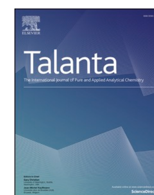
Talanta 242 (2022) 123326

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



DMTMM-mediated methylamidation for MALDI mass spectrometry analysis of N-glycans with structurally conserved sialic acid residues in biological fluids "via direttissima"

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ARTICLE INFO

Keywords:
N-Glycan
MALDI
DMTMM
Biomarker
Sialic acid

ABSTRACT

Characterization of serum glycoprotein N-glycans with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in positive-ion mode needs a derivatization step to stabilize and neutralize the negative charge on sialic acids. The acidic sugars are attached to the end of glycoproteins, glycolipids or gangliosides. Here, we present a method for sialic acid stabilization via modification based on derivatization of carboxylic acid group activated with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) with methylamine. DMTMM substitutes in many processes N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS) as activation reagent due to its better performance and higher stability in water. Glycosylated proteins are used as solid phase support for glycan derivatization and purification from excess of derivatization reagents. We evaluated our glycan analysis method in murine sera and intestinal lavages. The stabilization of sialic acid enables a complete conservation of the glycan structures, in contrast to other methods where sialic acids are partially lost. In BALB/c mouse sera, we detected predominantly mono- and di-sialylated N-glycans with mostly N-Glycolylneuraminic acid (Neu5Gc) and only trace amounts of N-Acetyl neuraminic acid (Neu5Ac). BALB/c mouse intestinal lavages glycoproteins contained asialo N-glycans. DMTMM-mediated methylamidation of N-glycans for MALDI mass spectrometry analysis is a fast and cheap method for structurally conserved glycan derivatization.

1. Introduction

Glycans are biomolecules essential for structure, energy storage and information storage in biological systems. These sugar chains, commonly referred to as glycans, are usually attached to proteins and lipids. Glycans are found in all living organisms and are specific for the bacterial, fungal, plant and animal domain. Their unique and complex structures make glycans one of the most information dense biopolymers on earth. Every organism, but also each kind of tissue, is characterized by a specific glycoconjugate profile providing a unique sample signature.

Glycans can be reshaped by post-translational modification, metabolic or catabolic processes assigning them to important functions in physiological and pathological processes. Hence, specific glycans identified in serum have been suggested as disease biomarker [1–3]. In particular, sialic acids are present in high concentrations on mammalian glycans and their content and composition changes during pathogenicity of diseases such as cancer [4–6], inflammatory disorders [7] and autoimmune diseases [8]. Recent data suggest the integration of glycan specific pattern to improve precise disease identification [9]. Still, there is an urgent need to identify new diagnostic and prognostic biomarkers that, in combination with already applied screening methods, are suitable to precisely identify a disease and reliably predict its pathogenesis. In particular, fluid-based biomarkers would be ideal to guide clinical decisions. Recent literature confirms the growing need and increasing attention to minimally invasive liquid biopsies, which enable the analysis of markers in patient fluids such as blood, urine and saliva [10,11].

With growing knowledge on disease mechanisms, the interest on glycome profiling has increased during the last years. Mass spectrometry is emerging as a powerful technique. Global glycan profiling of human

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https://doi.org/10.1016/j.talanta.2022.123326
Received 2 November 2021; Received in revised form 14 February 2022; Accepted 16 February 2022
Available online 17 February 2022
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serum based on mass spectrometry has already led to several potentially promising markers for diverse types of cancer [12–14]. MALDI offers several advantages with respect to other N-glycan analysis techniques such as lower sample amount, higher throughput, faster analysis and lower costs. However, to enable the analysis of glycans by MALDI in positive mode, the neutralization of the negative charge of the carboxylic group of the sialic acid is required. Many different strategies exist to address this issue. Some protocols use methylation, esterification or amidation. Unfortunately, many of these derivatization procedures or glycan sample preparation approaches require conditions, which totally or at least partially degrade sialic acid residues present on the terminal position of N-glycans. Sialic acid is very unstable at pH levels lower than 5. Avoiding an acidic environment is essential for the preservation of the original glycan structure. Methylamidation of N glycans [15] carried out with DMTMM activation [16] stabilizes the sialic acid and allows a correct analysis with MALDI.

This study aimed to combine the above mentioned knowledge for analysis of N-glycans with a conserved structure using mouse sera and intestinal lavages as biological sample fluids from different body sites. Special attention was directed to develop a method able to conserve the very labile sialic acid residues on the antennary position of the N-glycans. Sialic acid was methylamidated using DMTMM as activation reagent and purified using a glycoprotein as a solid support for analysis with MALDI-MS. This easy conversion and efficient protocol can be used for any mammalian biological fluid containing protein-bound glycans.

2. Experimental section

2.1. Chemicals and reagents

PNGaseF (N-glycanase) was obtained from Promega (Madison, USA). The maltodextrine glycan ladder was provided by AGRANA GmbH (Tulln, Austria). 2.4.6-Trichloro-1.3.5-triazine was purchased from Sigma-Aldrich (Vienna, Austria). Bio-Rad Protein Assay Dye Reagent Concentrate was obtained from Bio-Rad (Hercules, USA). All other reagents were of analytical grade or the best grade available. Murine serum and intestinal lavage samples (n = 6) derived from naïve, adult, female BALB/c mice. The animals were purchased from the Core Facility for Biomedical Research, Division for Laboratory Animal Science and Genetics (Himberg, Austria) and housed under conventional conditions (circadian rhythm of 12 h light and dark cycles at 22 °C). Mice were kept in groups of six at the animal facility of the Institute of Pathophysiology and Allergy Research in polycarbonate Makrolon type II cages (Ehret GmbH, Emmendingen, Germany) with aspen wood bedding (Ehret GmbH, Emmendingen, Germany) sealed with filter tops. Cages were enriched with red transparent plastic nest boxes and nesting material of cellulose. Mice were treated according to the European Union guidelines of animal care and with approval of the animal ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (approval number: BMWF-66.009/0270-II/3b/2013) [17].

2.2. Activation reagent preparation

Synthesis of 2-chloro-4.6-dimethoxy-1.3.5-triazine (CDMT): A mixture of MeOH (64.48 ml), H_2O (6.59 ml) and $NaHCO_3$ (34.16 g) was cooled to 10–15 °C. 2.4.6-Trichloro-1.3.5-triazine (25 g) was added and the solution was slowly warmed to 35 °C under stirring. During the reaction the production of bubbles due to the neutralization of hydrochloric acid with sodium bicarbonate was observed. After 13 h of stirring, 270 ml of water were added to precipitate 2-chloro-4.6-methoxy-1.3.5-triazine (CDMT) and to solubilize the sodium carbonate salts. The white crystalline product was isolated by filtration and washed with 200 ml water [18]. The precipitate was dried under vacuum at room temperature. Yield: 66%, Melting point: 73.9–79 °C, GC-MS: M^+ = 175.01 *m/z*; M^+(-MeO) = 145.0 *m/z*, NMR: ^{1}H NMR (200 MHz, CD_2Cl_2) δ 4.15 (s, 2H). The reaction scheme can be found in the supporting information Fig. S1.

Synthesis of DMTMM: 6.0 g (3.52 mmol) of CDMT were dissolved in 60 ml of tetrahydrofuran (THF). The reaction did not work if anhydrous THF was used. 4.14 ml (3.87 mmol) of N-methylmorpholine (NMM) were slowly added and a white precipitate of DMTMM formed immediately. After 45 min of stirring, white crystals were filtered, washed two times with 80 ml THF and dried under vacuum [18]. Yield: 100%, NMR: ^{1}H NMR (400 MHz, methanol-d_4) δ 4.54 (s, 2H), 4.19 (s, 6H), 4.09 (d, J = 9.5 Hz, 2H), 3.86 (q, J = 11.7, 10.9 Hz, 4H), 3.54 (s, 3H). The reaction scheme can be found in the supporting information Fig. S2.

2.3. Serum protein quantification in microliter plates

Proteins were quantified using the Coomassie brilliant blue method. One part of Bio-Rad Protein Assay Dye Reagent Concentrate was diluted with four parts of distilled water. Bovine serum albumin with a concentration range from 5 to 100 mg/ml was prepared. 5 μl of serum sample or BSA standard were diluted with 195 μl of water into a 96-well microliter plate. 10 μl of each diluted standard or diluted sample were pipetted into separate wells and mixed with 200 μl of diluted dye reagent. Air bubbles were removed by centrifugation of the plate for 1 min at 100 g. The absorbance was read with an automatic plate reader at 595 nm. Samples and standard were assayed in triplicates.

2.4. N-glycan sample preparation

For delipidating and precipitating glycoproteins the volume corresponding to 4.00 mg of serum protein or 0.5 mg of lavage protein was pipetted in a 1.5 ml reaction tube. The volume was adjusted to 100 μl by adding PBS buffer to a final protein concentration of 40 mg/ml. The samples were delipidated and proteins were precipitated by addition of 600 μl 2-propanol and 900 μl hexane. To improve the precipitation process, tubes were put on ice and gently homogenized for 30 min. The solution was centrifuged at 11 000 g for 10 min. Thereafter, three different fractions were visible. The top layer represented the organic phase, the middle layer was constituted by aqueous/alcoholic phase and precipitated glycoproteins were clearly visible in the bottom layer. The liquid organic phase and the aqueous/alcoholic phase were discarded. For derivatization, the solid precipitate was treated with 200 μl of a solution of DMTMM 0.25 M, methylamine hydrochloride 0.44 M in a 10 mM phosphate buffer at pH 6. The reaction tube was vortexed and incubated at 70 °C for 1 h. 200 μl of MeOH were added, vortexed and centrifuged 11 000 g for 10 min. This step was repeated for two times to remove excess salt and byproducts of the glycoprotein precipitation.

2.5. N-glycan release

The precipitate was suspended with 100 μl of denaturing solution consisting of phosphate buffer solution 20 mM at pH 6.2, 0.1 M dithiothreitol (DTT), 0.2% of SDS and 12 μl NP-40 (10% in water). 0.5 μl of enzyme PNGase F was added to the sample and incubated for 24 h at RT. Thereafter, the sample was centrifuged at 11 000 g for 10 min. 100 μl of the supernatant were transferred in a new reaction tube.

2.6. HILIC cotton solid phase extraction (SPE)

Purification and sample concentration with cotton HILIC SPE microextraction was performed by addition of 488 μl acetonitrile (ACN) to 100 μl of supernatant to set the concentration of ACN to 83% [19], required for HILIC purification. Thus, 3 mg of cotton were put in a 200 μl polypropylene pipette tip and washed three times with 50 μl water. Subsequently, the tip was equilibrated three times with 50 μl ACN 83%. Aliquots of 100 μl were transferred in a new reaction tube and pipetted up and down ten times. Afterwards, the tip was washed with 100 μl ACN 83% three times. Glycans were eluted from the cotton by pipetting five

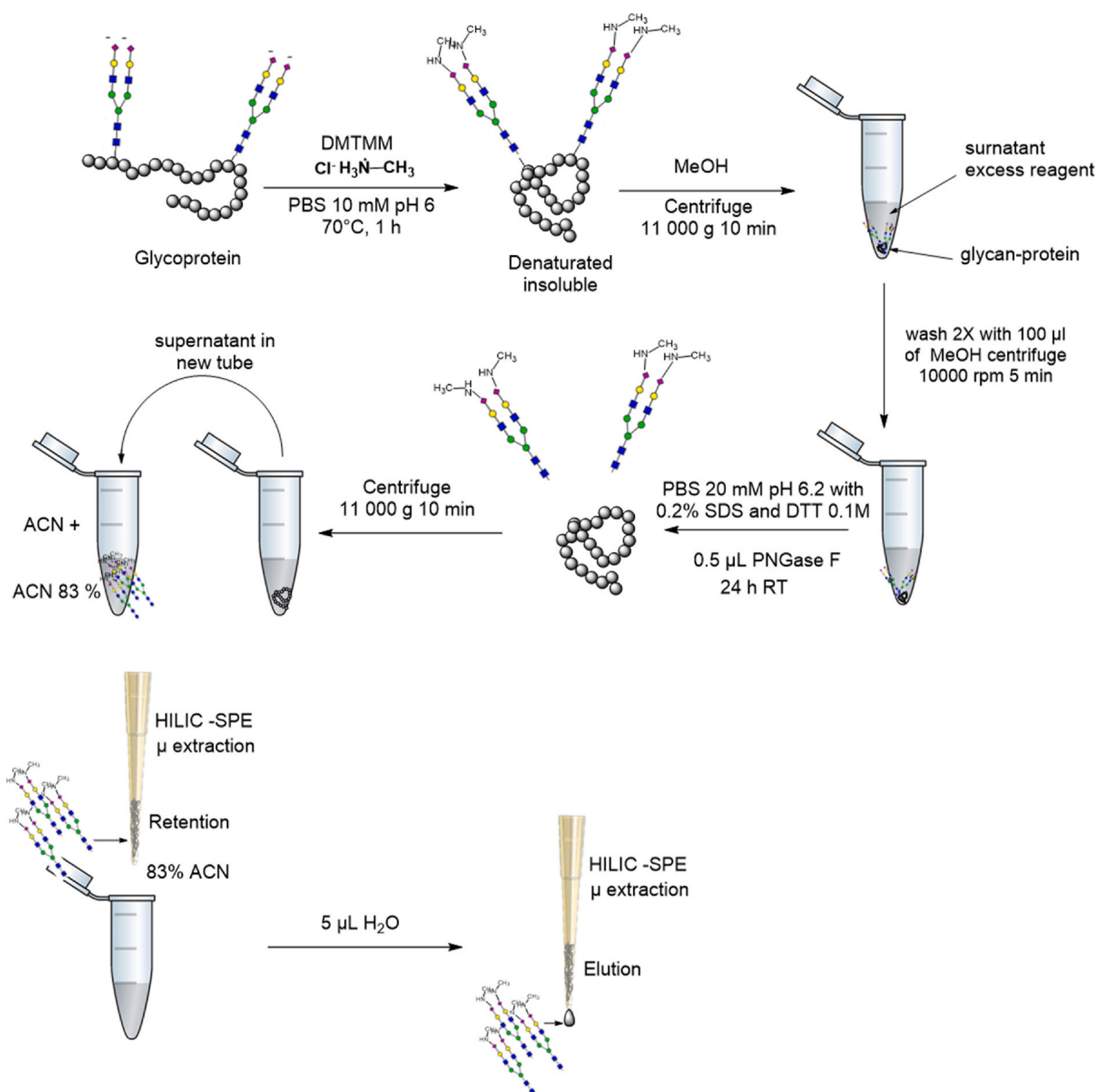

Fig. 1. Schematic representation of the sample preparation.

times up and down with 5 μL of water. The samples were stored at −20 °C until further analysis. A schematic illustration of the sample preparation can be seen in Fig. 1.

2.7. MALDI-MS measurement

The 2,5-dihydroxybenzoic acid (DHB) matrix solution was prepared by dissolving 5 mg of 2,5-DHB to 500 μl of ACN and diluting it with 500 μl of 2 mM NaCl solution. One μl of matrix solution was mixed with 1 μl of purified glycan sample. Then, 1 μl of the matrix-glycan-mix was spotted on a MALDI-MS steel target and dried at room temperature. For a typical MALDI analysis, solutions of a sample (pure or a mixture; ~10 μM) and matrix (~10 mM) were pre-mixed and a small volume (~1 μl) was applied directly to the target [20]. The shape of the matrix crystals was uniformed by addition of 0.2 μL EtOH causing a rapid crystallization. MALDI/MS measurements were performed using a MALDI-TOF Microflex (Bruker Daltonics, Bremen, Germany), equipped with 20 Hz laser (nitrogen) and operating in reflection positive ion modes. The instrumental conditions employed to analyze molecular species in the *m/z* range 1000–3500 were: ion source 1: 20.00 kV; ion source 2: 18.00 kV, lens: 9.00 kV, pulsed ion extraction: 140 ns; laser power: 97%. Each sample was recorded with 2500 shots. Maltodextrin was used as external mass calibration ladder.

2.8. MS-data preprocessing

The obtained spectra where exported as text format using flex analysis version 2.4 (Bruker Daltonics). The mass spectra were analyzed using mMass 5.5.0 software (www.mmass.org). In the supporting information Table S1, the list of the calibration mass is shown. Glycan structures and their expected mass were sketched and calculated using the GlycoWorkbench 2.1 software [21].

3. Results and discussion

3.1. Charge neutralization of sialic acids by methylamidation

N-Glycans of mammalian origin contain sialic acids, Neu5Ac or

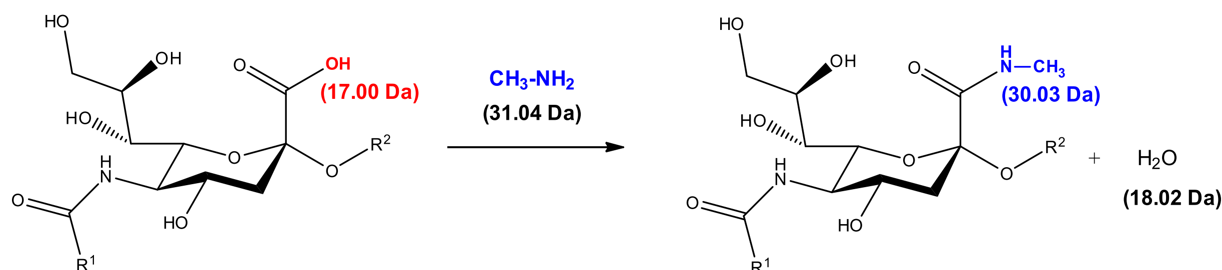

Fig. 2. Sialic acid methylamidation. After modification, each sialic acid has an increase of 13.03 Da molecular weight.

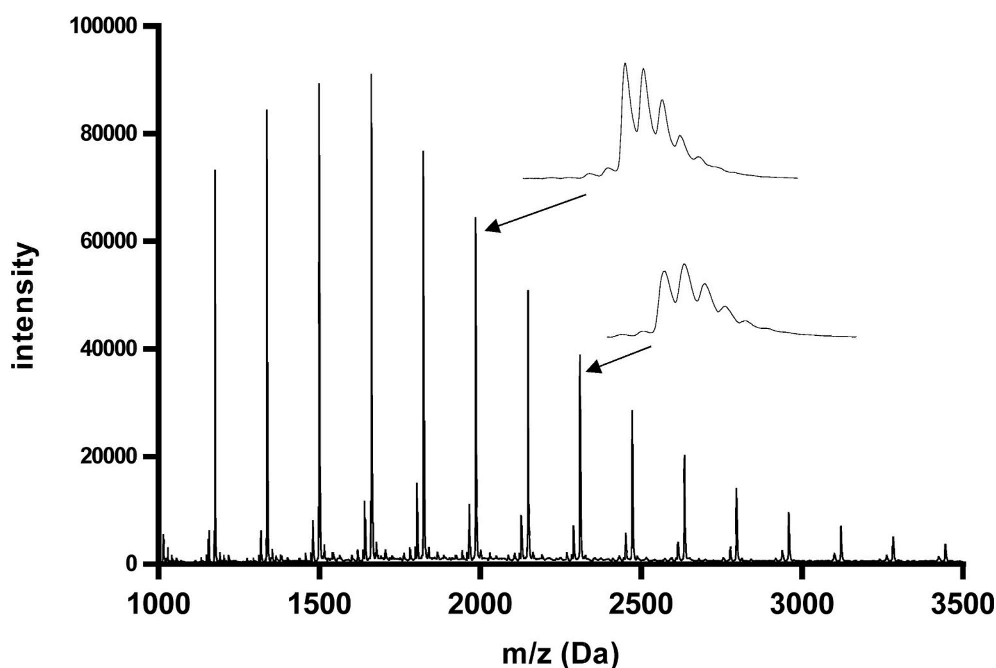

Fig. 3. Glycan ladder used for mass calibration.

Neu5Gc, on the terminal position. For MALDI-MS analysis in positive-ion mode, negative charges of the sialic acid's carboxyl group had to be neutralized. This was achieved by methylamidation after activation of the carboxylic acid with the DMTMM. DMTMM was more efficient than the standard activation method using EDC/NHS for amide formation [16]. In addition, DMTMM could be easily synthetized from cheap reagents in high yield making this method cost effective. During the methylamidation reaction the amine donor, in our case methylamine, had to be present in excess to avoid a reaction of the activated carboxylic acid with free amino- or hydroxyl-groups of the protein, which would hinder glycan release by PNGaseF.

Table 1
Protein concentrations of murine serum and intestinal lavage samples determined with the Comassie brilliant blue method.

serum sample	mean protein concentration (mg/ml)	standard deviation	input volume (μl)
mouse 1	33.89	2.02	118.0
mouse 2	40.61	0.98	98.5
mouse 3	43.51	5.37	91.9
mouse 4	27.89	6.25	143.4
mouse 5	29.33	9.69	136.4
mouse 6	32.49	4.07	123.1
lavage sample	**mean protein concentration (mg/ml)**	**standard deviation**	**input volume (μl)**
mouse 1	2.15	0.31	232
mouse 2	1.65	0.14	302
mouse 3	1.47	0.24	340
mouse 4	1.49	0.09	334
mouse 5	2.05	0.17	244
mouse 6	1.19	0.20	420

3.2. *Usage of proteins as solid support*

MALDI analysis is very sensitive to impurities and excess of reagents. Impure samples would lead to reduced signal intensity and poor MALDI mass spectra. To obtain high sample quality, we used a protein backbone as a solid support. The glycans were derivatized while being still bound to the protein. After extensive washing, the glycans were separated from their protein backbone with PNGaseF and enriched by cotton HILIC SPE microextraction.

3.3. *Calibration of molecular weight*

Maltodextrin mixtures, or any other debranched starch, can be used as a mass calibration standard for MALDI-MS analysis. These mixtures consist of a short glucose chain with a polymerization degree ranging

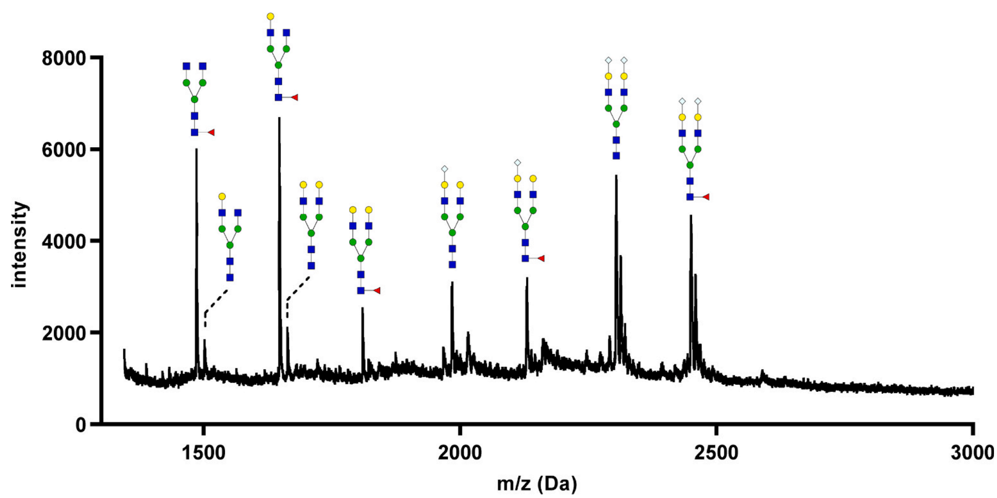

Fig. 4. MALDI-TOF/MS spectra of methylamidated serum N-glycan profile of a representative BALB/c mouse sample. The glycans cartoons were created using the standard consortium for functional nomenclature, i.e., GlcNAc, N-acetylglucoseamine [blue square]; hex, hexose (mannose) [green circle], galactose [yellow circle]; Neu5Ac, 5-N-acetylneuraminic acid [purple diamond]; and Neu5Gc, 5-N-glycolylneuraminic acid [white diamond].

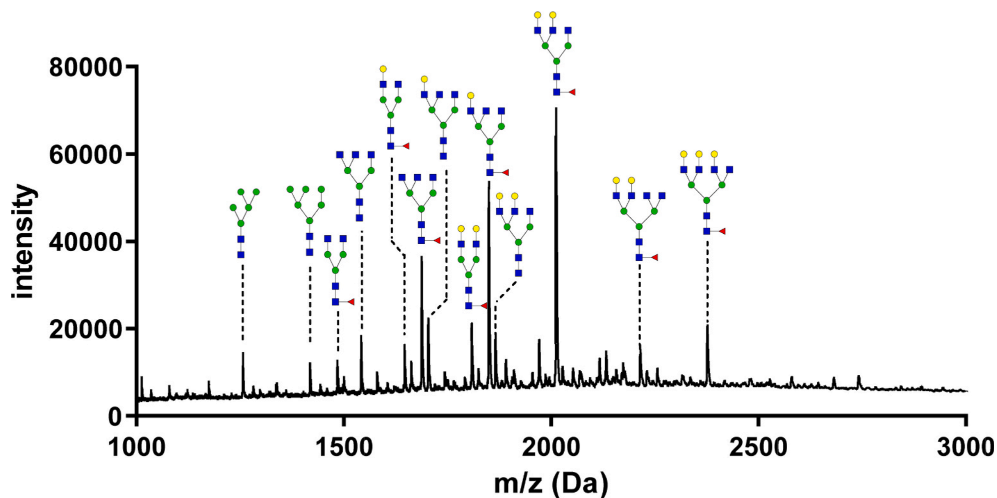

Fig. 5. MALDI-TOF/MS spectra of methylamidated intestinal lavages N-glycan of a representative BALB/c mouse sample.

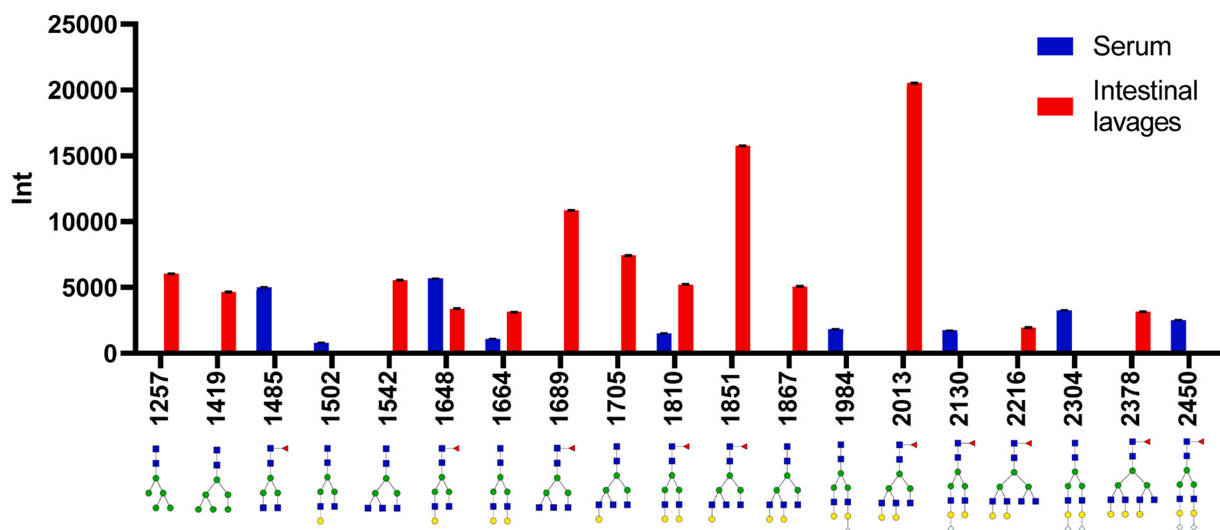

Fig. 6. Comparison of MALDI-TOFglycan profiles from sera and intestinal lavages of BALB/c mice. Only three glycan structures (1648,1664,1810 Da) are in common.

from 1 to approximately 25. The advantage of using a maltodextrin glycan ladder over a peptide standard is the regular distribution of mass covering the whole mass range, as it can be observed in Fig. 3. Attention must be given during the integration of the signal due to the inversion of the isotopic abundance at ~14 glucose units (GU). At GU > 14 the isotope species with the highest abundance are the [M + Na^++1 Da]. The molecular weight of each peak for glycan ladder calibration can be found in the supplementary material Table S1.

3.4. Protein concentrations in mouse sera and intestinal lavages

As our biological model fluid, murine serum and intestinal lavage samples were analyzed in triplicates. The average protein concentrations of each sample are shown in Table 1. Sample concentrations were adjusted to ensure analysis of the same concentration of glycoproteins. Exactly 4.00 mg of protein was used for serum glycan analysis. Intestinal lavage samples were normalized to 0.5 mg of protein content.

3.5. N-glycan analysis in BALB/c mouse sera and intestinal lavages

Structural assignments showed methylamidated N-glycans in murine sera [M + Na^+]. Glycans were assigned based on published literature on mouse plasma N-glycome pattern and established biosynthetic pathways [2,22]. The molecular weight of methylamine is 31.05 Da. Themethylamidation product produced an increase of molecular weight of sialic acid of 13.03 Da (Fig. 2). In our study DMTMM was used as activation reagent for sialic acid methylamidation during sample preparation. DMTMM allowed the methylamidation of sialic acid under very mild conditions and conserved the sialic acid bound on the N-glycans without degradation. A representative glycan profile detected in murine serum samples is depicted in Fig. 4 and the glycan profile of intestinal lavages is depicted in Fig. 5.

The most abundant glycans examined in murine biological fluids are compared in Fig. 6 (Table S2). Only three glycan structures are in common between mouse sera and mouse intestinal lavages. The study identified a high degree of terminal glycolylneuraminic acid (Neu5Gc) in N-glycans of mouse sera. Acetylneuraminic acid (Neu5Ac) was present only in very low amounts on mouse N-glycans (less than 5%). In contrast to other study [22,23] were also BALB/c mice serum N-glycome is analyzed, we found a higher concentration of di-antennary N-glycans displaying Neu5Gc. With regards to conserved sialic acids on N-glycans, our method is, to the best of our knowledge, superior to the current state of the art. Of interest, in intestinal lavage samples we did not observe any sialylated N-glycans. A possible explanation might be hydrolysis caused by low pH of gastric fluids and neuraminidases produced by intestinal microbiota. From the signal intensity of the mass spectra it was possible to observe a substantial site specific difference between sera and intestinal lavages. Even though protein concentrations from serum samples were 8 times higher than the protein concentrations of intestinal lavages, the amount of glycans was one order of magnitude higher in the intestinal lavages compared to serum samples.

6. Conclusion

In this study we introduce a robust, cheap and high-throughput MALDI-MS method for N-Glycan analysis of mammalian biological fluids. Our method allows profiling structurally conserved N-glycans in an cost and time effective way providing the fundament for a standardized high-throughput method compared to the current state of the art. Special attention was paid to derivatize sialic acid using very mild reaction conditions. This was achieved by using DMTMM as carboxylic acid activation reagent for conservation of sialic acids present on the terminal position of N-glycans. As the activation reagent DMTMM is stable in water, it is possible to perform this derivatization in aqueous solvents. The N-glycan profile of mouse serum showed high concentrations of Neu5Gc-terminal glycans. The N-glycans determined in the intestinal lavages revealed no sialic acid content. We are well aware of the study's limitation as BALB/c mice glycome pattern are not directly comparable to the human system. Neu5Gc is an endogenous sugar moiety in mice, but not in humans. Therefore, models for human diseases should consider the usage of mouse strains with similar sugar structures as humans, like the $CMAH^{-/-}$ knock-out mice. Also other animals such as ferrets or hedgehogs lacking the ability to produce cytidine monophospho-N-acetylneuraminic acid hydroxylase and therefore, only expressing Neu5Ac like humans may be considered for analysis.

Credit author statement

Davide Ret: Conceptualization, Methodology, Investigation, Visualization, Writing. Linda Stefenatti: Investigation. Alessio Gentile: Investigation, Reviewing. Johanna Rohrhofer: Writing- Reviewing and Editing. Simone Knaus: Supervision. Eva Untersmayr: Supervision, Writing- Reviewing and Editing.

Novelty statement

The newly adapted method described in this article allows sample preparations with a high structural integrity for a high throughput analysis of N-glycans. All steps in the sample preparation procedure take in consideration the lability of terminal sialic acids aiming to minimize their loss for a comprehensive N-glycan analysis. DMTMM is described as excellent activator reagent with high water stability and high activity, which allows a simple and cost effective synthesis.

Bullet points.

* MALDI mass spectrometry analysis for characterization of N-glycans
* Stabilization and neutralization of sialic acids via methylamidation
* DMTMM mediated modifications for cost-effective high yield synthesis
* Site specific N-glycan profile analysis in sera and intestinal lavages of BALB/c mouse

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors acknowledge the TU Wien Bibliothek for financial support through its Open Access Funding Program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123326.

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