

Microfluidics-Mass Spectrometry of Protein-Carbohydrate Interactions: Applications to the Development of Therapeutics and Biomarker Discovery

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Abstract

The functional interactions of carbohydrates and their protein receptors are the basis of biological events critical to the evolution of pathological states. Hence, for the past years, such interactions have become the focus of research for the development of therapeutics and discovery of novel glycan biomarkers based on their binding affinity. Due to the high sensitivity, throughput, reproducibility, and capability to ionize minor species in heterogeneous mixtures, microfluidics-mass spectrometry (MS) has recently emerged as a method of choice in protein-glycan interactomics. In this chapter, a straightforward microfluidics-based MS methodology for the assessment of protein-glycan interactions is presented. The general protocol encompasses: (1) submission of the interacting partners to a binding assay under conditions mimicking the in vivo environment; and (2) screening of the reaction products and their structural characterization by fully automated chip-nanoelectrospray (nanoESI) MS and multistage MS. The first section of the chapter is devoted to describing a method that enables the study of protein-oligosaccharide interactions by chip-nanoESI quadrupole time-of-flight (QTOF) MS and top-down complex analysis by collision-induced dissociation (CID). This section provides the protocol for the determination of the complex formed by standard β -lactoglobulin (BLG) with maltohexaose (Glc_6) and recommends as a concrete application the study of the interaction between BLG extracted from human milk with Glc_6 , considered a ligand able to reduce the allergenicity of this protein. The second part is dedicated to presenting the protocols for the binding assay followed by chip-nanoESI ion trap (ITMS) and electron transfer dissociation (ETD) in combination with CID for protein-ganglioside interactions, using as an example the B subunit of cholera toxin (Ctb5) in interaction with commercially available GM1 species. The methodology described may be successfully applied to native ganglioside mixtures from human brain, in particular for discovery of biomarkers on the basis of their binding affinity.

Key words Chip-nanoelectrospray, Multistage mass spectrometry, Noncovalent interactions, Proteins, Glycans, Gangliosides, Top-down fragmentation

1 Introduction

Carbohydrates are ubiquitous biopolymers of high structural and functional complexity. They may occur in nature as free oligo- and polysaccharides or as glycoconjugates, in which the glycan is

covalently linked to a protein or a lipid forming the known classes of glycoproteins, proteoglycans, and glycolipids. Carbohydrates are found mostly displayed on the surface of the cells participating in essential biological processes among which protein folding, stability, and trafficking [1–3]. The noncovalent interactions between carbohydrates and their protein receptors lie at the basis of the antigen recognition machinery and other biological functions [4, 5] such as signal transduction and cellular adhesion of bacteria and viruses. Protein-carbohydrate interactions mediate also the specific host-pathogen recognitions, which are of critical importance in the evolution of various diseases [4]. As a consequence, in the past decade, these interactions came into the focus of research for the development of therapeutics, and, in particular, for designing carbohydrate-based or protein-based inhibitors [6]. Knowledge of the structure of the interacting glycan-protein partners and of the interaction mechanism, which very often encompasses a cascade of complex processes, is a fundamental requirement for the development of such therapeutics.

On the other hand, particular carbohydrate structures were shown to represent valuable biomarkers of severe pathologies [7–9]. However, there is a large discrepancy between the need for natural glycoforms of extreme structural variability representative of biological information, the limited availability of samples from natural sources, and the ability to analyze and characterize such complex glycoform extract mixtures. These challenges resulted in substantial efforts dedicated to the development of sensitive and specific analytical methods for mapping the glycan mixtures, identifying biomarker or disease-associated species, and characterizing the detailed structure of a marker in a multicomponent sample. An efficient approach in this regard targets the discovery of novel structures based on their high binding affinity to specific protein receptors [10]. Hence, protein-glycan interactions may be efficiently exploited also for biomarker discovery in complex extracts.

Mass spectrometry (MS) with either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) has developed over the past two decades into one of the most proficient methods, which greatly advanced the fields of genomics, proteomics, and glycomics [11–13]. The continuous technical improvements in the MS instrumentation in terms of resolution, mass accuracy, analysis throughput, and sensitivity have expanded the applicability of this technique from simple molecular mass determinations of individual compounds, to challenging *de novo* identifications, biomarker discovery, drug development, and structural analyses of heterogeneous mixtures, molecular aggregates, whole cells and tissues [14–16]. In the same context of performance augmentation, in the past years, modern achievements in chip technology and robotics were introduced also in MS [17, 18], in particular for replacing the classical ion sources with microfluidic

devices [18–20]. Among these technologies, chip-based nanoESI with automatic sample infusion (NanoMate robot) exhibits multiple advantages, making this approach highly suitable for the characterization of protein-glycan complexes: a several-fold increase in sensitivity; capability to ionize minor components in heterogeneous mixtures; compatibility with a wide range of solvents; reported in-run and run-to-run reproducibility of the experiments between 98% and 100%; elevated signal-to-noise ratio; high-throughput; low flow rates; steady spray beneficial for fragmentation analysis using either collision-induced dissociations (CID), electron transfer dissociation (ETD) or their combination; and, potential to discover, on the basis of their binding affinity [10, 21], novel species, undetected before because of their reduced expression.

In this chapter, a straightforward MS-based methodology for the assessment of protein-glycan functional and structural interactions is described. The general protocol requires the conjunction of biochemical tools for purification and binding assays, with a modern analytical system based on fully automated chip-nanoESI (NanoMate robot) successively coupled to a high-resolution quadrupole time-of-flight (QTOF) MS and a high performance ion trap (IT) MS. The described methodologies are divided into two parts (Fig. 1): (1) a binding assay under conditions mimicking the *in vivo* environment; and (2) a detailed chip-nanoESI MS and multistage MS compositional and structural analysis protocol of the reaction products for confirming the noncovalent complex formation and its

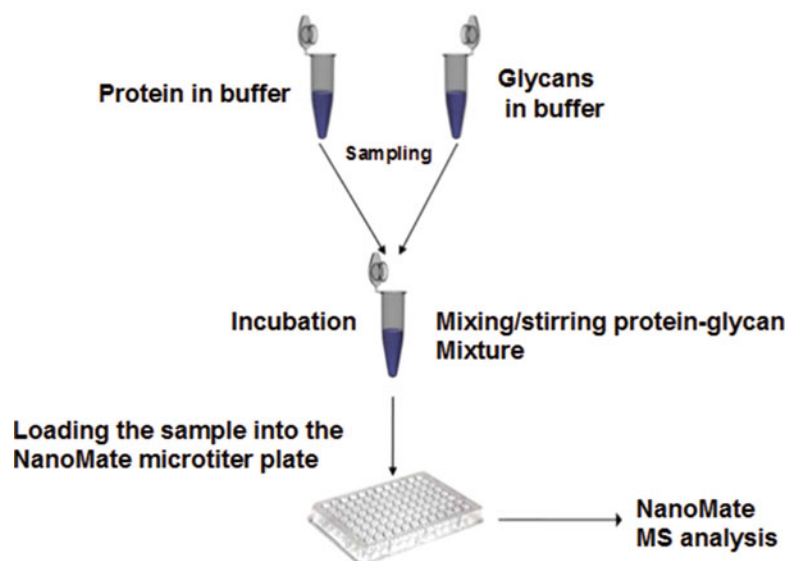


Fig. 1 Schematic of the workflow for protein-glycan noncovalent interaction assay and chip-nanoESI analysis of the reaction products (adapted from ref. 10 with permission from Taylor and Francis)

detailed characterization in terms of binding site(s) and structure of the bound glycan/glycoconjugate species. The chapter describes in detail the procedures to be followed for the assessment of protein-oligosaccharide interactions by chip-nanoESI QTOF MS and CID MS/MS, and of protein-ganglioside interactions by chip-nanoESI ITMS and ETD/CID MSⁿ. The standards chosen for exemplification are β -lactoglobulin (BLG) in interaction with maltohexaose (Glc₆) and the B subunit of cholera toxin (Ctb5) in interaction with GM1 ganglioside. Concrete applications, to be carried out following the described procedures, are also suggested. The first proposed application is related to the complex formed by BLG extracted from human milk with Glc₆, considered among the ligands able to reduce the allergenicity of this protein. The second recommended application is the interaction of Ctb5 with a native mixture of human brain gangliosides, which is able to offer data on novel species, of potential biomarker value, on the basis of their binding affinity.

2 Materials

2.1 Binding Assays

Prepare all solutions using HPLC or MS grade solvents and deionized water from a Milli-Q or other similar water purification systems. Follow the manufacturer's recommendations for storing reagents.

1. Ammonium acetate buffer: 10 mM, pH 6.0. Prepare 1 mL solution and adjust the pH with either 98% formic acid or acetic acid (*see* **Notes 1–3**). Filter the buffer solution using Millipack disposable filter units, 0.22 μ m pore size (or other similar performance products).
2. Maltohexaose (Glc₆): $\geq 65\%$ by HPLC (MW 990.86). The tested product was from Sigma-Aldrich, but other suppliers may be used.
3. Maltohexaose Glc₆ solution: 10 pmol/ μ L. Prepare the Glc₆ solution in filtered ammonium acetate buffer and vortex. Amount needed for analysis is 20 μ L.
4. β -Lactoglobulin (BLG): $\geq 90\%$ by PAGE. The tested product was from Sigma-Aldrich, but other suppliers may be used.
5. BLG from human milk: Extracted and purified as described in ref. [21].
6. BLG solution: 5 pmol/ μ L. Prepare the BLG protein solution in filtered ammonium acetate buffer and vortex. Amount needed for analysis is 50 μ L.

7. Subunit B (Ctb5) of cholera toxin from *V. cholerae* (*see* **Note 4**). The tested product was from Sigma-Aldrich, but other suppliers may be used.
8. Ctb5 protein solution: 1 pmol/ μ L. Prepare the ganglioside solution in filtered ammonium acetate buffer and vortex. Amount needed for analysis is 120 μ L.
9. Monosialoganglioside GM1 fraction from bovine brain: $\geq 95\%$, lyophilized powder (*see* **Note 5**). The tested product was from Sigma-Aldrich, but other suppliers may be used.
10. Native ganglioside mixture from human brain: Extracted and purified as described in ref. 22.
11. Ganglioside solution: 10 pmol/ μ L (*see* **Note 6**). Prepare the ganglioside solution in filtered ammonium acetate buffer and vortex. Amount needed for analysis is 120 μ L.
12. SpeedVac concentrator for drying the samples coupled to a vacuum pump.
13. Thermomixer Eppendorf Comfort (or other similar devices).
14. pH meter.
15. Digital microbalance.
16. Micro DispoDialyzer system with 500 Da cutoff dialysis membrane (5–100 μ L).
17. Dialysis membranes for protein purification, 10,000 Da cutoff.

2.2 Chip-Based nanoESI MS and MS/MS Analysis

1. NanoMate™ 400 or NanoMate™ TriVersa 400 robot from Advion BioSciences with commercial bracket from Advion BioSciences for NanoMate robot coupling to QTOF mass spectrometer.
2. Silicon nanoESI Chip™ with 400 nozzles from Advion BioSciences.
3. Microtiter sample plates with 96 wells, V-bottom, 200 μ L, thermal cycler-compatible, polypropylene, or glass-coated from Beckman Coulter Inc. or Eppendorf AG.
4. Rack of 96 disposable conductive pipette tips from Advion BioSciences.
5. ChipSoft 8.1.0 software operating under Windows system from Advion BioSciences for controlling and manipulating the NanoMate robot.
6. Orthogonal hybrid quadrupole time-of-flight (QTOF) mass spectrometer (Micromass or Waters instruments) equipped with electrospray ion source in Z-spray geometry. Instrument characteristics: (1) resolution 5000 (full width at the half maximum, FWHM) for m/z 500; (2) average mass accuracy 20 ppm for m/z (150–4000); (3) scan rate from 2 to 10 scan/s; (4)

mass range: m/z 250–4500 for MS, m/z 150–4000 for CID MS/MS (*see* Note 7).

7. MassLynx software v. 4.1. from Waters/Micromass running on a PC under Windows to control the QTOF MS instrument, acquire the signal, and process the MS and MS/MS data.
8. Ion trap or Orbitrap mass spectrometer equipped with electrospray ionization source and with an ETD module using fluoranthene as anionic reagent and methane as the supporting gas. For example, high capacity ion trap ultra (HCT Ultra, PTM discovery) mass spectrometer (Bruker Daltonics) or amaZon speed ETD ion trap (Bruker Daltonics).
9. Compass™ Software (Bruker Daltonics) for HCT Ultra mass spectrometer.
10. Commercially available bracket from Advion BioSciences, for Bruker ion trap coupling with the NanoMate robot.
11. MS calibration solution: standard G2421A electrospray “tuning mix” from Agilent Technologies, 1:100 dilution in acetonitrile; or, alternatively, sodium iodide (2 $\mu\text{g}/\mu\text{L}$) or cesium iodide (50 $\text{ng}/\mu\text{L}$) in 2-propanol/water, 50:50 (v/v). Prepare fresh before analysis.
12. Nitrogen for QTOF mass spectrometer and NanoMate robot, purity $\geq 99.999\%$.
13. Argon for CID MS/MS experiments, purity $\geq 99.9995\%$.
14. Helium for ion trap mass spectrometer, purity $\geq 99.999\%$ vol.
15. Methane as the supporting gas for anion production in ETD experiments, purity $\geq 99.995\%$ vol.

3 Methods

3.1 Protein-Glycan Interactions.

Application:

β -Lactoglobulin in Interaction with Maltooligosaccharides

β -Lactoglobulin is a lipocalin protein highly expressed in cow's milk as the major whey proteic component. This protein is known as one of the most aggressive allergens for newborns, triggering a complex symptomatology and possibly life-threatening complications. BLG may incidentally occur in human milk by cow's milk intake. As no prophylactic treatment is presently available, finding ligands able to reduce the BLG allergenicity is currently the only possible option to address this problem. Earlier work conducted in the field [23] has shown that some classes of oligosaccharides represent ligands with such properties. A 2016 report [21] on chip-nanoESI MS and MS/MS application, with significant biomedical impact, revealed that maltohexaose binds BLG under conditions mimicking the in vivo environment and therefore might be considered among the ligands able to reduce the allergenicity of this protein.

3.1.1 Binding Assay

1. Incubate 30 μL standard BLG solution with 6 μL Glc₆ solution in the Eppendorf thermomixer under constant stirring at 37 °C (*see* **Note 8**).
2. Collect 5 μL of the reaction products after 1, 5, 10, 15, 20, 25, and 30 min incubation time and submit the aliquots immediately after collection to MS analysis by loading the solution directly into the vial of the NanoMate robot (*see* **Notes 9** and **10**).
3. For the binding assay using BLG extracted from human milk, follow the same procedure as described above in **steps 1** and **2** for the standard BLG solution.

3.1.2 Chip-Based nanoESI MS Detection of the Noncovalent Complex

1. Remove the original ESI source of the QTOF mass spectrometer and couple the NanoMate robot via the Advion commercial bracket.
2. Turn on the gas supplies, the mass spectrometer, and the NanoMate robot.
3. Tune the QTOF MS instrument for operating in positive ESI mode and set the parameters in the Mass Lynx software 4.1 as follows: acquisition in MS mode; cone voltage: 40 V; nitrogen nebulizer: 60 psi; source block temperature: 80 °C; mass range: m/z 250–4500.
4. Tune the NanoMate robot in the positive ion mode by setting on the ChipSoft software the following values for the operating parameters: sample aspiration volume 5 μL ; air aspiration volume: 2 μL (*see* **Note 11**); nanoESI voltage on the pipette tip: 1.8 kV; nitrogen back pressure: 0.40 psi.
5. Slide the 400 ESI Chip in the chip holder of the robot with the frame notches oriented down and the chip frame tag upward, whereas the serial number faces the robot.
6. Select in the ChipSoft the chip with the corresponding serial number.
7. Place the rack of the 96 disposable conductive pipette tips and the sample microtiter plate in their corresponding holders.
8. Load the collected aliquot of the reaction products into a well of the microtiter plate and select in the ChipSoft program the corresponding well position (*see* **Note 12**).
9. Set on the ChipSoft the option “return to the initial well the unused sample.”
10. Select “deliver sample” on the ChipSoft software of the NanoMate robot and “Acquire” in the MS mode on the Mass Lynx software of the QTOF MS (*see* **Note 13**).
11. After initiation of the electrospray, optimize the signal intensity and stability by fine adjustment of the chip position with respect to the mass spectrometer inlet. Select the back-forward,

right-left options in the “spray optimization” of the ChipSoft software.

12. Acquire the signal and switch to View Chromatogram (Total Ion Chromatogram, TIC). Generate the spectrum by “combining in progress” the TIC scans. Accumulate scans until a fair signal-to-noise ratio is obtained. Recommended acquisition time is above 2 min.
13. Load the calibration solution in a new well of the microtiter plate and infuse it by chip-based nanoESI. Generate the calibration spectrum. Apply the calibration file to the analyte spectrum (*see* **Note 14**).
14. Subtract, smooth, and center the mass spectrum using these options in the “Process” portal of Mass Lynx. Deconvolute the protein-related signals and determine the protein molecular weight by applying Process/Component/Find Auto or Find Manual.
15. Keep the same parameters and apply an identical procedure for the measurement of all seven reaction product aliquots collected.
16. Apply the **steps 1–15** described above in this section for detecting the noncovalent complexes formed by BLG extracted from human milk and maltooligosaccharides (*see* Fig. 2).

3.1.3 Top-Down Analysis of the Formed Noncovalent Complex by CID MS/MS

1. Identify in the screening mass spectrum, the ions that, according to the calculated mass, correspond to the protein-glycan noncovalent complex (*see* **Notes 15 and 16**).
2. Leave the chip nanoESI settings unaltered, turn on the Ar and set the LM Res and HM Res parameters for precursor ion isolation at 10 and 10 respectively (*see* **Note 17**).
3. Select “Acquire,” set Function MS/MS and introduce the m/z of the chosen precursor ion.
4. Select the mass range with “start mass” 150 and “end mass” 4000, “scan time” 10 min.
5. Start the signal acquisition and acquire the scans while increasing gradually the collision energy from 40 to 100 eV (*see* **Note 18**).
6. Accumulate scans until a fair signal-to-noise ratio is obtained and vary the collision energy within the (40–100) eV range to produce fragment ions confirming the complex formation and the structure of the bound oligosaccharide (*see* **Notes 18–20**).
7. Stop the acquisition, switch to View Chromatogram, generate the MS/MS by combining over the entire TIC, and further calibrate the CID tandem mass spectrum as described in Subheading 3.1.2.

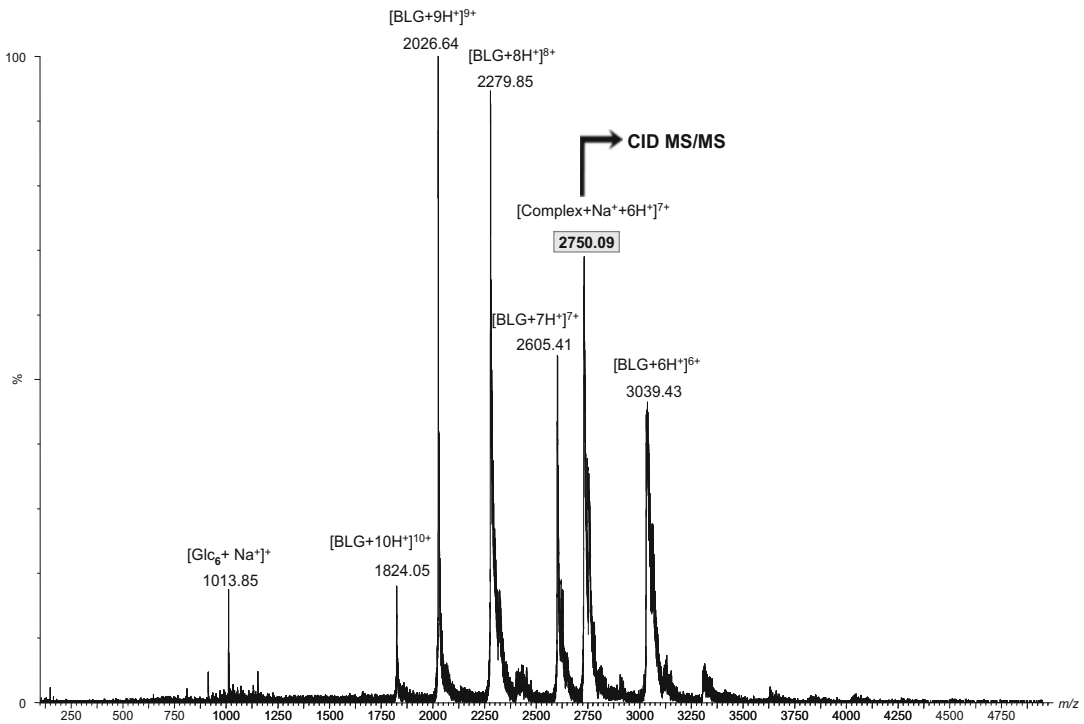


Fig. 2 (+) Chip-nanoESI QTOF MS of BLG-Glc₆ reaction products after 10 min incubation in buffer at 37 °C. Interaction partners: BLG-extracted from human milk upon deliberate intake of 300 mL cow's milk and standard Glc₆. Buffer: 10 mM ammonium acetate/formic acid pH 6.0. (+) Chip-based nanoESI QTOF MS conditions: nanoESI voltage 1.8 kV. Cone voltage 40 V. Nitrogen back pressure 0.40 psi. Nitrogen nebulizer pressure 60 psi. Acquisition time 2 min (reproduced from ref. 21 with permission from Springer)

8. Apply the same protocol for top-down analysis of the complexes formed by BLG extracted from human milk and maltooligosaccharides (*see* Fig. 3).

3.2 Protein-Ganglioside Interaction.

Application: Cholera Toxin in Interaction with Human Brain Gangliosides

Gangliosides, sialylated glycosphingolipids highly expressed in the central nervous system (CNS), are valuable biochemical markers in early diagnosis of CNS pathologies [25–28], being in the focus of research as potential therapeutic targets [28–30] in particular for designing anticancer vaccines. While the hydrophobic ceramide (Cer) moiety anchors the molecule into the cell membrane, the glycan chain of gangliosides may interact freely with the soluble extracellular molecules and with the hydrophilic segments of other membrane components [31, 32]. Being enriched in specialized microdomains, gangliosides interact with signal transducers. They mediate carbohydrate-dependent cell adhesion, induce cell activation, motility and growth and participate in cell–cell and cell–matrix interactions. The interest in the study of ganglioside interactions with proteins was lately stimulated by findings indicating that the formed complexes might play a role in: (1) the molecular

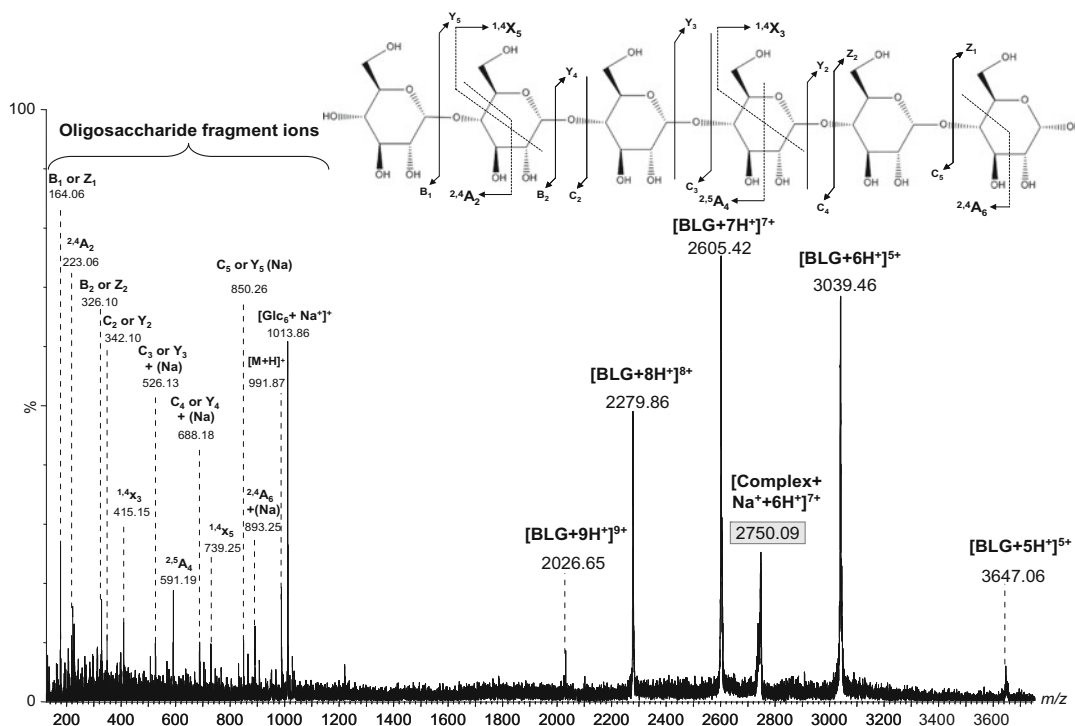


Fig. 3 Top-down by (+) chip-nanoESI QTOF CID MS/MS of the $[\text{Complex} + \text{Na} + 6\text{H}]^{7+}$ detected at m/z 2750.09, corresponding to formed BLG-Glc₆ noncovalent complex. Variable collision energy within (40–100) eV. Acquisition time 5 min. Other MS conditions as in Fig. 1. Inset: ion fragmentation scheme of Glc₆ in top-down experiment. Glc₆ fragment ions are designated following the nomenclature introduced by Domon and Costello [24] (reproduced from ref. 21 with permission from Springer)

mechanisms of Alzheimer disease (AD), with an emphasis on the functions of amyloid β -protein-ganglioside complexes [33]; (2) the action of bacterial toxins, for which several gangliosides were found as specific receptors [23]; (3) the progression of malignant brain tumors [34]; and, (4) the discovery of novel species in complex mixtures based on their high binding affinity [10].

Since gangliosides are readily embedded on hydrophobic surfaces, their interactions with water-soluble proteins were studied by specific methods such as high performance thin layer chromatography, enzyme-linked immunosorbent assay, and surface plasmon resonance [25]. Flow cytometry [35] and carbohydrate microarray systems [36] were also implemented for the determination of ganglioside-cholera toxin complexes, which hitherto is the most studied interaction of gangliosides. The major drawbacks of these methods relate to reduced sensitivity and the type of environment in which the interactions are studied. To overcome these shortcomings, the lipid domain of gangliosides was used for integrating the molecule into liposomes [37], which allowed the interaction under conditions mimicking better the *in vivo* environment.

Nevertheless, the sensitivity issue and the impossibility to structurally characterize the complex still represent the limitations of all approaches. Due to its sensitivity, reproducibility, data accuracy, wealth of structural information and compatibility with aqueous solutions, ESI MS became a method of choice in ganglioside interatomics. A few valuable ESI MS approaches developed for glycolipid-protein interactions, and introduced to some extent also to ganglioside analysis, are available [38–41]. However, besides the significant gain in sensitivity and other benefits described, in the case of gangliosides, chip-nanoESI was shown to reduce the *in-source* fragmentation with particular loss of the labile Neu5Ac residues. Also, the steady spray provided by chip-nanoESI is beneficial to ETD/CID MSⁿ experiments on the protein-ganglioside complex since multiple fragmentations require stable signal of relatively high intensity.

3.2.1 Binding Assay

1. Incubate 100 μ L Ctb5 solution with 100 μ L standard GM1 ganglioside solution in the Eppendorf thermomixer under constant stirring at 37 °C (*see* **Note 21**).
2. Collect directly into the 96-well plate of the NanoMate 15–20 μ L aliquots of the reaction products after 10, 30 min, and 60 min of incubation and proceed with the MS analysis (*see* **Notes 9** and **10**).
3. Proceed with the **steps 1** and **2** described above for the binding assay of Ctb5 and the native human brain ganglioside extract.

3.2.2 Chip-nanoESI MS Analysis

1. Dismantle the original ESI source of the ITMS and the connections to the nitrogen supply and couple the mass spectrometer and NanoMate robot via the Bruker commercial interface. Follow the HCT-NanoMate coupling principles (*see* ref. 22).
2. Connect the nitrogen supply to the NanoMate, turn on all gas supplies, the mass spectrometer and the NanoMate robot.
3. Tune the ion trap mass spectrometer for operating in the positive ion mode. Set on the Compass™ software the following parameters: “positive ion mode”; HCT capillary exit: 50 V; source block temperature: 80 °C; skimmer voltage: 50 V; nebulizer nitrogen pressure: 40 psi; detection in a range of (100–3000) m/z , scan speed: 8000 m/z per sec.
4. Tune the NanoMate robot in positive ion mode. Set on the ChipSoft program the following values of the electrospray: pipette tip voltage: 0.8 kV; nitrogen back pressure: 0.30 psi; sample aspiration volume: 10 μ L; air aspiration volume: 2 μ L (*see* **Notes 11** and **12**).
5. Proceed with **steps 5–9** described in Subheading 3.1.2.

6. Select “deliver sample” on the ChipSoft software of the Nano-Mate robot and “start acquisition” on the Compass software of the ion trap MS (*see* **Note 13**).
7. Proceed with **step 11** described in Subheading 3.1.2.
8. Acquire the spectrum until a fair *signal-to-noise* ratio is obtained. Recommended acquisition time is above 1 min.
9. After stopping the acquisition, switch to DataAnalysis portal of Compass™ software, import the TIC and generate the mass spectrum by combining it over all TIC scans.
10. Proceed with **step 13** described in Subheading 3.1.2.
11. Deconvolute the signals related to the protein and the protein-ganglioside complex and determine their MW and the standard deviation using the Compound Mass Spectra and Charge State Ruler options available in the Data Analysis program (*see* **Note 22**).
12. Keep the same parameters and apply an identical procedure for the measurement of the other aliquots collected.
13. Apply the **steps 1–12** described above in this section for detecting the noncovalent complexes formed by Ctb5 and native gangliosides extracted from human brain.

3.2.3 Top-Down Noncovalent Complex Analysis by ETD/CID MSⁿ

The ETD method is highly suitable for the characterization of noncovalent protein-ganglioside complexes in top-down experiments. By ETD, c and z type fragment ions are produced; N–C_α bonds within the protein are cleaved [42] with the preservation of other linkages [43, 44], among which, certainly, the noncovalent bonds. Currently, the most efficient and modern procedure for structural elucidation of noncovalent protein–carbohydrate complexes is based on multistage fragmentation analysis with alternate ETD and CID fragmentation stages. ETD provides information on the protein structure and site(s) of the noncovalent carbohydrate attachment, while CID generates data on the structure of the linked glycan. In the case of gangliosides, the ETD/CID methodology encompasses the following steps: (1) the noncovalent complex is first subjected to ETD MS² which will produce fragment ions corresponding to peptides and ganglioside-linked peptides; and (2) following ETD sequencing, the fragment ions related to the noncovalent peptide-ganglioside complex are isolated and further submitted to CID MSⁿ; CID analysis will offer valuable data on the structure of the bound ganglioside species in terms of carbohydrate sequence, sialylation, possible identification of the Neu5Ac positional isomers and on the ceramide composition (*see* Figs. 4a, b, and 5). In some instances CID is also able to discover or confirm the noncovalent attachment of the ganglioside species to a certain aminoacid of the peptide [10]. Hence, in a single run an exhaustive structural analysis of the molecules forming the noncovalent complex can be achieved.

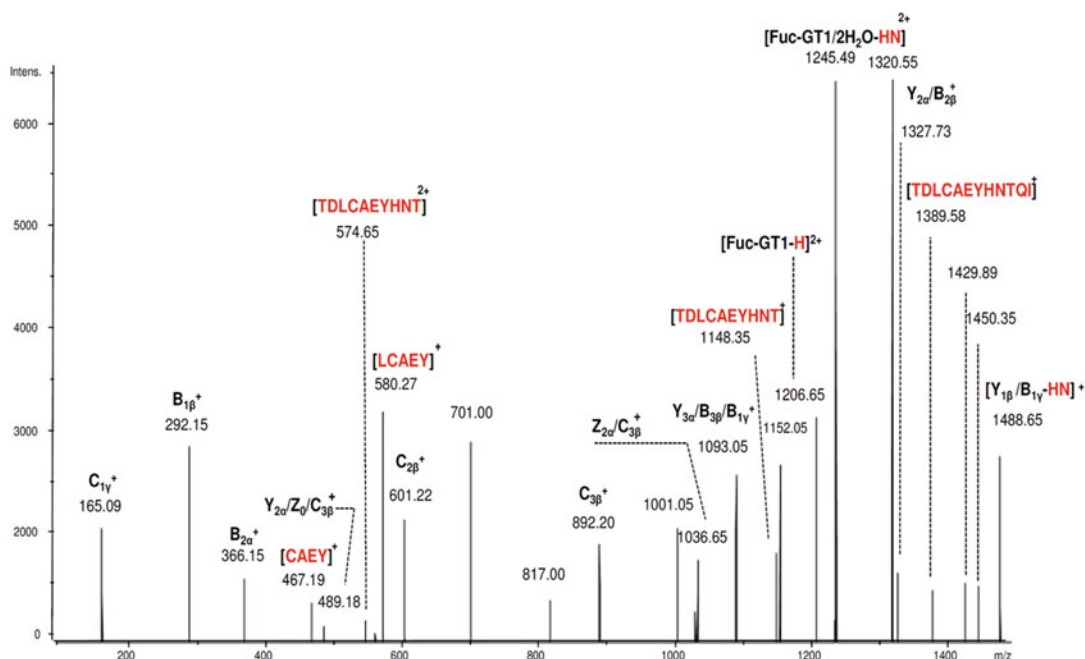


Fig. 4 ETD/CID MS³ analysis of the $[M + 2H]^+{}^{2+}$ at m/z 1832.55 having the composition (Fuc-GT1) TDLCAEYHNTQI. (a) m/z range (100–1500); (b) m/z range (1500–3000). ETD: 75 eV ionization energy; 20 ms interaction time. CID: RF amplitude: 0.4–0.8 V. The signal at m/z 1206.65 corresponding to $[Fuc-GT1-His]^+{}^{2+}$ indicates the noncovalent attachment of the ganglioside to His. Assignment of the glycan fragment ions follows the nomenclature of Domon and Costello [24]. Assignment of the peptide fragment ions follows the nomenclature of Roepstorff and Fohlman [45] (reproduced from ref. 10 with permission from Taylor and Francis)

1. Identify in the screening mass spectrum, the ions that, according to the calculated mass, correspond to the protein-ganglioside noncovalent complex (*see Note 15*).
2. Leave the MS settings unaltered, switch to ETD, set the isolation window to 2u, introduce the m/z value of the precursor ion and restart the acquisition. ETD parameters: 600 °C reactant temperature; 2.5 μ A emission current; – 4.5 V ionization chamber; 75 eV ionization energy; 20 ms interaction time between the fluoranthene anions and analyte cations (*see Note 23*).
3. Acquire the ETD MS² signal for at least 2 min.
4. For CID MS³, identify in the ETD MS² the ion of interest for additional fragmentation (the GM1-linked peptide).
5. Do not stop the acquisition, leave unchanged the settings for MS and ETD MS², select CID MS³, and introduce the m/z value of the fragment ion chosen for further fragmentation.
6. Acquire the CID MS³ signal for at least 2 min while increasing gradually the fragmentation amplitude from 0.4 V to 0.8 V

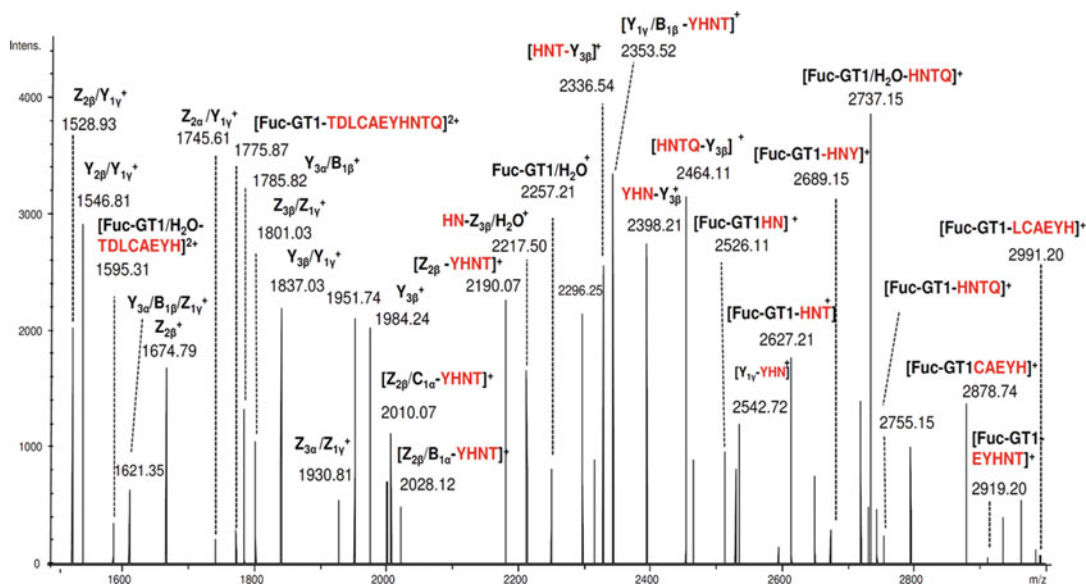


Fig. 4 (continued)

with a ramp from 30% to 200% within 40 ms per single spectrum and a fragmentation cutoff default of 27% of the precursor ion m/z (see Note 24).

7. For superior fragmentation stages, keep unaltered the settings and selections related to the MS screening and the previous fragmentation stages and proceed further as indicated above in **steps 1–3** for ETD and **steps 4–6** for CID (see Note 25).
8. Stop the acquisition, switch to DataAnalysis portal of Compass™ software, import the chromatogram, and generate the ETD and separately the CID mass spectra.
9. Calibrate the fragmentation mass spectra as described in Sub-heading 3.1.2.
10. Proceed with the **steps 1–9** described above in this section for the top-down analysis of the novalent complexes formed by Ctb5 and the native ganglioside mixture extracted from human brain (see Fig. 4a, b).

4 Notes

1. A higher ionic strength influences positively the interaction; however, it impedes the chip-nanoESI process. Therefore, 10 mM ammonium acetate concentration at pH 6.0 is a fair compromise between the complex formation and the solvent system requirements for chip-electrospray.

2. Perform this step under properly ventilated chemical fume hood to avoid formic or acetic acid inhalation.
3. Although of low toxicity, formic acid in 98% concentration is harmful for skin and eyes. Concentrated formic acid may irritate, even burn the skin and is dangerous for the eyes. Therefore, it is recommended to wear chemical splash goggles, laboratory coat and gloves (nitrile, or natural rubber). Avoid wearing shorts or open-toed shoes.
4. According to the specification of the producer, Ctb5 exhibits a purity above 95%, as determined by SDS-PAGE. However, prior to the interaction assay followed by chip-nanoESI, the protein should be desalted by overnight dialysis at 4 °C using a 10,000 Da cutoff membrane.
5. Prior to the interaction assay desalt the gangliosides by overnight dialysis at 4 °C against water using Micro DispoDialyzer systems with 500 Da cutoff dialysis membrane.
6. In the case of the native human brain ganglioside mixture an average molecular weight of 2200 g/mol should be considered for establishing the solution concentration.
7. Other high-resolution instruments with ESI and CID capabilities such as Orbitrap or FTMS may be used.
8. Other volumes of the solutions containing the interaction partners dissolved in buffer may be prepared and considered if the proper stoichiometry is chosen. The stoichiometry of 1:2.5 Glc₆:BLG was so far found to be the best suited for both complex formation and detection by chip-based nanoESI MS.
9. To reduce the interference in the reaction process during the collection procedure, do not remove the vial from the Thermo-mixer. Collect the aliquot with the pipette, close the vial and leave it for further incubation.
10. In some cases the noncovalent protein-glycan complex is formed rapidly so that after only 1 min incubation time the ions of the complex might be already visible in the mass spectrum. Nevertheless, as the reaction might be still ongoing, the aliquots of the reaction products should be further monitored at different time intervals.
11. In order to prevent sample dripping during manipulation, the robot should always be programmed to aspirate a few µL of air after sample aspiration.
12. As a general rule, prior to the binding assay, collection of the reaction products and their MS analysis, measure always the interaction partners separately in buffer for finding the conditions for their optimal ionization and detection and for assessing their individual spectra. The glycan/glycoconjugate

molecular ion should be also sequenced by CID. Knowing the spectrum of the protein in buffer and the induced charge states will allow a straightforward identification of those signals corresponding to the protein-glycan/glycoconjugate complex. On the other hand, prior knowledge of the glycan/glycoconjugate screening mass spectrum and the fragmentation pattern by CID will greatly facilitate the evaluation of the top-down experiment results.

13. If no ESI signal is detected, or the ESI process is unstable or stops immediately, proceed with the following steps: (1) at first, change the chip position with respect to the MS entrance orifice by fine adjustment “forward/back,” “right/left” in the ChipSoft; (2) increase slightly the nitrogen back pressure; (3) alter slightly the ESI voltage.
14. On a QTOF mass spectrometer, under appropriate calibration conditions, a mass accuracy of at least 30 ppm can be achieved for BLG or proteins of similar MW.
15. The most efficient fragmentation by either CID or ETD will be obtained for the precursor ion exhibiting the highest charge state. On the flip side, usually, the highly charged ions have a reduced intensity signal, which results in a low yield of fragment ions. For this reason, in selecting the precursor ion, the best compromise between the charge state and the signal intensity is to be considered.
16. In the case of oligosaccharides, the ions may also be formed as adducts with alkali metals; hence the formed noncovalent complex could be detected as an alkali adduct ion.
17. HM Res and LM Res define the resolving DC on the quadrupoles and are set to give constant resolution across the mass range. Though a broader isolation window (lower HM and LM values) appears initially beneficial to the total ion current intensity, it provides a diminished selectivity that results in simultaneous isolation of other ions (exhibiting m/z values close to those of the chosen precursor).
18. Values of the collision energy lower than 40 eV (E_{lab}) are insufficient for any fragmentation, while values above 100 eV (E_{lab}) induce multiple ring cleavages of the oligosaccharide, which complicates the spectrum and makes difficult its interpretation.
19. Under properly optimized fragmentation conditions, a top-down CID MS/MS experiment, carried out using as precursor the ion corresponding to the protein-glycan complex, yields two events (*see* Fig. 5): (1) breakdown of the protein-glycan complex; this event, crucial for the confirmation of the complex formation, is observed through the occurrence in the mass spectrum of the signals corresponding to the m/z of the

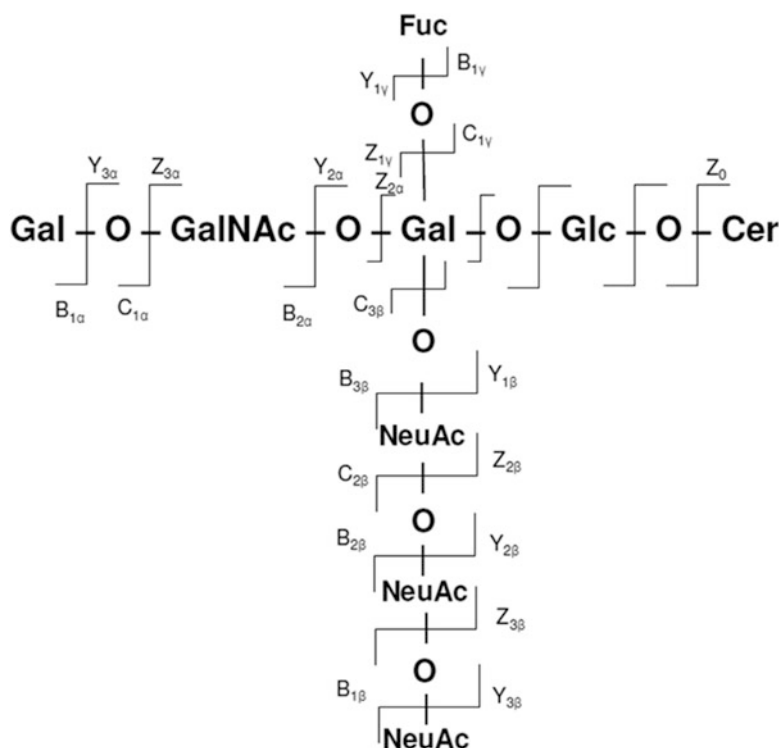


Fig. 5 CID fragmentation pathway and the structure of the bound Fuc-GT1 species. $C_{3\beta}$ corresponding to $(\text{NeuAc})_3$, $Z_{2\alpha}/Y_{1\gamma}$ having the composition $(\text{Neu5Ac})_3\text{GalGlcCer}$, and $Z_{3\alpha}/Z_{1\gamma}$ corresponding to $\text{GalNAc}(\text{Neu5Ac})_3\text{GalGlcCer}$ are diagnostic for GT1c isomer. Assignment of the glycan fragment ions follows the nomenclature of Costello et al. [46] (reproduced from ref. 10 with permission from Taylor and Francis)

protein molecular ion and the m/z of the bound glycan molecular ion; and (2) the sequencing of the released glycan ligand, indicated by the detection of glycan fragment ions in the low m/z region.

20. If instruments with ETD or ECD capabilities such as Orbitrap, FTMS, or advanced ion traps are available, a more structurally informative top-down analysis can be performed by the ETD/CID protocol described in Subheading 3.2.3.
21. Other solution volumes containing the interaction partners dissolved in buffer may be prepared, with the preservation of the stoichiometry. With the exception of GM3 [47], gangliosides form micelles in aqueous solutions. Therefore, higher concentrations are not recommended.
22. On a HCT mass spectrometer, under appropriate calibration conditions, a mass accuracy of at least 100 ppm can be achieved for Ctb5 or proteins of similar MW (58 kDa).
23. Prior to ETD fragmentation, always check the production of the fluoranthene anions. For this purpose generate the

spectrum of the fluoranthene by selecting “reactant only” option. Check the presence of the corresponding ion. The MW of fluoranthene is 202.26 g/mol; the signal of the fluoranthene radical anion is at m/z 202.26.

24. RF amplitudes below 0.40 V will result in an excitation energy insufficient for fragmentation. RF amplitudes above 0.80 V will induce extensive desialylation of the ganglioside glycan core which will impede the identification of the ganglioside isomer bound to the protein.
25. In MSⁿ experiments the intensity of the signal corresponding to the fragment ions decreases after each sequencing event. No further fragmentation is recommended after the intensity dropped below 100 counts/s.

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