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Evaluation of ion mobility for the separation of glycoconjugate isomers due to different types of sialic acid linkage, at the intact glycoprotein, glycopeptide and glycan level

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ABSTRACT

The study of protein glycosylation can be regarded as an intricate but very important task, making glycomics one of the most challenging and interesting, albeit under-researched, type of "omics" science. Complexity escalates remarkably when considering that carbohydrates can form severely branched structures with many different constituents, which often leads to the formation of multiple isomers. In this regard, ion mobility (IM) spectrometry has recently demonstrated its power for the separation of isomeric compounds. In the present work, the potential of traveling wave IM (TWIMS) for the separation of isomeric glycoconjugates was evaluated, using mouse transferrin (mTf) as model glycoprotein. Particularly, we aim to assess the performance of this platform for the separation of isomeric glycoconjugates due to the type of sialic acid linkage, at the intact glycoprotein, glycopeptide and glycan level. Straightforward separation of isomers was achieved with the analysis of released glycans, as opposed to the glycopeptides which showed a more complex pattern. Finally, the developed methodology was applied to serum samples of mice, to investigate its robustness when analyzing real complex samples.

Biological significance: Ion mobility mass spectrometry is a promising analytical technique for the separation of glycoconjugate isomers due to type of sialic acid linkage. The impact of such a small modification in the glycan structure is more evident in smaller analytes, reason why the analysis of free glycans was easier compared to the intact protein or the glycopeptides. The established methodology could be regarded as starting point in the separation of highly decorated glycoconjugates. This is an important topic nowadays, as differences in the abundance of some glycan isomers could be the key for the early diagnosis, control or differentiation of certain diseases, such as inflammation or cancer.

1. Introduction

Glycosylation is by far one of the most common and complex posttranslational modifications, with more than half of all secretory and cellular proteins being glycosylated [1–3]. Carbohydrates enhance the functional diversity of proteins, but they can also define their destination or elicit an immune response. The presence of glycans in the surface of eukaryotic cells is vital, as they take part in important cellular events, such as cell–cell interactions and receptor recognition [4]. Notwithstanding its importance and the major role of glycosylation in a multitude of biological processes [5–7], the analysis and characterization of carbohydrates is usually difficult due to their inherent complexity - the main reason why advances in glycomics have been scarcer compared to other "omics" sciences [8,9]. Very often, in contrast to

Abbreviations: ATD, arrival time distribution; CCS, collision cross section; CIA, collagen-induced arthritis; hAGP, human α-1-acid glycoprotein; hTf, human transferrin; IM-MS, ion mobility–mass spectrometry; mTf, mouse transferrin; nano-ESI, nanoelectrospray ionization; nano-UPLC, nanoultraperformance liquid chromatography; TOF, time-of-flight; TWIMS, traveling wave ion mobility spectrometry

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more linearly assembled biological molecules such as proteins or oligonucleotides, carbohydrates can form complex structures, severely branched, with many monosaccharide constituents, which usually results in a multitude of isomers [10].

Mass spectrometry (MS)-based techniques are the prime option for the characterization of glycoproteins, as reliable structural information can be obtained [7,11]. MS is frequently used in conjunction with chromatographic or electrophoretic separation techniques, as this allows high sensitivity profiling and accurate characterization of heterogeneous glycan structures [12–14]. However, when analyzing isomeric glycan structures, MS often fails to separate them [8,15–17], as they have identical mass and atomic composition. Some authors have suggested alternative strategies to separate isomeric glycoconjugates based on their derivatization, the use of capillary electrophoresis (CE) or hydrophilic interaction liquid chromatography (HILIC) [18–23]. But even then, derivatization protocols can be time-consuming, expensive or hinder the ionization of some glycans, or, in the case of CE or HILIC, the unambiguous identification is still impossible when different isomers coelute. Moreover, in the last few years, several tandem mass spectrometry (MS/MS) methods have been reported that allow the identification of glycan isomers and the characterization of their structure [24–26]. However, few authors have studied the fragmentation of glycans with different sialic acid linkages. Even then, distinguishing by MS/MS between isomeric glycans due to sialic acid linkage is not trivial and, quite often, is based on differences in the relative abundance of certain fragment ions [27,28]. Therefore, a straightforward technique that helps to separate and differentiate those isomeric compounds is much needed.

In this regard, ion-mobility (IM) spectrometry coupled with MS has aroused some interest in the last years, not only in the glycomics field but also in other omics sciences, as a proficient analytical technique for the separation of isomeric compounds [3,8,10,15–17,29–31]. Ion mobility provides an additional dimension for the separation of compounds, where ions are not only separated due to their mass and charge, but also on the basis of their shape and size - thereby resolving ions that would be otherwise indistinguishable solely by MS, such as, for instance, isomers [32–36]. Particularly, IM measures the time (drift time) that a particular ion takes to cross a cell filled with an inert, neutral background gas (N_2 and He are most commonly used) at a controlled pressure under the influence of a weak electric field. The drift time of a specific ion is mainly due to ion-gas collisions; therefore, ions are separated due to their ion-neutral collision cross-section (Ω), related to the overall shape and topology of the ion [32–36]. Small compact ions have the shortest drift times, i.e. they arrive first, as a result of their smaller Ω. Moreover, the higher the charge of the ion, the greater the accelerating electric force, and therefore the more quickly the ion will cross the chamber. Consequently, the drift time of an ion is often described as being determined by the collision cross-section-to-charge ratio (Ω/z) [35]. When coupled on-line with MS (IM-MS), ion mobility provides three-dimensional analytical information for each detected species, i.e. shape-to-charge, mass-to-charge and abundance, thus allowing reliable analyte identification.

Nowadays, there are several IM methods next to the classical drift-time ion mobility spectrometry (DTIMS), such as field asymmetric waveform ion mobility spectrometry (FAIMS), but among them, traveling wave ion mobility spectrometry (TWIMS) is the one that has seen a major growth in the last years [37,38]. In TWIMS, ions are propelled thanks to a sequence of symmetric potential waves continually propagating through a cell, each ion with its own velocity, thus different species transit the cell in different times. One of the main advantages of TWIMS is that it disperses ion mixtures, allowing the simultaneous measurement of multiple species. This, in conjunction with a high sensitivity obtained when TWIMS is coupled to certain analyzers in MS, such as time-of-flight (TOF), has made this platform an alluring option for structural analysis and isomer separation [38–40]. This platform, along with other IM methods, has been recently explored for the analysis of glycans or glycoconjugates by several authors [8,15,16,41–47].

In this work, TWIMS combined with TOF-MS was used for the study of glycoconjugate isomers which differ in the type of sialic acid linkage, with mouse transferrin (mTf) as a model glycoprotein. Sialic acid, an important monosaccharide residue of complex type N-glycans, may form primarily two types of linkages: α2–3 or α2–6. We aim to assess the capacity of TWIMS-TOF-MS (from now on referred to as IM-MS) as an analytical platform to separate α2–3 and α2–6 isomeric glycoconjugates at the intact glycoprotein, glycopeptide and glycan level. The developed methodology was also applied to serum samples of mice, to confirm its robustness when analyzing real complex samples.

2. Materials and methods

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Isopropanol (iPrOH), hydrochloric acid (HCl, 37% (w/v)), formic acid (FA, 98–100%), ammonium acetate (NH_4Ac, ≥ 98.0%) and glycine (≥ 99.7%) were supplied by Merck (Darmstadt, Germany). CNBr-activated-Sepharose 4B was provided by GE Healthcare (Waukesha, WI, USA) and "NP-40 alternative" by Calbiochem (Darmstadt, Germany). Sodium chloride (NaCl, ≥ 99.5%), DL-dithiothreitol (DTT, ≥ 99%), sodium cyanoborohydride ($NaBH_3CN$), 2-mercaptoethanol (β-ME), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), ammonium hydrogencarbonate, sodium azide (NaN_3, ≥ 99.5%) water (LC-MS grade), acetonitrile (LC–MS grade) and mouse apotransferrin (mTf, reference: T0523) were supplied by Sigma–Aldrich (St. Louis, MO, USA) and Tris(hydroxymethyl) aminomethane (TRIS, ≥ 99.5%) by J.T. Baker (Deventer, Holland). Trypsin (Sequencing grade modified) was provided by Promega (Madison, WI, USA). RapiGest® from Waters (Bedford, MA, USA) was used to facilitate the enzymatic digestion. Goat polyclonal antibody against human transferrin (hTf) (immunogen affinity purified) was purchased from Abcam (Cambridge, UK). Human transferrin (hTf) and human α-1-acid glycoprotein (hAGP) were used as additional examples of other glycosylated proteins and were also supplied by Sigma–Aldrich.

2.2. Mice and induction of arthritis

Wild-type (WT) mice were from Harlan Ibérica (Barcelona, Spain). All studies with live animals were authorized by the Institute of Parasitology and Biomedicine "López-Neyra" (IPBLN) and Universidad de Cantabria Institutional Laboratory Animal Care and Use Committees. For the induction of collagen-induced arthritis (CIA), 8–12 weeks-old male mice were immunized as described elsewhere [48,49].

2.3. Purification of serotransferrin from mouse serum samples by immunoaffinity chromatography (IAC)

In order to isolate mTf from the rest of serum proteins, an immunoaffinity (IA) purification was carried out using a cyanogen-bromide sepharose column where a polyclonal antibody against human transferrin (hTf) was bound, as detailed previously [50]. The IA procedure consisted of: first, a conditioning step with two washes of 10 mM Tris-HCl; second, approximately 25 μL of serum were diluted 1:8 in 10 mM Tris-HCl (pH 7.6–7.7) in order to improve antigen-antibody interaction, and passed through the column ten times. After washing with 10 mM Tris-HCl and 0.5 M NaCl (pH 7.6–7.7), retained mTf was eluted with 100 mM glycine-HCl (pH 2.5). Eluted mTf was immediately neutralized with 0.5 M Tris. Afterwards, glycine-HCl buffer was exchanged for water by ultracentrifugation, using Microcon YM-10 (MW cut-off 10 kDa, Millipore, Bedford, MA, USA). Then, samples were evaporated to dryness using a SpeedVac™ concentrator (Thermo Fisher Scientific, Waltham, MA, USA) and stored at − 20 °C until use. Finally,