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Technical Note

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Laser Microdissection of Paraffin Embedded Tissue as a Tool to Estimate the Sialylation Status of Selected Cell Populations

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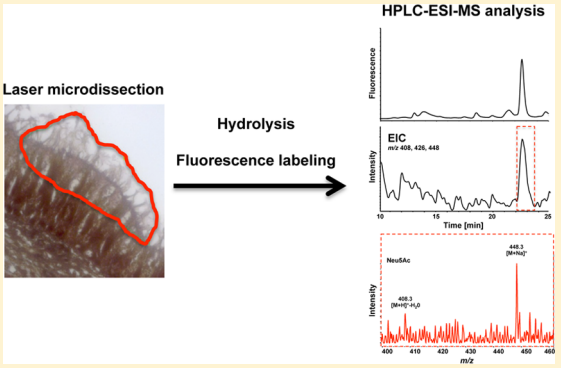
Supporting Information

ABSTRACT: In vertebrates, sialic acids occur at the terminal end of glycans mediating numerous biological processes like cell differentiation or tumor metastasis. Consequently, the cellular sialylation status under healthy and pathological conditions is of high interest. Existing analytical strategies to determine sialylation patterns are mostly applied to tissue samples consisting of a mixture of different cell types. Alterations in the sialylation status in a distinct area of tissues or in a specific cell population may, therefore, be easily overlooked. Likewise, estimated variations in sialylation in tissue homogenates might be simply the result of a changed cell composition. To overcome these limitations, we employed laser microdissection to isolate defined cell types or functional subunits and cell populations of paraffin embedded specimens which represent the most abundant supply of human tissue associated with clinical records. For qualitative and quantitative estimation of the sialylation status, sialic acids were released, fluorescently labeled, and analyzed by an online high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) system. As a proof of principle, this strategy was successfully applied to characterize the sialylation of the apical region of epididymal epithelial cells. Furthermore, it was possible to detect an impaired sialylation during kidney maturation in a transgenic mouse model, which was restricted to glomeruli, whereas no differences in sialylation were observed when whole kidney homogenates were used. Thus, starting from paraffin embedded tissue samples, the outlined approach offers a sensitive method to detect and quantify sialic acids on defined cell populations, which may be useful to explore novel sialic acid dependent roles during physiological and pathological processes.



Sialic acid residues are frequently present at the outermost position (nonreducing end) of eukaryotic glycoconjugates.[1,2] This family of 9-carbon atoms containing α-keto acids consists of more than 50 members among which *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) represent the most abundant species in mammals.[3] Sialic acids are essential for vertebrate development, and their biological role has been demonstrated in a large number of studies.[1−6] A recent finding, e.g., is the demonstration that correct sialylation of nephrin is essential for the formation of the glomerular filtration barrier during kidney development.[7]

In addition, sialic acids have been implicated in pathological events. Several cancer cells were reported to increase their sialylation status, thus supporting cancer metastasis.[8−12] Another example is the incorporation of Neu5Gc from red meat and milk products into human tissues. As humans have lost the enzyme responsible for Neu5Gc synthesis,[13] antibodies are generated against Neu5Gc containing epitopes and have been connected with chronic inflammation.[5] All these examples highlight the need of diagnostic tools that are able to determine the cellular sialylation status at qualitative and quantitative level. However, an in depth characterization of the sialylation status of specific cell types is very often impossible in tissue samples since organs usually comprise a heterogeneous cell composition. A tool to isolate distinct types of cells from paraffin embedded tissue sections is laser microdissection, which is a frequently used method for cell specific proteomics and mRNA analysis.[14−20]

In the present study, we conducted experiments to evaluate a combination of laser microdissection of paraffin embedded tissue sections and high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis of fluorescently labeled sialic acids to analyze the sialylation patterns of selected cell populations. Labeling of sialic acids was achieved using the α-keto acid specific 4,5-methylene-dioxybenzene (DMB) reagent because analysis of such compounds by HPLC (DMB-HPLC analysis) is very

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robust and, besides GC/MS applications and a very recently optimized 2-aminoacridone (AMAC)-capillary electrophoresis approach, the most sensitive strategy (20 fmol) to analyze sialic acids including *O*-acetylated and sulfated species.[21−26] The combination of liquid-chromatography (LC) with electrospray ionization mass spectrometry (ESI-MS) allows, in addition, an unambiguous identification of the analyzed sialic acid residues and the localization of potential substituents.[27−29] Using the outlined strategy, we were able to examine changes in the sialylation status of selected cell populations, which would have been impossible using tissue lysates of complete organs.

EXPERIMENTAL SECTION

Materials. The monosaccharide standards Neu5Ac, Neu5Gc, and KDN were purchased from Sigma-Aldrich (Taufkirchen, Germany). All reagents used were of analytical grade. Doubly distilled water was used throughout the experiments.

Laser Microdissection. Murine epididymis, lung, and kidney tissue was fixed, dehydrated, and embedded in paraffin as previously described.[7,30,31] Paraffin embedded tissue was sliced (4 μm) with a rotary microtome RM2245 (Leica, Wetzlar, Germany) and mounted on membrane slides (Zeiss, Munich, Germany). Resultant tissue slides were deparaffinated with xylene or RotiClear (Carl Roth, Karlsruhe, Germany) and rehydrated in absolute ethanol, 70% ethanol, and water. Rehydrated sections were stained with Mayer's hematoxylin for 30−40 s and rinsed again with water. After dehydration in 70% ethanol followed by 100% ethanol, sections were air-dried.

Cell populations were dissected with a PalmRobo system (Zeiss, Munich, Germany). Tissue areas were marked using 20−40× objectives and dissected in Robo-LPC-mode. Dissected tissue was collected in *Adhesive Caps* (Zeiss, Munich, Germany) (Figure S1A, Supporting Information). The number of isolated cells was always calculated by the nucleus staining with Mayer's hematoxylin.

DMB-Labeling of Sialic Acids. For the release of sialic acids, the cap containing the dissected tissue was cut and transferred into a 2 mL Eppendorf cup (safe lock) (Figure S1B, Supporting Information). Samples were hydrolyzed in 500 μL of 2 N acetic acid for 90 min at 80 °C, transferred into glass vials, and dried. For DMB-labeling, hydrolysates were dissolved in 80 μL of DMB-reaction buffer (9 mM sodium hydrosulfite, 1 M β-mercaptoethanol, 20 mM trifluoroacetic acid (TFA), and 2.7 mM DMB (Dojindo, Kumamoto, Japan)) and incubated for 2 h at 55 °C as described previously.[32,33] Reactions were stopped by adding 20 μL of 0.2 M NaOH. For quantification of Neu5Gc and Neu5Ac, a four-point calibration was used (Figure S2, Supporting Information). Since the yield of DMB-labeling slightly differs between Neu5Gc and Neu5Ac, two independent calibration curves were generated.

Online DMB-HPLC-ESI-MS. DMB-labeled samples were analyzed on a Superspher 100 C-18e-RP(reversed-phase) column (250 × 4 mm, Merck-Hitachi, Darmstadt, Germany) at 40 °C using an Ultimate LC system, which was directly coupled with an Esquire 3000 ESI-ion trap (IT)-MS (Bruker Daltonik).[34] Mobile phases of acetonitrile/water/formic acid (5:95:0.1) and acetonitrile/methanol/water/formic acid (45:45:10:0.1) (M2) were used for separation. A linear gradient was applied from 0% to 15% M2 in 30 min at a flow rate of 200 μL/min. Typical ESI source conditions were: spray voltage 1.4 kV, capillary temperature of 250 °C, end plate offset of −500 V, and capillary exit of 140 V.

RESULTS AND DISCUSSION

Combination of Laser Microdissection and DMB-HPLC-ESI-MS Analysis of Paraffin Embedded Tissue. The quantification of sialic acids using DMB as an α-keto acid specific reagent was already established twenty years ago.[21] Until now, this application represents one of the most powerful methods to study the sialylation status of tissues, because the method (1) requires only femtomolar amounts of material, (2) needs no sample purification, and thus (3) represents a high-throughput method, which (4) can be combined with MS-techniques. So far, this technique was mainly applied to tissue homogenates consisting of a mixture of different cell types. With the aim to investigate the sialylation status of specific cell populations in a given tissue, the preservation of the tissue morphology by methods that do not break down sialo-glycans is of outmost importance. A way to reach this goal is the release of distinct cell types via laser microdissection from paraffin embedded tissue sections.

In a pilot study, the possibility to combine laser microdissection with DMB-HPLC-ESI-MS sialic acid analysis was investigated with paraffin embedded epididymidis from mice. First, tissue slices were mounted on membrane slides and after deparaffination and rehydration were air-dried. As shown in Figure 1A, exclusively, the apical regions of approximately 290 epithelial cells were isolated in this way. The accurate binding of the dissected cells to the *Adhesive Caps* was controlled after each cutting step, to avoid the risk that excised cells did not bind to or reach the *Adhesive Cap* during the catapult procedure (Figure S1A, Supporting Information).

Subsequently, the adhesive cap was cut and transferred into a 2 mL Eppendorf cup (safe lock) (Figure S1B, Supporting Information) to release sialic acids under acidic conditions. Subsequently, fluorescence-labeling was performed and resulting DMB-labeled sialic acids were separated by reversed phase HPLC leading to a fluorescent signal at the predicted retention time of DMB-Neu5Ac (Figure 1B). The unambiguous assignment of this compound could always be achieved by monitoring one or more of the monoisotopic pseudomolecular masses of DMB-Neu5Ac at m/z 408 ($[M + H]^+ - H_2O$), 426 ($[M + H]^+$), and 448 ($[M + Na]^+$) in the extracted ion chromatogram (EIC) mode (Figure 1C) in agreement with the ESI-MS spectrum recorded during the elution time of DMB-Neu5Ac (Figure 1D). In the selected example, no signal at m/z 426 occurred. In contrast to its dehydrated form at m/z 408, the monoisotopic pseudomolecular mass $[M + H]^+$ at m/z 426 was only detectable if more than 0.01 nmol DMB-Neu5Ac was applied. Additional experiments using a Neu5Ac standard demonstrated that only sodium adducts are stable during ESI-MS analysis (Figure S3, Supporting Information). If necessary, additional fragmentation analyses can be performed for identification of distinct sialic acid species as shown previously.[27−29,34]

Thus, a combination of laser microdissection from paraffin-embedded tissue and DMB-HPLC-ESI-MS analysis was suitable for the qualitative analysis of sialic acids in a distinct region of less than 300 epithelial cells.

Analysis of Alveolar Areas to Test the Reproducibility of the Protocol Used. In order to address the question as to whether the described strategy is also useful for a quantitative comparison, its reproducibility was tested. To this end, alveolar regions were isolated from paraffin embedded mouse lung (Figure 2A). The number of collected cells was calculated by

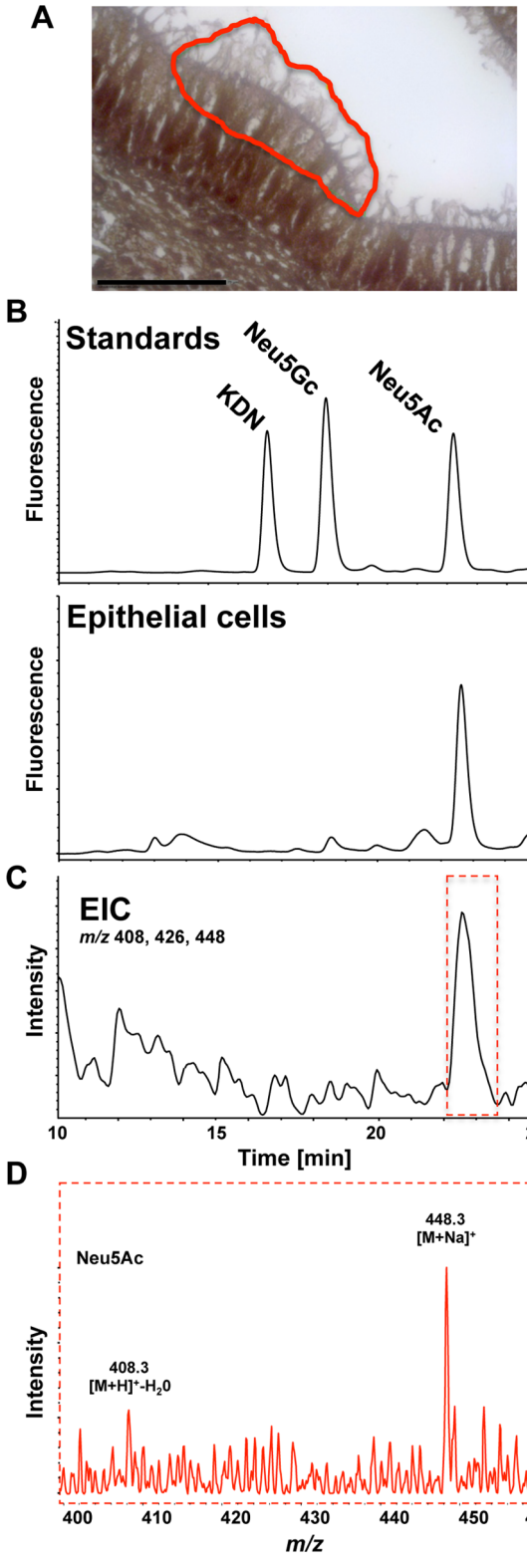

Figure 1. Determination of the sialylation status of murine epididymal epithelial cells. (A) Paraffin embedded mouse epididymidis was used for the isolation of the apical region of epithelial cells by laser microdissection (red circle). Scale bar = 50 μm. (B) Following hydrolysis and fluorescent labeling, DMB-sialic acid residues were separated by RP-HPLC. (C) Extracted ion chromatogram (EIC) was generated using the most prominent DMB-Neu5Ac adducts at m/z 408, 426, and 448. (D) ESI-MS spectrum was recorded during the elution time of DMB-Neu5Ac.

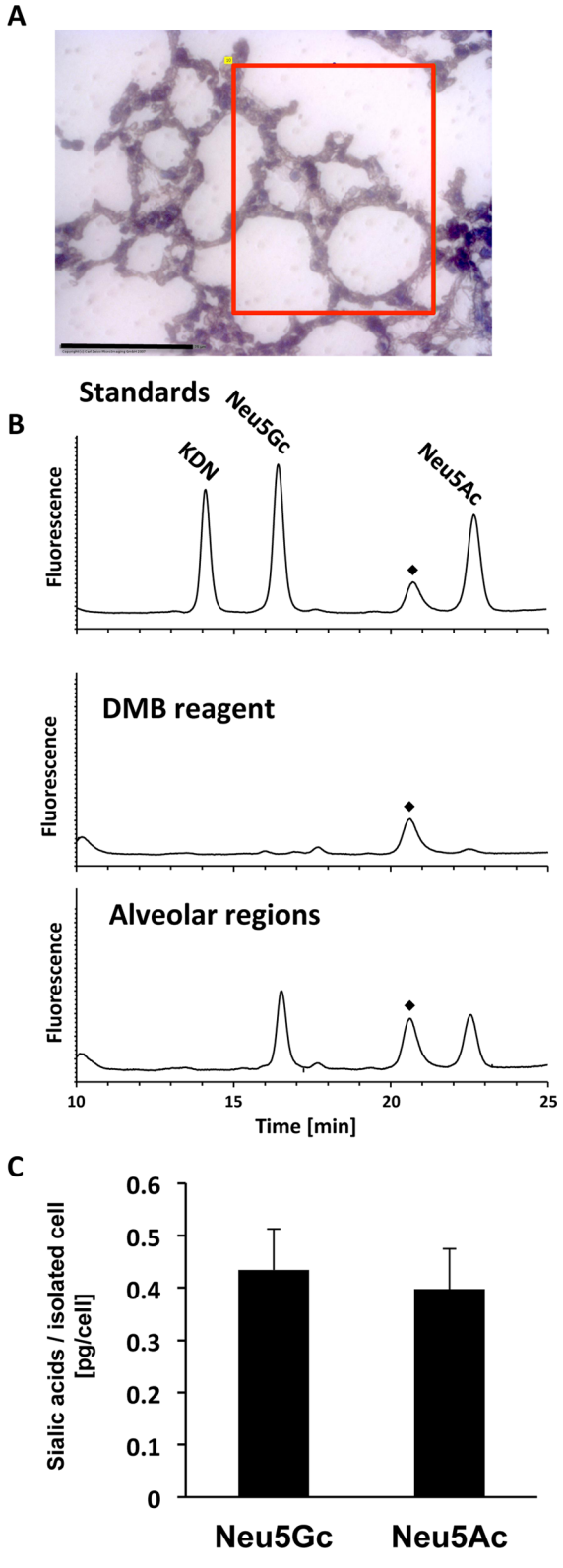

Figure 2. Estimation of the sialic acid content of alveolar regions. (A) To isolate alveolar areas, paraffin embedded mouse lungs were subjected to laser microdissection (red box). Scale bar = 75 μm. (B) DMB-labeled sialic acids were separated by RP-HPLC. (C) The amounts of Neu5Gc and Neu5Ac and the respective standard deviations (S.D.) of five independent experiments were calculated. Diamonds, DMB-impurity: In comparison to Figure 1, a different batch of DMB has been used.

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nucleus staining. Starting from approximately 300 cells, recorded DMB-HPLC-chromatograms displayed 2 strong peaks representing Neu5Gc and Neu5Ac among which the signal intensity of Neu5Gc was slightly more pronounced (Figure 2B and Table S1, Supporting Information). The comparison of the calculated values of five independent experiments demonstrated that the outlined workflow is reproducible (Figure 2C). The calculated relative standard deviations (%RSD) of Neu5Gc (17%) and Neu5Ac (16%) represent the sum of deviation, which occurred during the cutting process (microtome), the laser dissection, and the hydrolysis as well as the DMB labeling, the injection system, and the measuring unit of the HPLC in addition to the general biological variability of the sialic acid contents of the analyzed cells. The %RSD values are comparable with data obtained for complete organs (%RSD > 10%).[7] In addition to the high coefficient of determination of the calibration curves (R^2 > 0.999) (see Figure S2, Supporting Information), the obtained results let one assume that the calculated %RSD mainly originates from biological variability rather than from the complex workflow.

Nevertheless, three critical steps have to be especially considered: (1) the accurate definition of the dissected area and (2) the transfer of the tissue slice to the *Adhesive Cap* (Figure S1A, Supporting Information). As outlined above, it is absolutely essential to control the recovery of all tissue areas after each dissection process. (3) Hydrolysis and fluorescence labeling: the snipped *Adhesive Caps* have to be completely surrounded by acetic acid to ensure complete hydrolysis (Figure S1B, Supporting Information). In conclusion, our results demonstrate that the described combination of laser microdissection and DMB-HPLC-ESI-MS analysis is a reproducible approach and is also suitable for quantitative studies.

Comparative Analysis of Wild-Type and Transgenic Mice with an Impaired Sialylation of Nephrin and Podocalyxin. In order to illustrate the power of the newly developed procedure over previous analyses of complete organ homogenates,[7] we reinvestigated kidney samples isolated from wild-type mice and from mice with a defect in the expression of CMP-sialic acid synthetase (CMAS). CMAS is the enzyme which catalyzes the activation of sialic acid to CMP-sialic acid in the nucleus.[35] CMP-activation of sialic acid is essential for sialylation since only the activated monosaccharide can be transported into the Golgi-apparatus and transferred onto nascent glycoconjugates. Mice expressing the so-called $CMAS^{nls}$ point mutation die from kidney failure caused by an impaired podocyte maturation.[7] Intriguingly, Western blot analyses demonstrated that two major podocyte proteins, nephrin and podocalyxin, are hyposialylated in $CMAS^{nls}$ mutant mice, whereas the sialylation of other glycoproteins (e.g., β-Integrin) was not influenced.[7] Trials to display the impaired sialylation by DMB-HPLC analysis in total kidney homogenates was unsuccessful.[7]

In order to find out whether the observed hyposialylation of nephrin and podocalyxin could be monitored by use of the current workflow, we isolated glomeruli of wild-type and mutant mice by laser microdissection (Figure 3A). As shown in Figure 3B, the achieved chromatographic profiles of DMB-HPLC analysis visualized a sharp peak for DMB-Neu5Ac in mutant as well as in wild-type mice. The comparison of the respective peak areas, however, showed that the Neu5Ac level in glomeruli of mutants was significantly reduced (Figure 3C

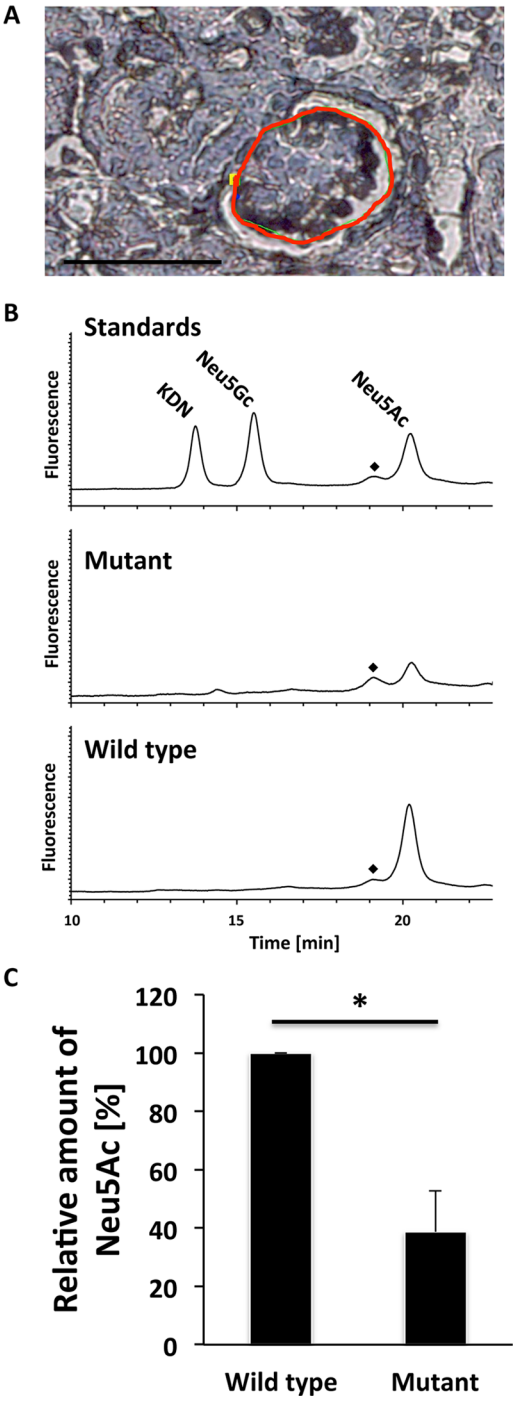

Figure 3. Analysis of the sialylation status of glomeruli in wild-type and $CMAS^{nls}$ mutant mice. (A) Paraffin embedded kidneys of wild-type and mutant mice were used for laser microdissection to isolate glomeruli (red circle). Scale bar = 50 μm. (B) Isolated cells (approximately 400 cells) were subjected to DMB-labeling after hydrolysis. Resulting DMB-sialic acid residues were separated by RP-HPLC. Diamonds, DMB-impurity: In comparison to Figure 1, a different batch of DMB has been used. (C) The amounts of Neu5Ac were compared between wild-type and mutant mice. Given mean values are based on 3 independent analyses of 3 wild-type donors as well as 3 mutants and were set to 100% for wild-type mice. The statistical evaluation was performed by Student's t test (unequal variances, two tailed). Significance levels are classified as follows: *, p < 5%.

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and Table S2, Supporting Information). In contrast to recent data obtained for whole kidney lysates,[7] a signal corresponding to DMB-Neu5Gc was not observed in the case of excised glomeruli. Thus, the specific analysis of the sialylation status of individual glomeruli verified the described hyposialylation in CMAS mutants and confirms the high value of our new method.

CONCLUSIONS

Up to date, analyses regarding the presence and quantities of different sialic acid species were mostly restricted to complete or bigger compartments of organs comprising a mixture of different cell types. Thus, the exact sialylation status of a defined area within an organ and/or of a distinct cell type could not be determined. The methodical approach established in this study provides a highly sensitive and reproducible way to solve this problem, because laser microdissection now allows analysis of well-defined cell populations. Moreover, online DMB-HPLC-ESI-MS provides a useful tool for the unambiguous identification and quantification of sialic acids including *O*-acetylated and sulfated species.[21−26] For the detection of the most abundant sialic acid species like Neu5Ac, less than 300 cells are necessary per analysis. However, in the case of sialic acid species existing at exceedingly low quantities, significantly more cells have to be isolated depending on the concentration of the sialic acid residues of interest.

The described combination of laser microdissection of paraffin embedded tissue with the robust as well as highly sensitive DMB-HPLC-ESI-MS approach could be further used to analyze human tissue samples associated with clinical records (e.g., biopsies) since such samples are prevalently available as paraffin embedded tissue and a lot of pathological processes like metastasis are discussed to come along with changed sialic acid levels.[8−12] Thus, the outlined strategy may allow the discovery of novel sialic acid dependent mechanisms during pathological events and possibly also the identification of sialic acids as corresponding biomarkers.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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