binding and regulatory events.^{1,2} It is also well known that Neu5Ac is typically overexpressed on the surface of cancer cells³ and many of the uniquely or excessively expressed glycans on cancer cells, known as tumor-associated carbohydrate antigens (TACAs),⁴ contain the Neu5Ac residue. Some sialo-TACAs, such as α -2,8-polysialic acid, sTn antigen, sialy Lewis antigens and the GM and GD gangliosides, are abundantly expressed by a number of tumors. Therefore, sialo-TACAs are very useful targets for the design and development of new strategies for cancer diagnosis and immunotherapy.^{5–7} However, most native TACAs are poorly immunogenic and tolerated by the patients' immune system; therefore, they may not be able to induce robust antitumor immune responses, especially T cell-dependent immune responses, in cancer patients.

To address the immunotolerance problem associated with TACAs, we have explored a novel strategy for cancer immunotherapy that is based on metabolic engineering of cancer cell surface sialo-TACAs.^{8,9} A key step of this strategy is to provide for cancer cells an unnatural analog of *N*-acetyl-D-mannosamine (ManNAc), the native biosynthetic precursor of Neu5Ac, to bioengineer cancer cell expression of an unnatural sialo-TACA derivative^{10,11} (Figure 2) that was proved to be more immunogenic than the natural TACA to enable effective cancer immunotherapy.⁸ By means of antigen-specific antibodies and bioassays such as ELISA and flow cytometry,^{12,13} we have demonstrated that *N*-phenylacetyl-D-mannosamine (ManNPhAc) could metabolically engineer cancer cells to express sialo-TACA analogs, such as that of sTn and GM3, carrying unnatural *N*-phenylacetylsialic acid (Neu5PhAc) residues. However, previous analyses were only qualitative or semiquantitative. To unambiguously determine the efficiency of ManNPhAc to metabolically engineer cancer cell, a reliable method to quantify the ratio of expressed Neu5Ac and Neu5PhAc is required, which is the aim of this research.

Results and Discussion

In the literature several methods have been described for the analysis of sialic acids, such as colorimetric and fluorometric assays after derivatization with thiobarbituric acid^{14,15} and liquid chromatography (LC) with UV,¹⁶ fluorescence^{17–27} and mass spectrometry (MS)^{28–32} detections. Except for LC-MS, sialic acids must be derivatized to be photometrically detectable. In addition to thiobarbituric acid,^{14,15} a number of reagents including 3,4-diaminotolene,³² 1,2-diamino-4,5-dimethoxybenzene,¹⁷ *O*-phenylenediamine,²⁰ and 1,2-diamino-4,5-methylenedioxybenzene (DMB),^{21–29,33,34} have been used for sialic acid derivatization, taking advantage of the special α -keto acid functionality. Among these methods, DMB derivatization for HPLC-fluorescence and LC-MS analyses was the most popular due to the high sensitivity and selectivity of the reaction involved. In this research, we aimed at a potentially broadly applicable method that uses simple instrument. Therefore, we became interested in a method that was based on HPLC separation and UV detection. To make sialic acids UV-detectable, we planned to employ DMB for sialic acid derivatization.^{29,33,34} As shown in Figure 3, the resulting DMB derivatives had strong maximum absorbance at 370 nm that can be utilized for quantitative analysis. Consequently, our design for sialic acid analysis was to first release total sialic acids from cells under mild acidic conditions, then derivatize them with DMB, and finally subject the reaction mixture to HPLC analysis with UV detection at 370 nm.

To verify that DMB derivatization of sialic acids can be used for quantitative analysis and that the HPLC signals of corresponding sialic acid derivatives have a linear relationship with the concentrations of Neu5NAc and Neu5PhAc and to establish proper calibration curves, different concentrations of standard Neu5NAc and Neu5PhAc samples were treated with DMB and then applied to HPLC-UV analysis. It was revealed that the reactions were clean and complete and the HPLC retention times [C18 column, 0.5 μ m, 250 $\times$ 4.6 mm; eluted with water and acetonitrile containing 0.1% trifluoroacetic acid (TFA): acetonitrile concentration changed from 5% to 25% in 10 min, kept at 25% for 10 min, then from 25% to 70% in 15 min; flow rate: 1 mL/min] for the Neu5Ac and Neu5PhAc derivatives **3** and **4**, which were confirmed with MS, were 9.87 min and 15.45 min, respectively. As shown in

Figure 4, under the tested conditions (that is, Neu5Ac and Neu5PhAc concentrations in the range of 15–130 μ M), the observed HPLC peak areas of **3** and **4** were in good linear relationship (coefficient: 0.9814 and 0.9763, respectively) with the Neu5Ac and Neu5PhAc concentrations in the samples. These results have demonstrated that the method could be employed for Neu5Ac and Neu5PhAc quantifications. Equations, $\text{HPLC peak area} = 2067 [\text{Neu5Ac}] + 18,835$ and $\text{HPLC peak area} = 1832 [\text{Neu5PhAc}] - 1,141$, were used to calculate the concentrations or quantities of Neu5Ac and Neu5PhAc in samples, respectively.

For the analysis of biological samples derived from cells, there are several issues that may be of concern. First, the detachment of sialic acids from cells may not be complete, which can affect the observed amount of sialic acids. However, this would be a systematic error that may affect sample recovery rate but not the ratio of Neu5Ac and Neu5PhAc, while the latter is actually our focus. Second, conditions used to detach sialic acids from cells may release other acid-sensitive components as well, thus sialic acids would be derivatized in the presence of potentially many impurities. Whether the derivatization reactions will be affected under this condition is another question, despite that the above studies have proved the reaction efficiency in the absence of impurities. Third, samples derived from metabolically engineered cells will contain variable ratios of Neu5Ac and Neu5PhAc, which can be immensely different. Whether this can be accurately measured is another question.

To address these issues, we then probed the reliability and accuracy of the proposed method under more complex situations, such as for the analysis of biologic samples or samples with big differences in the Neu5Ac and Neu5PhAc concentrations. To probe the reliability of the method, we added different amounts of Neu5PhAc in the culture of SKMEL-28, a melanoma cell line, and then examined the recovery upon a series of treatments involved in the real sample analysis, including incubation with acetic acid employed to detach sialic acids from cells, lyophilization, reaction with DMB, and eventually HPLC analysis. As shown in Table 1, the recovery rates of the externally added Neu5PhAc in various samples were excellent (97–99%, average $98 \pm 1\%$). In this study, the recovery of Neu5Ac from cells was not examined, as the added Neu5Ac would interfere with naturally existing Neu5Ac. However, we anticipated that its recovery rate should not be lower than that of Neu5PhAc. To probe the accuracy of the method, we prepared a series of Neu5Ac and Neu5PhAc mixtures in ratios ranging from 100:0 to 100:8 and applied them to analysis by the proposed method. As shown in Table 2, the experimental results were in good agreement with the expected ratios, and in average the accuracy was above 93%. These results have clearly demonstrated the reliability and accuracy of the proposed method for the analysis of Neu5Ac and Neu5PhAc expressed by metabolically glycoengineered cells.

After the proposed method was verified with standard samples under different conditions, it was applied to the analysis of metabolically engineered cancer cells. In these studies, SKMEL-28 cells were incubated with various concentrations of ManNPhAc (0, 5, 10, 20, and 30 mM) for 2–4 days. Then, *ca.* 1.0×10^6 cells were harvested from each culture, washed, and treated with 2 M acetic acid at 80 °C for 2 h to release sialic acids from cells, a condition that was employed to hydrolyze sialic acids in a number of literature reports^{22,24,28,29} and therefore was not further optimized. This was followed by

lyophilization and reaction with excessive DMB at 50 °C for 3 h.²⁹ After the reaction mixtures were filtered off through a 0.22 µm film, they were subjected to analysis by RP-HPLC using gradient mixtures of water and acetonitrile containing 0.1% TFA as eluents. Figure 5 shows the typical HPLC diagram of a sample. As labeled in the figure, the DMB derivatives of Neu5Ac and Neu5PhAc **3** and **4** had the same retention times as that of the standard samples. To further prove their identity, **3** and **4** peaks were collected and subjected to MALDI-MS analysis, which gave the correct molecular masses [m/z : calculated, 425.2 and 501.2; observed, 426.3 and 502.5 ($M+H^+$), respectively]. Evidently, the samples also contained some impurities, which appeared at different positions and did not obviously affect the analysis.

The amounts of Neu5Ac and Neu5PhAc in samples obtained from 10⁶ SKMEL-28 cells were calculated based on the HPLC peaks of **3** and **4** according to the standard curves shown in Figure 4, and the results are presented in Figure 6. As anticipated, significant Neu5PhAc expression was observed for cells incubated with 5 mM of ManNPhAc (Figure 6B), and the amount of expressed Neu5PhAc increased proportionally with the increase of ManNPhAc concentration in the cell culture. However, Neu5PhAc expression was still much lower than that of Neu5Ac even at a quite high concentration (30 mM) of ManNPhAc. Furthermore, it was found that natural Neu5Ac expression was also elevated in the presence of high concentrations of ManNPhAc. Typically, one should expect natural product biosynthesis to be depressed on the addition of an unnatural biosynthetic precursor due to either competition or inhibition. In contrast, it was very interesting to notice that the presence of ManNPhAc seemed to promote not only Neu5PhAc expression but also Neu5Ac biosynthesis, which is rather unique and can be of biological significance. The results conclusively suggest that ManNPhAc could effectively glycoengineer SKMEL-28 cell to express unnatural Neu5PhAc in a ManNPhAc concentration-dependent manner in the range of 5–30 mM, whereas Neu5Ac expression was only slightly affected under the same condition. It was also evident that only a rather small fraction of Neu5Ac on the cell surface was substituted with Neu5PhAc upon metabolic engineering using ManNPhAc, a process that had to compete with the endogenous biosynthetic pathway.

To further define the efficiency of cell metabolic engineering using ManNPhAc, the percentage of Neu5PhAc expressed on the cell surface in terms of total sialic acids was calculated according to the following equation, $\text{Neu5PhAc\%} = \frac{\text{Neu5PhAc}}{\text{Neu5Ac} + \text{Neu5PhAc}} \times 100\%$, and the results are shown in Figure 7A. The percentage was about 2.2% and 5.8% for cells incubated with 5 mM and 30 mM of ManNPhAc for 3 days, respectively.

Finally, we examined the influence of incubation time on the cell engineering efficiency. In these studies, we incubated cancer cells with 5 mM and 20 mM of ManNPhAc for 2, 3, 4 and 9 days, and then examined their Neu5PhAc expression. The results (Figure 7B) indicated that the percentage of Neu5PhAc increased during the first 3 days to reach peak values at day 3 and then decreased to eventually retain a rather consistent level of Neu5PhAc for a long period after day 4. The reason for decreased Neu5PhAc expression after day 3 was not clear, but it might be due to the change of metabolic activities of the cell, as we noticed that after 3 days of incubation cell proliferation and growth became slower.

Nevertheless, it was ultimately disclosed that cancer cells could be glycoengineered to express a significant level of unnatural Neu5PhAc to make the cells distinct for a quite long period.

Conclusion

We have developed a convenient and reliable method for quantitative analysis of sialic acids expressed by cells. This method was based on the specific reaction between DMB and the α -keto acid functionality of sialic acids to generate products that had strong UV absorption, enabling easy HPLC analysis with UV detection. After the consistency and accuracy of this method were verified with standard samples under various conditions, it was utilized to analyze metabolically glycoengineered cancer cells, which expressed both natural Neu5Ac and unnatural Neu5PhAc.

The protocols for this analysis were rather straightforward. After incubation with ManNPhAc, cells were subjected to acetic acid-promoted hydrolysis to release sialic acids. Then, the sample was lyophilized and treated with DMB. The reaction mixture was directly applied to HPLC analysis to determine the amounts and ratio of Neu5Ac and Neu5PhAc. The method is not only applicable to the analysis of Neu5Ac and Neu5PhAc but also generally useful for the analysis of other sialic acids.

While this is a simple, reliable and quantitative method, it does have limitations. For example, it is not easily applicable to the analysis of cells incubated with μM concentration of ManNPhAc, probably because the Neu5PhAc expression level was too low. However, Neu5PhAc expression on these cells was detected by flow cytometry with the help of antibody-labeling.¹³ Evidently, flow cytometry is more sensitive, albeit not precisely quantitative, than HPLC analysis. Besides not being strictly quantitative, another problem for the flow cytometry method is that it relies on antigen-specific antibodies that are not always readily available. Consequently, a combination of these two analytical methods can portrait the whole picture concerning the metabolic engineering of cell surface sialic acids with unnatural ManNAc derivatives as biosynthetic precursors.

This research has revealed that more than 2% of Neu5Ac was substituted for Neu5PhAc on metabolic glycoengineering with ManPhNAc. We have previously demonstrated by means of flow cytometry¹³ and antibody-mediated cytotoxicity¹² that cancer cells could be metabolically engineered to express Neu5PhAc at low μM concentrations (20–50 μM) of ManNPhAc. Under these conditions, the Neu5PhAc expression level should be lower than 2%, but the engineered cancer cells could still be recognized by Neu5PhAc-specific antibodies and undergo functional immune reactions. This information is useful not only for our immunotherapy but also for other studies that are based on metabolic engineering of cell surface sialic acids or other sugars. A particularly interesting but unexpected discovery was that natural sialic acid expression also increased in the presence of ManNPhAc, which could be only disclosed from quantitative analysis. This may be of biological significance, as sialic acid expression is related to various biological activities; therefore, it is worthy further investigation.

Experimental Section

Materials, reagents and animals

SKMEL-28 cell line, Dulbecco's Modified Eagle's Medium (DMEM) for cell culture, and fetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC). Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen. ManNPhAc was synthesized by a procedure previously reported by our laboratory.¹³ DMB, sodium hydrosulfite, β -mercaptoethanol and other chemicals were purchased from Sigma.

Metabolic engineering of tumor cell

SKMEL-28 cells were cultured in DMEM containing 10% of FBS, 1% of penicillin-streptomycin and various concentrations (0 to 30 mM) of ManNPhAc at 37 °C. After a certain number of days of incubation, cells were harvested upon treatment with trypsin-EDTA solution, and finally washed twice with PBS to be readied for further analysis.

Hydrolysis of sialic acids

A cell sample (containing about 1.0×10^6 cells) was dissolved in a 2 M solution of acetic acid (2 mL). The mixture was stirred at 80 °C for 2 h, and then lyophilized to get a powder product that contained sialic acids released from cells.

DMB derivatization of sialic acids and HPLC analysis

The powder product obtained above was mixed with DMB (0.5 mg, excessive) in 1.4 M acetic acid containing 18 mM of sodium hydrosulfite and 0.75 M of β -mercaptoethanol.²⁹ After the reaction mixture was stirred at 50 °C for 3 h, it was filtered off through a 0.22 μ m film, and the filtrate was analyzed by RP-HPLC (C18 column, 250 $\times$ 4.6 mm, 0.5 μ m) using gradient eluents of water (A) and acetonitrile (B) containing 0.1% TFA at flow rate of 1 mL/min. Elution started with 5% B, which was increased gradually to 25% B in 10 min. Elution of the column with 25% B was kept for 10 min, followed by gradual increase of B composition to 70% within 15 min, and the elution was kept under this condition to the end of an experiment. Finally, the column was washed and balanced with 5% B for 40 min before the next experiment. The eluents were monitored with a UV detector at 370 nm, at which wavelength DMB derivatives of sialic acids had the maximum absorption. All of the HPLC peaks were separately collected, and the fractions containing the anticipated DMB derivatives of sialic acids were further analyzed and verified by MALDI-MS.

Establishment of standard calibration curves and equations used to quantify Neu5Ac and Neu5PhAc

A Neu5Ac or Neu5PhAc standard sample (1, 2, 4 or 6 μ g) was mixed with a DMB reaction solution (150 μ L containing 0.5 mg DMB, excessive) in 1.4 M acetic acid, 18 mM of sodium hydrosulfite and 0.75 M of β -mercaptoethanol. The reaction mixture was stirred at 50 °C for 3 h, and then filtered off through a 0.22 μ m film. The filtrate (10 μ L) was analyzed by RP-HPLC (C18 column, 250 $\times$ 4.6 mm, 0.5 μ m) using gradient eluents of water and acetonitrile containing 0.1% TFA at flow rate of 1 mL/min as described above. The eluents

were monitored with a UV detector at 370 nm. The peak areas were plotted against the sample concentration to obtain the calibration curves and equations.

Determining the recovery rate of Neu5PhAc from cell culture

SKMEL-28 cell was cultured, harvested, and washed twice with PBS. To the cell sample (about 1.0×10^6 cells, in 1 mL PBS solution) was added 65 or 130 nmol of a Neu5PhAc standard sample. The cell sample was added to an acetic acid solution (final volume 2 mL and final concentration 2 M). The mixture was stirred at 80 °C for 2 h. The reaction mixture was lyophilized to obtain a powder product that was reacted with a DMB solution (150 μ L, containing 0.5 mg DMB) in 1.4 M acetic acid containing 18 mM of sodium hydrosulfite and 0.75 M of β -mercaptoethanol. The reaction mixture was finally subjected to HPLC analysis as described above.

Determining the Neu5Ac and Neu5PhAc ratios in standard samples

A series of standard samples containing different ratios of Neu5Ac and Neu5PhAc (100:0, 100:2, 100:4, 100:6, 100:8 mol/mol) were prepared. About 20 nmol of each sample was added to a DMB solution (150 μ L, containing 0.5 mg DMB) in 1.4 M acetic acid containing 18 mM of sodium hydrosulfite and 0.75 M of β -mercaptoethanol. After the reaction mixture was stirred at 50 °C for 3 h, it was filtered off through a 0.22 μ m film, and 10 μ L of the filtrate was finally subjected to RP-HPLC analysis as described above.

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Figure 1.
Structure of Neu5Ac

Figure 2.
Metabolic engineering of sialic acids on the cancer surface

Figure 3.
Reactions between sialic acids and DMB and structures of the reaction products

Figure 4.

A linear correlation between the peak areas of Neu5Ac or Neu5PhAc derivative and the concentrations of Neu5Ac or Neu5PhAc was demonstrated. The coefficient of determination (R^2) is displayed for each graph.

Figure 5.
HPLC diagram of a sample derived from 10^6 SKMEL-28 cells treated with 30 mM of ManNPhAc for 3 days. The retention times for the DMB derivatives of Neu5Ac and Neu5PhAc **3** and **4** were about 9.90 and 15.50 min, respectively.

Figure 6.

The amounts of Neu5Ac (A) and Neu5PhAc (B) expressed by SKMEL-28 cells (10⁶) that were incubated with 0, 5, 10, 20 and 30 mM of ManNPhAc for 3 days, determined by HPLC. Error bar represents the standard deviation of three parallel experiments.

Figure 7.

The percentages of Neu5PhAc expression on SKMEL-28 cells incubated with 0, 5, 10, 20, and 30 mM of ManNPhAc for 3 days (A), and the percentages of Neu5PhAc on SKMEL-28 cells incubated with 5 and 20 mM of ManNPhAc for 2, 3, 4 and 9 days (B), respectively. The error bar represents the standard deviation of three parallel experiments.

Table 1

Recovery analysis of Neu5PhAc from the SKMEL-28 cell culture

Added Neu5PhAc (nmol)	65	130	130	130
Detected Neu5PhAc (nmol)	63	128	127	129
Recovery rate (%)	97	98	98	99

Table 2

Analysis of Neu5Ac and Neu5PhAc mixtures prepared in different ratios

Sample number	1	2	3	4	5
Standard samples Neu5Ac:Neu5PhAc	100:0	100:2	100:4	100:6	100:8
Experimental results Neu5Ac:Neu5PhAc	100:0	100:1.9(±0.1)	100:3.7(±0.1)	100:5.9(±0.3)	100:7.1(±0.4)