Metabolic Glycoengineering with Azide- and Alkene-Modified Hexosamines: Quantification of Sialic Acid Levels

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Dedicated to Professor Horst Kessler on the occasion of his 80th birthday

Metabolic glycoengineering (MGE) is an established method to incorporate chemical reporter groups into cellular glycans for subsequent bioorthogonal labeling. The method has found broad application for the visualization and isolation of glycans allowing their biological roles to be probed. Furthermore, targeting of drugs to cancer cells that present high concentrations of sialic acids on their surface is an attractive approach. We report the application of a labeling reaction using 1,2-diamino-4,5-methylenedioxybenzene for the quantification of sialic acid derivates after MGE with various azide- and alkene-modified ManNAc, GlcNAc, and GalNAc derivatives. We followed the time course of sialic acid production and were able to detect sialic acids modified with the chemical reporter group – not only after addition of ManNAc derivatives to the cell culture. A cyclopropane-modified ManNAc derivative, being a model for the corresponding cyclopropene analog, which undergoes fast inverse-electron-demand Diels-Alder reactions with 1,2,4,5-tetrazines, resulted in the highest incorporation efficiency. Furthermore, we investigated whether feeding the cells with natural and unnatural ManNAc derivative results in increased levels of sialic acids and found that this is strongly dependent on the investigated cell type and cell fraction. For HEK 293T cells, a strong increase in free sialic acids in the cell interior was found, whereas cell-surface sialic acid levels are only moderately increased.

Introduction

Cell-surface glycans play a vital role in numerous cell recognition processes.[1] Sialic acids for example are located at the outer end of glycans and are involved in inflammatory processes and serve as docking point for influenza viruses that bind to them by hemagglutinin. Metabolic glycoengineering (MGE) is an established method that enables the incorporation of unnatural functional groups (so-called chemical reporter groups) into glycans.[2] Cells are cultivated in the presence of an unnatural carbohydrate derivative with a chemical reporter group that is metabolically incorporated into the glycans. Subsequently, it can be reacted in a bioorthogonal ligation reaction.[3] Labeling of sialic acids can be achieved with *N*-acetylmannosamine (ManNAc) derivatives that are modified in the acetyl side chain. These derivatives are converted into the corresponding sialic acid and incorporated into glycans in five enzymatic steps.[2a] MGE has found broad application for the visualization and isolation of glycans thereby probing their biological roles.[2d] Furthermore, the in vivo targeting of drugs to cancer cells that present high concentrations of sialic acids on their surface has been reported.[4] Recently, we showed that MGE-based quantification of plasma membrane sialylation can be used as a readout for cell function and neurotoxicity.[5]

Numerous reporter groups have been employed[2] that can be ligated by various chemistries including Staudinger ligation,[6] copper-catalyzed[7] and strain-promoted[8] azide-alkyne cycloadditions. Beside these azide-based ligation reactions, more recently the inverse-electron-demand Diels-Alder (DA_{inv}) reaction of terminal or strained cyclic alkenes has been added to the MGE toolbox.[9] Terminal alkenes are small, easily accessible, and robust reporter groups[10] whereas strained cyclic alkenes, such as cyclopropenes,[11] norbornenes[12] or *trans*-cyclooctenes[13] allow faster bioorthogonal ligation reactions. The DA_{inv} reaction can be orthogonal to azide-alkyne cycloadditions[9a,14] opening the possibility to label two different carbohydrates within the same MGE experiment.[9b] Furthermore, the DA_{inv} reaction does not require catalysis by heavy metals allowing the visualization of glycosylation inside living cells.[15]

A characteristic feature of MGE is the observation, that different monosaccharides can be interconverted by epimerases raising the question where the sugars end up during an MGE experiment. For example, *N*-acetylglucosamine (GlcNAc) can be converted to ManNAc by the GlcNAc 2-epimerase encoded by the RENBP gene.[16] Furthermore, uridine diphosphate-activated GlcNAc (UDP-GlcNAc) can be metabolized to ManNAc by the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202000715

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kinase (GNE)[17] representing another possibility for GlcNAc and potentially derivatives thereof to enter the sialic acid biosynthesis pathway. UDP-activated *N*-acetylgalactosamine (UDP-GalNAc) can be epimerized to UDP-GlcNAc and vice versa by the UDP-glucose 4-epimerase (GALE).[18] Thus, also GalNAc and derivatives might be converted to sialic acids in a multi-step process. For MGE experiments, it is of high interest to know whether these processes take place and whether they are influenced by the nature of the reporter group. As cells are fed unnaturally high concentrations of monosaccharides during MGE experiments, it is also of concern whether the carbohydrate metabolism, for example the biosynthesis of sialic acids is increased under these conditions. The processes described above might also be dependent on the used cell type.

Here we report the application of the DMB labeling reaction (DMB = 1,2-diamino-4,5-methylenedioxybenzene) for the detection and quantification of sialic acid derivates after MGE with various azide- and alkene-modified ManNAc, GlcNAc, and GalNAc derivatives. We follow the time course and efficiency of the production of modified sialic acid derivatives after cells have been cultivated in the presence of synthetic monosaccharide derivatives. We show that Ac_4GlcNAz feeding results in the expression of the corresponding sialic acid Neu5Az whereas neither an alkene-modified GlcNAc derivative nor the investigated GalNAc derivatives do so. Furthermore, we investigated whether feeding of the cells with natural and unnatural ManNAc derivatives results in increased levels of sialic acids. It turned out that this is dependent on the investigated cell type and cell fraction.

Results and Discussion

Figure 1 depicts the carbohydrate derivative that have been used in this study. Beside peracetylated natural ManNAc (Ac_4ManNAc), the azide and alkene derivatives Ac_4ManNAz and Ac_4ManNBtl, respectively, as well as the corresponding GlcNAc and GalNAc derivatives have been employed. In addition, we used the cyclopropane derivative Ac_4ManNCp(H_2). This derivative serves as model for the corresponding cyclopropene derivative Ac_4ManNCp[11d,19] that has a high DA_{inv} reactivity and leads to intense cell-surface staining. The cyclopropene moiety, however, does not survive the DMB labeling procedure that includes the release of sialic acids under acidic conditions.[11d,19] Ac_4ManNCp(H_2) on the other hand is stable and has a comparable size, for which reason we expect it to have a similar behavior in MGE. We first carried out MGE experiments with several monosaccharide derivatives and compared the intensity of the resulting cell-surface labeling. Subsequently, we determined the resulting sialic acid levels after cells had been cultivated in the presence of these monosaccharides.

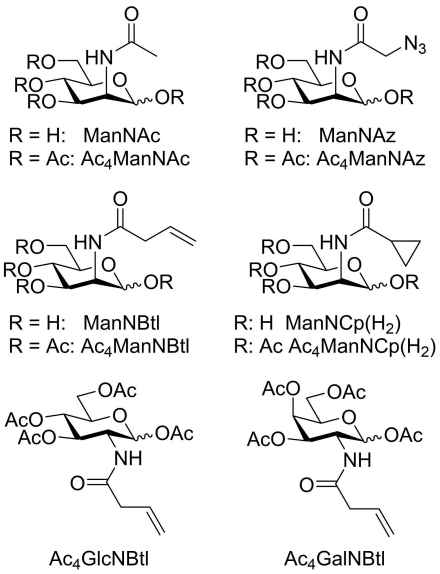
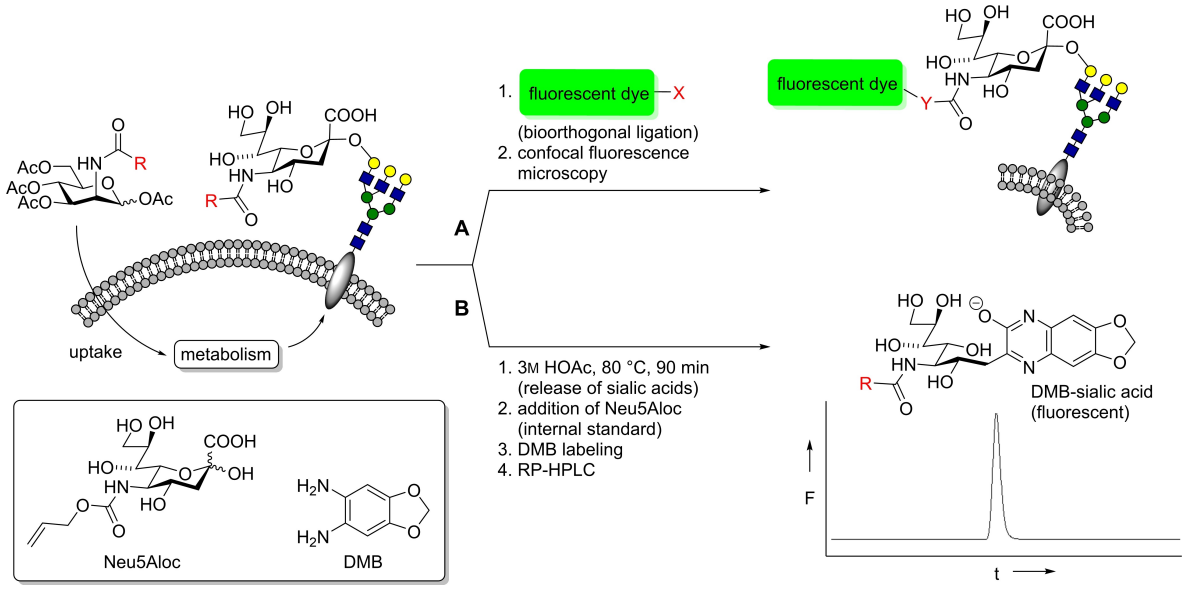


Figure 1. Monosaccharide derivatives used in the MGE experiments.

MGE experiments

For MGE experiments, cells were cultivated in presence of the respective sugar derivative (Scheme 1). To detect the incorporated sugar derivative on the cell surface, the treated cells were labeled by a bioorthogonal ligation reaction (path A). For the azido derivatives, the strain-promoted azide-alkyne cycloaddition (SPAAC) was performed with DIBO-AlexaFluor488 (DIBO-AF488). For the butenoyl modified sugar derivatives, the DA_{inv} reaction was performed using a tetrazine-biotin conjugate (Tz-biotin) followed by treatment with streptavidin-AlexaFluor555 (streptavidin-AF555; see Figure S1 in the Supporting Information for formulae of the reagents). This two-step labeling procedure resulted in lower background staining compared to a one-step labeling with a tetrazine-AlexaFluor conjugate. Confocal fluorescence microscopy was used to read out the staining.

To determine how well the used sugar derivatives are incorporated as sialic acid and to quantify absolute sialic acid levels, we used a previously described technique, the DMB labeling reaction.[20] For this purpose, metabolically engineered cells were incubated with 3 M acetic acid at 80 °C for 90 minutes to detach the sialic acids and lyse the cells (Scheme 1, path B). After concentration using a vacuum centrifuge, *N*-(allyloxycarbonyl)neuraminic acid (Neu5Aloc) was added as internal standard and the sample was labeled with DMB. DMB selectively reacts with α-keto carboxylic acids such as neuraminic acids, forming a quinoxaline fluorophore. The resulting fluorescent sialic acid derivatives were analyzed by RP-HPLC using a fluorescence detector ($\lambda_{ex} = 372$ nm, $\lambda_{em} = 456$ nm) enabling the detection of even small amounts of α-keto carboxylic acids in complex mixtures. Incorporation efficiencies (IE), that is, the percentage of sialic acids modified by the chemical reporter group, were calculated from the integral of the RP-HPLC signals of DMB-labeled natural Neu5Ac (I_{Neu5Ac}) and the integral of the respective DMB-labeled modified sialic acid (I_{Neu5R}) according to the formula $IE = I_{Neu5R} \times (I_{Neu5Ac} + I_{Neu5R})^{-1} \times 100\,\%$.[10b]



Scheme 1. Principle of MGE depicted with an example ManNAc derivative. To detect the reporter group R on the cell surface, cells are fluorescently labeled in a bioorthogonal ligation reaction and investigated by confocal fluorescence microscopy (pathway A). To quantify sialic acid levels, sialic acids are cleaved off by treatment with acetic acid and – after addition of internal standard Neu5Aloc – labeled by DMB allowing sialic acids to be quantified by RP-HPLC (pathway B).

Cell-surface staining

Cell-surface staining with Ac_4ManNAz, Ac_4GalNAz and Ac_4GlcNAz has been previously reported.[11a,21] When we carried out experiments with HEK 293T cells under identical experiments, MGE with Ac_4ManNAz results in a very intense staining (Figure 2). Ac_4GalNAz led to a slightly weaker staining and Ac_4GlcNAz to a significantly weaker staining, which nevertheless was positive compared to the solvent control. It has been reported that Ac_4GlcNAz is essentially excluded from incorporation into glycoproteins on the cell surface.[21a] This raises the question whether the staining achieved with Ac_4GlcNAz might result from isomerization to ManNAz and incorporation as Neu5Az which is indeed supported by competition experiments.[16,21a]

In addition to the azido modification, which can be labeled by click chemistry, we also investigated cell-surface staining of alkene-modified sugar derivatives that can be stained by the DA_{inv} reaction. For this purpose, we employed a GlcNAc, GalNAc and ManNAc derivative with a butenoyl (Btl) modification (Figure 3). The butenoyl residue is even smaller than an azidoacetyl residue and Ac_4ManNBtl has been shown to have a high IE of 62%.[10b] The staining intensity obtained with HEK 293T cells treated with Ac_4ManNBtl shows a very intense staining. No staining was observed for cells treated with Ac_4GalNBtl and Ac_4GlcNBtl. As shown previously, the staining intensity depends on the combination of the rate of the bioorthogonal labeling reaction and the efficiency by which the modified sugar derivative is incorporated in the cellular glycans.[10b] Taking the previously determined relatively low reaction rate of butenoyl moiety in the DA_{inv} reaction into account (second-order rate constant $k_2 = 0.0011\ M^{-1}\ s^{-1}$),[10b] we

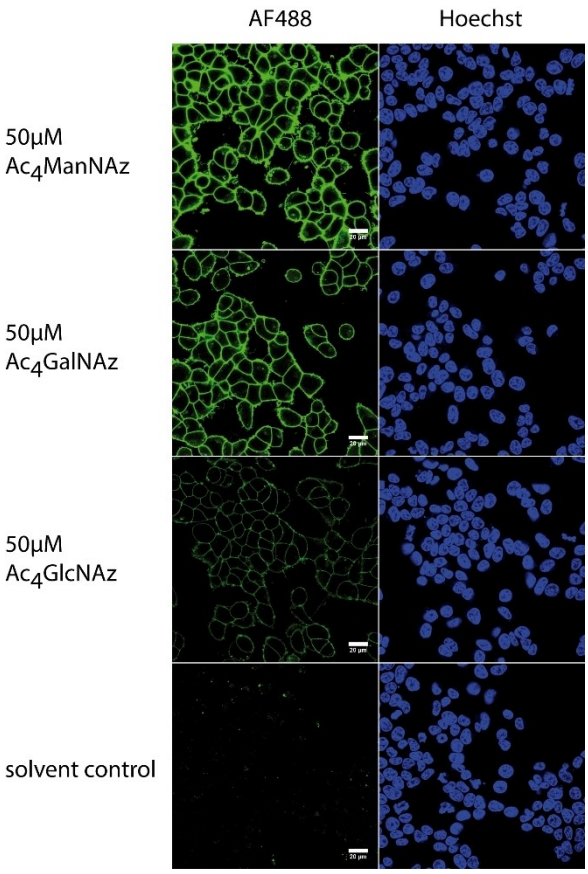


Figure 2. HEK 293T cells were incubated with 50 μM each of Ac_4ManNAz, Ac_4GalNAz, or Ac_4GlcNAz or DMSO only (solvent control) for 48 h. Subsequently, the cells were treated with DIBO-AF488 and Hoechst 33342 for 30 min and analyzed by confocal fluorescence microscopy. Scale bars: 20 μm.

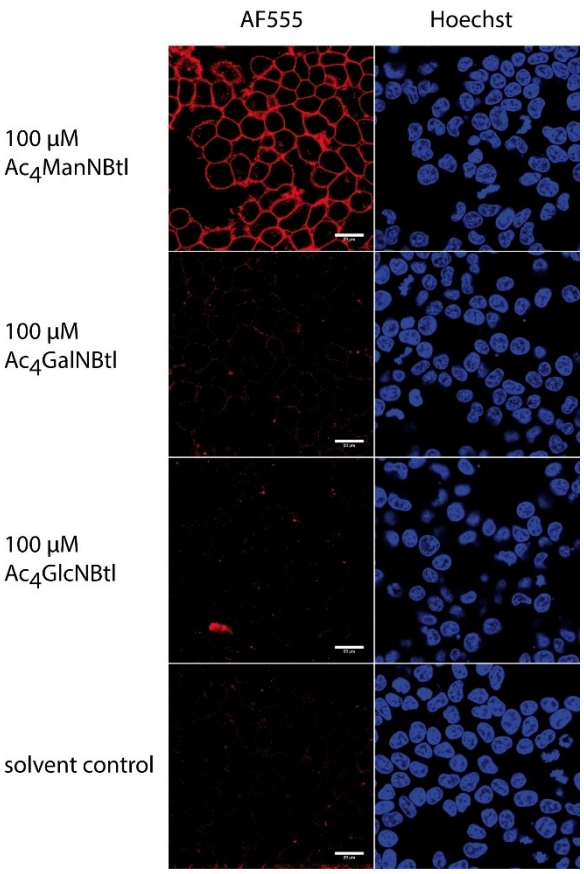


Figure 3. HEK 293T cells were incubated with each 100 μM Ac_4ManNBtl, Ac_4GalNBtl, or Ac_4GlcNBtl or DMSO only (solvent control) for 48 h. Subsequently, the cells were incubated with Tz-biotin for 6 h, followed by treatment with streptavidin-AF555 and Hoechst 33342 for 20 min and analyzed by confocal fluorescence microscopy. Scale bars: 20 μm.

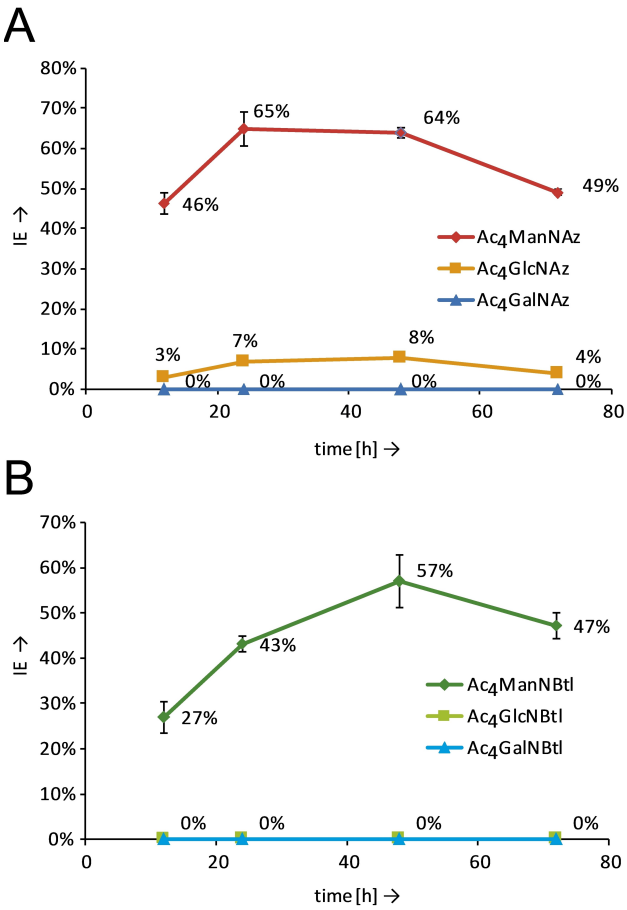


Figure 4. Time-dependency of the incorporation efficiency after MGE with various monosaccharide derivatives. HEK 293T cells were incubated with 100 μM A) Ac_4ManNAz, Ac_4GlcNAz, or Ac_4GalNAz or B) Ac_4ManNBtl, Ac_4GlcNBtl, or Ac_4GalNBtl for 12, 24, 48, or 72 h. The sialic acids were released with HOAc, treated with DMB, and quantified by RP-HPLC.

assume that the combination of reaction rate and metabolic incorporation of GlcNBtl and GalNBtl results in a staining intensity below the detection limit of fluorescence microscopy.

Are GlcNAc and GalNAc derivatives converted to modified sialic acids? Time dependency of the incorporation efficiency

To determine whether GlcNAc and GalNAc derivatives are converted to sialic acids and how long such a process takes, we followed the temporal course of the formation of any corresponding sialic acids and compared the determined IE values to the ones obtained when the cells were grown with the corresponding ManNAc derivative (Figure 4). For Ac_4ManNAz, an IE of 46% was determined after 12 h which increased to 65% after 24 h, remained constant (64%) until 48 h, before dropping to 49% after 72 h (Figure 4A). We suspected that the drop in IE results from the consumption of sugar in the cell medium. Accordingly, we replaced the cell medium every 24 h with fresh cell medium containing 100 μM Ac_4ManNAz and – as expected – the IE stayed constant once the maximum had been reached (Figure S2).

Next, we investigated MGE in the presence of Ac_4GlcNAz and could show its conversion to sialic acid with an IE that reaches a maximum of 8% after 48 h, before decreasing again to 4% after 72 h. This demonstrates that GlcNAz can enter the sialic acid biosynthesis pathway likely by the GlcNAc 2-epimerase RENBP and/or GNE accepting the modified sugar and transforming it to ManNAz which is then further transformed to the modified sialic acid. Therefore, the cell-surface staining with Ac_4GlcNAz is at least partly caused by Neu5Az. In cells treated with Ac_4GalNAz, no conversion to sialic acid could be measured. Obviously, the cell-surface staining after treatment with Ac_4GalNAz is not caused by sialic acids but presumably by incorporation of GalNAz into mucins.[21b]

For the butenoyl modified sugar derivative Ac_4ManNBtl we determined an IE of 27% after 12 h which increased to 43% after 24 h and to its maximum of 57% after 48 h, before dropping to 47% after 72 h (Figure 4 B). We did not observe any conversion of GlcNBtl or GalNBtl to the sialic acid Neu5Btl. From these experiments we conclude that the conversion of GlcNAc and GalNAc derivatives into the corresponding ManNAc derivative is strongly influenced by the modification of the *N*-

acetyl group. Thus, the azido derivative $Ac_4GlcNAz$ can be converted to the sialic acid Neu5Az, but the butenoyl derivative $Ac_4GlcNBtl$ cannot although the ManNAc derivatives with these modifications ($Ac_4ManNBtl$ and $Ac_4ManNAz$) show a similar incorporation efficiency after 48 h (64 and 57%, respectively). The Btl modification generally leads to a comparatively low staining of the cell surface, whereby GalNBtl and GlcNBtl are below the detection limit.

Influence of metabolic glycoengineering on sialic acid levels

IE values as defined above reflect only relative abundancies of sialic acids. Increased IE values could arise from both an expression of modified sugars in addition to the natural ones or from the partial replacement of natural sugars by the modified ones. Therefore, we next determined absolute amounts of sialic acids for a defined number of cells before and after MGE. Previously, it was shown that the addition of $Ac_4ManNAc$ leads to increased sialic acid levels of HeLA, Jurkat and PC 12 cells.[22] In the following experiments, we included $Ac_4ManNCp(H_2)$.

HEK 293T cells were treated with solutions of $Ac_4ManNBtl$, $Ac_4ManNAz$, $Ac_4ManNCp(H_2)$, or $Ac_4ManNAc$ as carried out in the previous experiments. To quantify sialic acid levels, we added an internal standard (Neu5Aloc) in a known concentration before DMB labeling which allowed the amount of sialic acids in the sample to be referenced. First, we treated the cells with $Ac_4ManNAc$ because it is often used as a control in MGE experiments. DMB labeling showed that the cells treated with this sugar have a strongly increased sialic acid level being 5.7 times the one of the solvent control (Figure 5A). Given in the figure are not only absolute sialic acid levels in terms of nmol per 400 000 cells (*y*-axis) but also relative levels (numbers in colored bars) referenced to the amount of Neu5Ac in the solvent control (0.46 nmol per 400 000 HEK 293T cells) which was set to 1. When feeding the unnatural sugar derivatives, the total sialic acid levels increased to more than twice the value of the solvent control. It is the level of the modified sugar that is mainly responsible for the increase. The IE values of $Ac_4ManNBtl$ and $Ac_4ManNAz$ are very similar 64 and 63%, respectively). Interestingly the IE of $Ac_4ManNCp(H_2)$ (81%) is significantly higher. This high IE value results from a decrease of the level of natural Neu5Ac to 0.4 compared to the solvent control, being the strongest reduction among the three ManNAc derivatives. A possible explanation could be that the cyclopropane modification is the smallest among the three derivatives and more efficiently competes with natural ManNAc.

To investigate whether the change in sialic acid level is dependent on the cell type, we next performed the same experiments with HeLa S3 cells (Figure 5 B). In general, the increase in sialic acid levels in HeLa S3 cells is significantly lower than in HEK 293T cells. HeLa cells treated with $Ac_4ManNAc$ showed an increase of the sialic acid level by a factor of 1.8. In case of treatment with $Ac_4ManNBtl$ and $Ac_4ManNAz$ no significant differences in the total sialic acid levels to the solvent control are observed. For these two sugars, the IE values were rather low (below 10%). Interestingly, the sialic acid level of

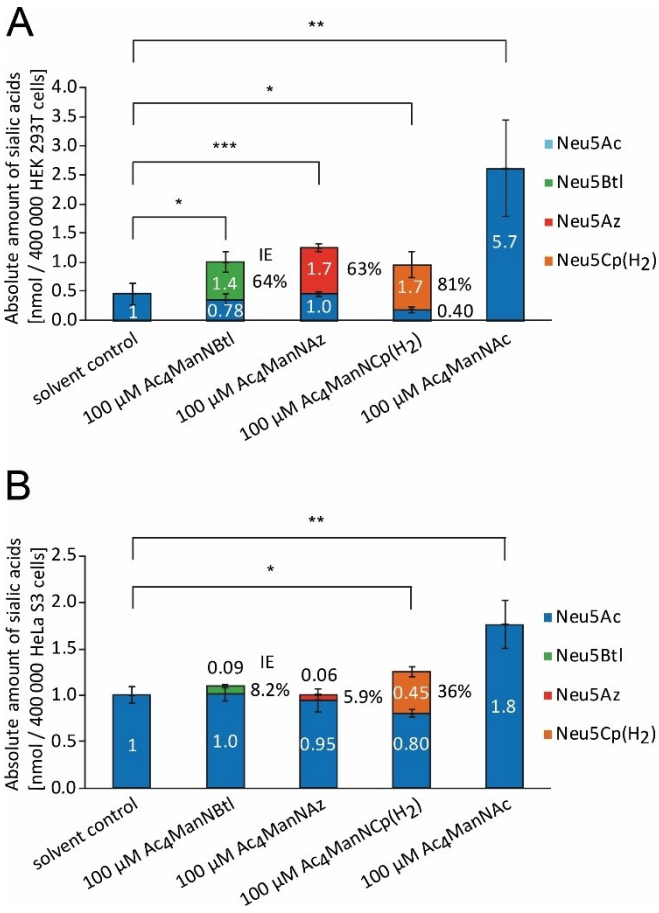


Figure 5. Absolute sialic acid levels of A) HEK 293T and B) HeLa S3 cells after MGE. In each experiment, 400 000 cells were incubated with 100 µM $Ac_4ManNBtl$, $Ac_4ManNAz$, $Ac_4ManNCp(H_2)$, or $Ac_4ManNAc$, or only with DMSO (solvent control), for 48 h. The sialic acids were released with HOAc, and after concentration a defined amount of Neu5Aloc was added as internal standard. Subsequently, the sialic acids were labeled with DMB and quantified by RP-HPLC. The numbers in the colored bars indicate sialic acid levels relative to the amount of Neu5Ac in the whole-cell lysate of the solvent control (0.46 nmol per 400 000 HEK 293T cells and 1.0 nmol per 400 000 HeLa S3 cells). The mean values of four independent cell experiments are shown. The standard deviation is given as an error bar, and the data were statistically analyzed with a t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

HeLa cells treated with $Ac_4ManNCp(H_2)$ is increased by the factor of 1.25 and the IE value is significantly higher (36%) than the one of the other two modified ManNAc derivatives.

The increase in sialic acid levels varies for different cell fractions

The experiments with HEK 293T cells have shown that whole cell sialic acid levels are significantly increased after MGE. We wondered if this increase is found all over the cell or specifically in certain cell fractions. Therefore, we developed a protocol for cell fractionation by modification of published protocols[22a,23] that allows the cell membrane fraction to be separated from the cell interior fraction including nucleosol and cytoplasm (cf.

Figure S3). Subsequently, we determined absolute sialic acid levels in both fractions by DMB labeling (Figure 6 A and B). The relative sialic acid levels given in Figure 6 as numbers on the colored bars are again referenced to the sialic acid level of the whole cell analysis of the solvent control shown in Figure 5A (0.46 nmol per 400 000 HEK 293T cells ≡ 1). In the solvent control, the amount of Neu5Ac in the membrane fraction and the cell interior fraction is the same. The sum of the relative Neu5Ac levels (0.56 for the cell membrane and 0.55 for the cell interior fraction) is 1.11 which nicely fits to the value of 1 of Figure 5A. This demonstrates that the cell fractionation protocol is effective and includes the whole material of the cell.

After MGE, we found only small changes of the sialic acid levels in the membrane fraction which are in the range of the measurement accuracy (Figure 6A). In contrast the sialic acid levels of the cell interior were significantly increased with all used sugar derivatives (Figure 6B). The most significant increase was observed for the cells that had been cultivated in the presence of Ac_4ManNAc leading to a Neu5Ac level that is 10 times higher compared to the solvent control. For Ac_4ManNBtl, Ac_4ManNAz, and Ac_4ManNCp(H_2), the sialic acid levels of the cell interior fraction are increased by factors between 2.9 and 4.5. In all cases, the IE in the cell interior fraction is higher than in the membrane fraction.

In the cell interior fraction, sialic acids could exist both in the form of the free sialic acids or as anomerically modified derivatives (e.g., CMP-activated or glycosidically linked). The DMB labeling procedure applied covers both forms because the acetic acid treatment converts the glycosidically modified derivatives into the corresponding free sialic acids.

To distinguish whether the measured sialic acid levels in the cell interior fraction originate from free sialic acids or glycosidically modified ones, we applied a reduction step[22a] before acid treatment and DMB labeling (Scheme 2). In this way, the open-chain α-keto acid is reduced to an α-hydroxy acid that does not react with DMB. Subsequent acid treatment to cleave glycosidic bonds of sialic acid and reaction with DMB results in selective labeling of glycosidically modified sialic acids.

The results of these experiments are shown in Figure 6C. In the solvent control, the reduction step did not lead to a decrease of the Neu5Ac level of the cell interior fraction determined by DMB labeling (relative level 0.6 vs. 0.55 in Figure 6B). This indicates that Neu5Ac is not present in its free form but mainly glycosidically modified. In contrast, for all cells that had been cultivated in the presence of added Ac_4ManNAc and derivatives thereof, the reduction step before DMB labeling resulted in a significant reduction of the measured sialic acid level. The most significant reduction by a factor of approximately 7 (5.5:0.77) is observed in the case of added Ac_4ManNAc. This means that the increased level of Neu5Ac in this case is mainly due to free Neu5Ac. Also, for the ManNAc derivatives Ac_4ManNBtl, Ac_4ManNAz, and Ac_4ManNCp(H_2), the increased sialic acid levels in the cell interior fraction are mainly due to the free sialic acids.

In all cases, addition of Ac_4ManNAc or its derivatives resulted in comparable levels of glycosidically modified sialic acids in the cell interior fraction (Figure 6C) with a slight

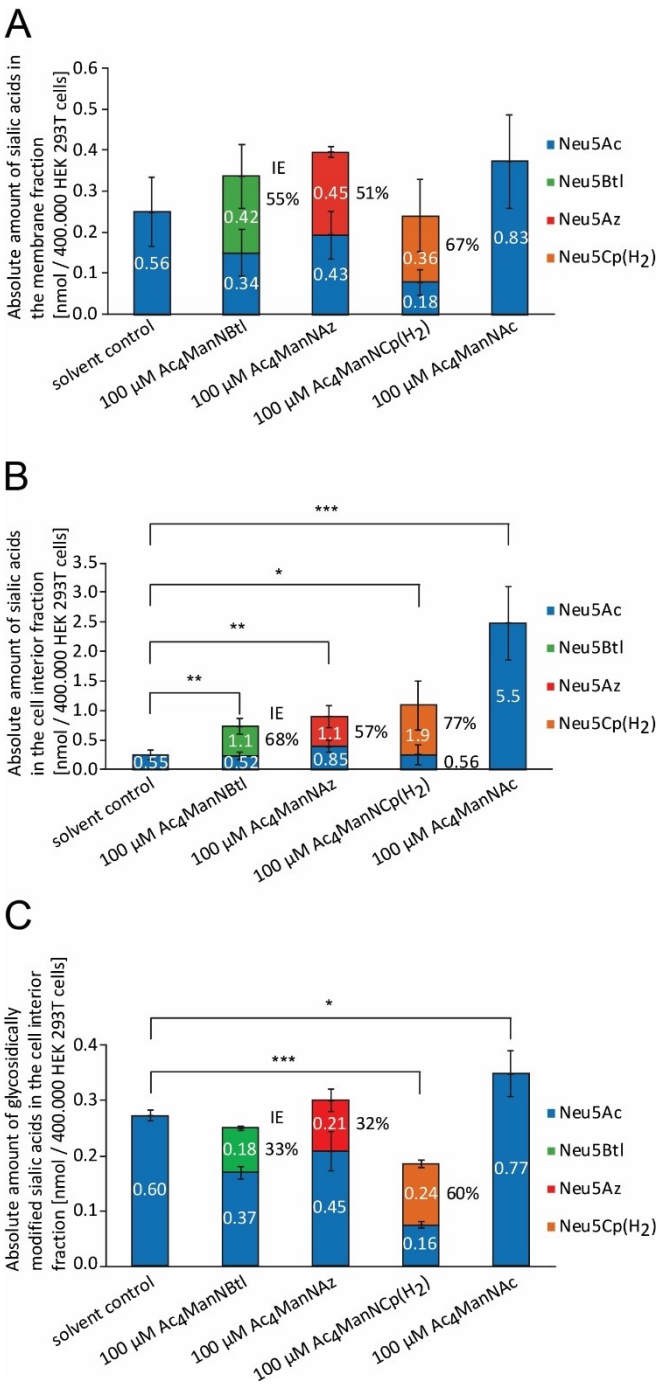
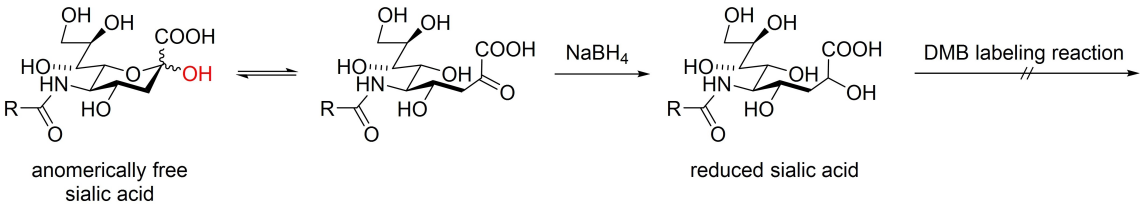


Figure 6. Absolute sialic acid levels in different cell fractions of HEK 293T cells. 400 000 cells were incubated with 100 µM Ac_4ManNBtl, Ac_4ManNAz, Ac_4ManNCp(H_2), or Ac_4ManNAc or only with DMSO (solvent control) for 48 h. The cells were fractionated into A) the cell interior and B) the cell membrane fractions, treated with HOAc to release sialic acids, and further analyzed as described in Figure 5. C) To determine the amount of glycosidically modified sialic acids in the cell interior fraction, a 0.2 M $NaBH_4$ solution (65 µL) was added to the concentrated cell interior fraction and incubated overnight at 4 °C. Excess $NaBH_4$ was quenched with concentrated TFA. Subsequently, the sample was treated with HOAc and further analyzed as described before. The numbers in the colored bars indicate sialic acid levels relative to the amount of Neu5Ac in the whole-cell lysate of the solvent control (0.46 nmol per 400 000 HEK 293T cells). The mean values of four independent cell experiments are shown. The standard deviation is given as an error bar, and the data were statistically analyzed with a t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



Scheme 2. $NaBH_4$ reduction of anomerically free sialic acid derivatives, which are in equilibrium with the open-chain keto form, results in the formation of α-hydroxy acids that do not react with DMB. In contrast, glycosidically modified sialic acids are fixed in the cyclic acetal form and cannot be reduced.

increase by a factor of 1.3 compared to the solvent control observed for Ac_4ManNAc. Only in the case of Ac_4ManNCp(H_2) addition, the level of glycosidically modified sialic acid is significantly lower.

Conclusion

MGE has proven to be a valuable technique to introduce chemical reporter groups into the glycome of a cell and thereby probe the functional roles of glycans. Prerequisite for such studies is the knowledge of how unnatural carbohydrates are metabolized by cells and how efficiently they are incorporated into glycans. For applications in which cell-surface sialic acids are used for drug targeting (e.g., to direct drugs to cancer cells),[4] it is furthermore of interest whether MGE causes an unnatural increase of sialic acid levels on cells. Our investigation included several ManNAc derivatives with different chemical reporter groups, and two GlcNAc and two GalNAc derivatives with an azidoacetyl and a butenoyl side chain, respectively.

We were able to show for all derivatives, which end up as sialic acid, that the maximal efficiency with which the modified sialic acid replaces natural Neu5Ac (i.e., the incorporation efficiency, IE) is achieved between 24 and 48 hours after addition of the monosaccharide derivative. The decrease of IE after that time is caused by consumption of the sugar within the medium which we could prove by replacing the medium with fresh sugar-containing medium every 24 h.

Furthermore, we could show that Ac_4GlcNAz, unlike Ac_4GlcNBtl, is converted to sialic acid, what causes at least part of the cell-surface staining. Ac_4GalNAz and Ac_4GalNBtl showed no conversion to the corresponding Neu5Ac derivative. This might be explained by the additional enzymatic steps that are required to convert the GalNAc derivatives to the corresponding GlcNAc derivatives, rendering this pathway inefficient.

When looking at absolute sialic acid levels, marked differences between HEK 293T and HeLa S3 cells were observed. Whereas all investigated sugars induced a strongly increased sialic acid biosynthesis in HEK 293T cells, the sialic acid levels of HeLa S3 were only increased with two monosaccharides, Ac_4ManNAc and Ac_4ManNCp(H_2). For the other sugars, Ac_4ManNBtl and Ac_4ManNAz, the total sialic acid levels did not change and IE values below 10% were determined from the whole-cell lysate. 0% cell surface incorporation of a diazirine-modified analog of sialic acid has been observed earlier with HeLa cells.[22b] Thus it is remarkable that Ac_4ManNCp(H_2) results in an IE of 36%.

As with HeLa S3 cells, also with HEK 293T cells the most significant increase of sialic acid levels (in this case by a factor of 5.7) resulted from addition of peracetylated natural mannosamine Ac_4ManNAc. Obviously, the natural precursor of Neu5Ac is well accepted by the cells and converted to sialic acid. Addition of the butenoyl- and azide-modified mannosamine analogs increased the total sialic acid level merely by additional formation of the modified sialic acids with only a slight (Ac_4ManNBtl) or no (Ac_4ManNAz) reduction of the level of natural Neu5Ac. Again, Ac_4ManNCp(H_2) resulted in the highest IE of 81% and the most pronounced reduction of natural Neu5Ac to a level of 40% of the solvent control making the corresponding cyclopropene derivative (Ac_4ManNCp) a promising candidate for high IE values in both cell lines.

Cell fractionation experiments uncovered that the cell interior fraction has the largest contribution to the observed increase in sialic acid levels whereas sialic acid levels in the membrane fraction are only moderately increased with the largest effect by a factor of 1.57 observed for Ac_4ManNAz. Ac_4ManNCp(H_2) leads to the largest IE within the membrane fraction (67%) with the total sialic level identical to that of the solvent control. By performing a reduction step before DMB labeling, we could show that the increase of sialic acid levels observed in the cell interior fraction is mostly caused by free sialic acids. This hints to a regulation of sialoglycoconjugate formation at a very early point of the biosynthesis pathway. To conclude, our investigation provides further insight into carbohydrate metabolism during metabolic glycoengineering and is of interest for various applications of MGE.

Experimental Section

General methods: Reagents were purchased from Sigma-Aldrich, Carbosynth, Acros Organics, ABCR, Roth, and New England Biolabs and used without further purification. 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride was purchased from Apollo Scientific (MFCD00037497), Ponceau S from Sigma-Aldrich (P7170), protein ladder from Thermo Fisher Scientific (26617), Neu5Az from Chemily Glycoscience (MP031), Trypsin from Gibco (TrypLe™ Express), and antibodies from Sigma-Aldrich (anti-rabbit, A0545; anti-GAPDH, G9545; anti-T-Cadherin, PRS3583; anti-RANBP3, SAB2101944). NMR spectra were recorded at room temperature on a 400 Avance III instrument from Bruker. Chemical shifts are reported relative to solvent signals ([D_6]DMSO: $\delta_H = 2.50$ ppm, $\delta_C =$

39.4 ppm; CD_3OD: $\delta_H = 3.31$ ppm, $\delta_c = 49.1$ ppm; D_2O: $\delta_H = 4.79$ ppm). Signals were assigned by two-dimensional NMR experiments (DQF-COSY, HSQC, and HMBC). Analytical RP-HPLC-MS was performed on a LCMS2020 Prominence system (high-pressure pumps LC-20 AD, auto sampler SIL-20AT HAS, column oven CTO-20AC, UV/Vis detector SPD-20A, fluorescence detector RF-20A, controller CBM-20, ESI detector, LCMS Software Solution) from Shimadzu under the following conditions. Mobile phase: gradient of acetonitrile with 0.1% formic acid (solvent B) in water with 0.1% formic acid (solvent A). Used columns: Nucleodur C18 Gravity, 3 μm, 125×4 mm from Macherey-Nagel, flow: 0,4 mL min^{-1}; Kinetex C18, 2.6 μm, 100 Å, 150×4.6 mm from Phenomenex, flow: 0.4 mL min^{-1}.

Preparation of reference compounds for DMB labeling experiments: As described earlier,[10b] the corresponding neuraminic acid derivative (Neu5Ac, Neu5Az, Neu5Aloc, Neu5Btl, or Neu5Cp(H_2); 0.032 mmol; see Figure S4, for formulae and sources) was dissolved in DMB solution (265 μL; 5.3 mM DMB·2 HCl, 16 mM $Na_2S_2O_4$, 40 mM TFA in Milli-Q water, 1 M β-mercaptoethanol). The mixture was incubated for 2.5 h at 56 °C in a thermomixer (300 rpm) in the dark. After incubation, the mixture was cooled on ice for 10 min and neutralized with aqueous NaOH (0.5 M, 21 μL). The solution was analyzed by analytical RP-HPLC-MS measurements. For fluorescence detection ($\lambda_{ex} = 372$ nm, $\lambda_{em} = 456$ nm), dilution (1:400) was necessary.

DMB-Neu5Ac: Analytical RP-HPLC (10–20% B over 40 min): t_R = 10.5 min (Kinetex C18); (10–25% B over 40 min): t_R = 12.2 min (Nucleodur C18 Gravity).

DMB-Neu5Az:[20d] Analytical RP-HPLC (10–20% B over 40 min): t_R = 5.1 min (Kinetex C18); (10–25% B over 40 min): t_R = 4.8 min (Nucleodur C18 Gravity).

DMB-Neu5Aloc:[10b] Analytical RP-HPLC (10–20% B over 40 min): t_R = 29.5 min (Kinetex C18).

DMB-Neu5Btl:[10b] Analytical RP-HPLC (10–20% B over 40 min): t_R = 21.5 min (Kinetex C18); (10–25% B over 40 min): t_R = 26.9 min (Nucleodur C18 Gravity).

DMB-Neu5Cp(H_2):[19] Analytical RP-HPLC (10–20% B over 40 min): t_R = 19.2 min (Kinetex C18).

Cell growth conditions: Cells were cultivated in cell culture medium (Dulbecco's modified Eagle's medium, DMEM, supplemented with 5 v/% fetal calf serum, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin) at 37 °C and 5% CO_2 atmosphere. All experiments were performed with living cells, which were 80 to 90% confluence. To achieve this, the medium was removed every third day, the cells were washed with phosphate-buffered saline (PBS), and then treated with 1–2 mL trypsin solution and incubated for 5 min. Then fresh cell medium (10 mL) was added, mixed and 9 mL of it were removed. The remaining cells were again supplied with fresh medium.

MGE experiments: HEK293T cells or HeLa S3 cells were seeded (450 000 cells in a 5 cm dish) and after 24 h the medium was exchanged with 4 mL DMEM containing labeled sugar. The sugars were prepared as stock solutions (100 mM) in DMSO and stored at −20 °C. They were freshly diluted into medium on the day of the experiment in the according concentrations. As a solvent control, the same amount of DMSO was added. Cells were trypsinated and resuspended in medium (5 mL) and pelleted by centrifugation (5 min, 500 *g*). The supernatant was discarded, and the pellet was washed with PBS (3×5 mL).

For time-resolved experiments, 800 000 HEK 293T cells were seeded in a 9 cm dish and incubated with 14 mL medium (DMEM) for 24 h. Afterwards the cells were incubated with 14 mL medium containing 100 μM sugar for 12, 24, 48, or 72 h. Cells were trypsinated and resuspended in medium (5 mL) and pelleted by centrifugation (5 min, 500 *g*). The supernatant was discarded, and the pellet was washed with PBS (3×5 mL). Cells were transferred to Eppendorf tubes.

Fluorescence microscopy: HEK 293T cells (15 000 cells cm^{-2}) were seeded in four-well ibiTreat μ-Slides (ibidi) Ph+ coated with and poly-L-lysine (0.0025%, 1 h, 37 °C) and allowed to attach for 24 h. Subsequently, cells were incubated with 50 μM or 100 μM sugar solution in DMEM for 48 h. As a solvent control, the same amount of DMSO was added. The cells were washed twice with PBS and in case of the DA_{inv} reaction incubated with Tz-biotin (1 mM) for 6 h. Excess Tz-biotin was removed by three washing steps with PBS. A solution of streptavidin-AF555 (6.6 μg mL^{-1}) and Hoechst 33342 (10 μg mL^{-1}, for nuclei staining) was added and incubated at 37 °C for 20 min. In case of the SPAAC reaction, cells were incubated with a solution of DIBO-AF488 (50 μM) and Hoechst 33342 (10 μg mL^{-1}) for 30 min at 37 °C in dark. Finally, in both cases the cells were washed again three times with PBS, and DMEM was added for fluorescence microscopy. Confocal fluorescence microscopy was performed with a Zeiss LSM 880 instrument.

Cell fractionation: After MGE, 400 000 HEK 293T cells were used to determine the total sialic acid level and incorporation efficiency. Lysis buffer [400 μL; Tris·HCl (10 mM) pH 7.9, NaCl (150 mM), EDTA (5 mM), PMSF (1 mM), aprotinin (0.462 μM), leupeptin (3 μM) and pepstatin A (4.36 μM)] was added to the cell pellet. The mixture was then ultrasonicated (20% intensity) 3 times for 30 s and cooled on ice for 1 min between each step. The suspension was centrifuged for 10 min at 4 °C with 21130 *g*. The supernatant was decanted and centrifuged again for 60 min at 4 °C with 21130 *g*. The united pellets correspond to the membrane fraction and the combined supernatant to the cell interior fraction (including cytoplasm and nucleosol). Lysis buffer (30 μL) was added to the membrane fraction and the suspension centrifuged for 60 min at 4 °C and 21130 *g*. The supernatant was decanted and added to the cell interior fraction. Fractions were evaporated using a SpeedVac vacuum concentrator.

Reduction step: Cell fractions were incubated with a freshly prepared 0.2 M $NaBH_4$ solution (pH 8.0, 65 μL) overnight at 4 °C to reduce the free sialic acids. After the reduction step, the excess $NaBH_4$ was quenched with concentrated TFA (until the end of gas formation) and the mixture concentrated using a SpeedVac vacuum concentrator.

DMB labeling of sialic acids: Treated cells (with or without cell fractionation and reduction step) were incubated with 3 M acetic acid (300 μL) at 80 °C and 300 rpm for 90 min. The samples were then cooled on ice for 10 min. The solution was diluted with Milli-Q water (400 μL) and neutralized with NH_3 (25%, in water, 20 μL). The solvent was removed in a SpeedVac vacuum concentrator, and the pellet was washed three times with EtOH (200 μL) and concentrated after every step. Then the pellet was solved in DMB-labeling solution [DMB·2 HCl (5.3 mM), $Na_2S_2O_4$ (16 mM), TFA (40 mM) in Milli-Q water, 265 μL]. For experiments in which the total sialic acid level was to be determined, Neu5Aloc stock solution (0.0028 mg mL^{-1}, 35 μL) was added as an internal standard. The mixture was incubated at 56 °C and 300 rpm for 2.5 h in a thermomixer in darkness. Then, the mixture was cooled on ice for 10 min, neutralized with NaOH solution (0.5 M, 25 μL) and analyzed by analytical RP-HPLC-MS with fluorescence detection.

SDS-PAGE and western blotting: Cell fractions were diluted with 60 μL Milli-Q Water and 20 μL 4x SDS buffer [100 mM Tris pH 6.8, 30 v/% glycerol, 0.21 M SDS, 2.84 M β-mercaptoethanol, 0.29 mM

Bromophenol Blue]. The solution was heated to 98 °C for 10 min. Acrylamide SDS gels (10 w/%) were prepared using the Mini-Protean tetra cell equipment from Bio-Rad. After loading the gels with the previously prepared samples, the gel was run with 0.05 A for approximately 60 min in running buffer [25 mM Tris pH 8.3, 192 mM glycerol, 3.45 mM SDS], until Bromophenol Blue and excess fluorescence dyes left the gel. For the western blot, a nitrocellulose membrane was placed on a filter and the gel was placed on top. The gel was also fixed with a filter and placed in a gel holder. The chamber was filled with ice-cold western blot running buffer [25 mM Tris pH 8.3, 192 mM glycerol, 20 v/% methanol] and in addition a cool pack and a stir fish were added. Blotting was performed at 120 V for 60 min under stirring. For immunoblotting the blot was blocked in 5 w/% milk powder in PBS–T [0.5 v/% Tween-20 in 1× PBS] by shaking for 1 h. The membrane was incubated with the 1st antibody (anti-GAPDH (1:1000), anti-T-Cadherin (1:500) or anti-RANBP3 (1:250)), at 4 °C overnight. The membrane was then washed 3 times with PBS-T by shaking for 15 min. The second antibody (anti-rabbit) was diluted in 5 w/% milk and incubated for 1 h by shaking at room temperature. ECL substrates were combined (500 μL each) and pipetted on the top of the membrane. Chemiluminescence was read out using the ChemiDocTMTouch Imaging System from Bio-Rad.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SPP 1623, SFB 969 project B05), the Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg, the University of Konstanz, and the Konstanz Research School Chemical Biology. We thank Nawid Albinger and Tjalda Falt for support with MGE experiments, the Bioimaging Center of the University of Konstanz for providing the fluorescence microscopy instrumentation, and the NMR Core Facility of the University of Konstanz for providing the NMR instrumentation. Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioorthogonal chemistry · carbohydrates · DMB labeling · metabolic engineering · sialic acids

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Manuscript received: October 14, 2020
Revised manuscript received: November 10, 2020
Accepted manuscript online: November 12, 2020
Version of record online: December 17, 2020