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RESEARCH ARTICLE

Cryogenic Vibrational Spectroscopy Provides Unique Fingerprints for Glycan Identification

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□ = GalNAc ◯ = Gal ■ = GlcNAc
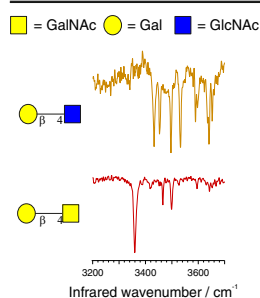
Abstract. The structural characterization of glycans by mass spectrometry is particularly challenging. This is because of the high degree of isomerism in which glycans of the same mass can differ in their stereochemistry, attachment points, and degree of branching. Here we show that the addition of cryogenic vibrational spectroscopy to mass and mobility measurements allows one to uniquely identify and characterize these complex biopolymers. We investigate six disaccharide isomers that differ in their stereochemistry, attachment point of the glycosidic bond, and monosaccharide content, and demonstrate that we can identify each one unambiguously. Even disaccharides that differ by a single stereogenic center or in the monosaccharide sequence order show distinct vibrational fingerprints that would clearly allow their identification in a mixture, which is not possible by ion mobility spectrometry/mass spectrometry alone. Moreover, this technique can be applied to larger glycans, which we demonstrate by distinguishing isomeric branched and linear pentasaccharides. The creation of a database containing mass, collision cross section, and vibrational fingerprint measurements for glycan standards should allow unambiguous identification and characterization of these biopolymers in mixtures, providing an enabling technology for all fields of glycoscience.

Keywords: Cryogenic vibrational spectroscopy, Mass spectrometry, Ion mobility, Glycans, Glycan analysis

Introduction

Glycans, which are biopolymers made up of monosaccharide units linked by glycosidic bonds, are ubiquitous in biological systems. Because they decorate the surface of cells, they play a central role in virtually all cellular recognition processes and are directly implicated in almost every major disease [1, 2]. The structural characterization of glycans is thus of utmost importance [3–5]. However, primary structure determination of glycans presents particular challenges for mass-spectrometric techniques [6–10]. Unlike linear sequences of monomers with distinct mass as found in proteins and DNA, many of the monosaccharide building blocks of glycans are

isomeric, differing only in the stereochemistry of the asymmetric carbon atoms. In addition, the carbon atom of the glycosidic bond linking monosaccharide subunits is also asymmetric, leading to isomeric α and β anomers. Moreover, two monosaccharides can have different attachment points, leading to different regioisomers. Because of their ability to support different linkage positions, a single monosaccharide unit can have multiple glycosidic bonds, leading to the formation of branched structures, which are isomeric with the corresponding linear chains of the same monosaccharide content. Finally, the large number of OH groups allow glycans to be functionalized at different locations without any difference in mass. For all these reasons, the structural characterization of glycans by mass spectrometry (MS) is substantially more complicated than that of proteins.

Mass spectrometry-based sequencing techniques, which rely on accurate measurements of the precursor ion mass and those of fragments produced either enzymatically in solution or by tandem MS (MS/MS), cannot easily determine the

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stereochemistry, monosaccharide content, and linkage positions of a glycan molecule. Information on such subtle structural details is obtained only after extensive sample preparation steps involving various enzymes and multistep MS/MS analyses. The fragmentation spectra obtained after such analyses are extremely complex and difficult to interpret. Moreover, only a limited set of enzymes that can selectively cut glycans after specific sequences are available. Because of these complicating factors, several groups have focused on the use of ion mobility spectrometry (IMS) [11–21] in combination with MS to characterize the structure of glycans. The mobility of an ion through a gas is related to its orientationally averaged collision cross section (CCS), which reflects its overall shape. Whereas in some cases the structures of different isomeric glycan species can be distinguished by their CCS, in many others cases the structural differences are too subtle to detect in this way [13]. To solve this fundamental problem, one needs to add an orthogonal dimension to these measurements that will allow the discrimination between any pair of isomeric glycans.

One possibility is to add a spectroscopic dimension to IMS–MS to achieve this discrimination, as was recently demonstrated for peptides [22]. Along these lines, Hernandez et al. [23] combined differential IMS (DIMS) with infrared multiphoton dissociation (IRMPD) spectroscopy to study monohydrated, methylated monosaccharides. Although they demonstrated small differences in the infrared spectrum of α and β anomers, the low resolution of differential IMS is unlikely to be able to separate larger species. Polfer et al. [24, 25] successfully combined IRMPD with high-resolution MS for the identification of disaccharides, but the room-temperature IRMPD spectra were too broad to uniquely identify isomeric disaccharides in a mixture. Gray et al. [26, 27] recently implemented a similar approach. Very recently, Mucha et al. [28] used helium droplets to obtain spectroscopic fingerprints of oligosaccharides, showing that sufficiently resolved spectra could indeed distinguish the various types of isomerism, albeit with an admittedly complex experimental setup. In the present work, we use messenger-tagging infrared spectroscopy in a cryogenic ion trap in combination with IMS–MS to identify and characterize glycans. The addition of the high-resolution spectroscopic dimension to mass and CCS measurements allows us to uniquely distinguish isomeric glycans that differ by their stereochemistry, linkage points, monosaccharide content, or primary sequence. The ultimate aim of this work is to acquire such data for standard glycans of known structure and create a database that can be further used to determine the composition of an unknown sample or mixture.

Experimental Methods

To achieve a vibrational spectrum of sufficiently high resolution, we use a unique instrument of our own design, shown schematically in Figure 1, in which we couple cryogenic, messenger-tagging spectroscopy in an ion trap with drift-tube IMS–MS [22].
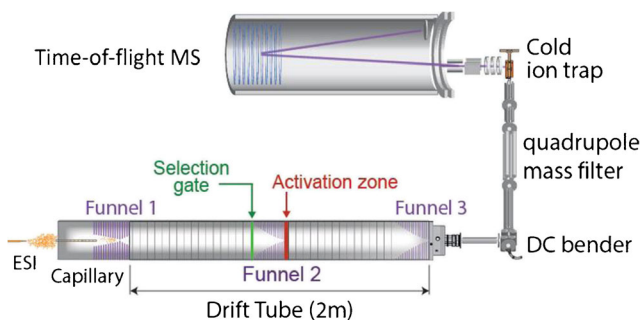

Figure 1. Our hybrid instrument, which combines an electrospray ion source with an ion mobility spectrometry drift tube, a cryogenic ion trap, and a time-of-flight mass spectrometer (*MS*), to measure the glycan mass, its average collision cross section, and its infrared spectrum. *ESI* electrospray ionization

Glycan molecules complexed with a sodium ion are transferred to the gas phase by means of nanoelectrospray. They are drawn into a vacuum through an ion funnel and injected into a 2-m drift tube, where we measure their drift time, which is related to their CCS. The central part of the drift tube is equipped with a selection region, where ions of a defined mobility can be selected and allowed to travel through the remainder of the instrument. The mobility-selected glycans, complexed with sodium, are then mass-selected by a quadrupole mass filter before being injected into a planar, cold ion trap, where they are confined and cooled by collisions with a cold buffer gas, in this case either hydrogen or nitrogen. The cold environment of the trap permits the formation of clusters in which the ion of interest is "tagged" with one or more messenger molecules [29]. Once the glycan ions have been cooled, trapped, and tagged, we extract them and interrogate the ion packet with an infrared laser pulse generated from a Nd:YAG-pumped optical parametric oscillator. If one of the tagged ions absorbs an infrared photon, the vibrational energy will be redistributed within the molecule, heating it up and leading to the evaporation of the tag(s). Using a time-of-flight mass spectrometer, we monitor the number of tagged ions as a function of the laser frequency, yielding a mass- and mobility-selected vibrational spectrum of the glycan. We thus characterize each glycan by three physical measurements: (1) its mass; (2) its rotationally averaged CCS from its drift time; and (3) its linear, high-resolution, infrared spectrum.

We have defined a protocol for data acquisition to help make such measurements reproducible across different laboratories and different instruments. The first aspect of this protocol is to produce the ions in the form of complexes with sodium cation in the gas phase. There are several practical reasons for this. First, protonation of glycans is generally difficult to achieve unless an amino monosaccharide is present in the sequence, which may or may not be the case, whereas cationization with sodium is universal. Second, sodium cations are naturally present in biological samples, and glycans tend to easily complex with them. Finally, the metal cation forms bonds with some of the hydroxy groups of the glycan and

shifts their vibrational bands outside the frequency region of the infrared laser, simplifying the remaining spectroscopic signature.

A second aspect of our protocol involves making sure that the distribution of the three-dimensional conformations that a glycan adopts in the gas phase is always the same, as this will influence the vibrational spectrum that we measure. Both IMS and cryogenic ion spectroscopy have shown that ions can adopt multiple stable conformations in the gas phase, some of which may be kinetically trapped [22, 30–33]. For this reason, we choose to anneal all the conformations produced in the nanoelectrospray ionization process, driving them to the lowest-energy gas-phase structure(s) before measuring the CCS and the vibrational spectrum (see the electronic supplementary material). In this way, our results can be reproduced without our being concerned about the gentleness of the ion source to preserve kinetically trapped conformations. It should be noted that we are not attempting to determine the three-dimensional structure of unknown glycans with this approach, but rather are attempting to make reproducible measurements that allow us to identify them from a mixture by comparing their mass, CCS, and vibrational spectrum with a database.

All the glycans used in our studies were purchased from Carbosynth and used without further purification (minimum purity guaranteed by Carbosynth 95%). Solutions containing each glycan were prepared at a concentration of 200–500 μM in a 50:50 solution of HPLC methanol (VWR Chemicals)–nuclease-free water (Ambion) with a similar concentration of sodium chloride.

Results and Discussion

To demonstrate that our technique is able to identify and characterize glycans, we performed proof-of-principle experiments on six standard isomeric disaccharides of known composition, stereochemistry, and primary structure, as depicted in Figure 2. Their drift-time distributions overlap, making them virtually indistinguishable by ion mobility, as reported previously for glycans of this size [13]. We then measured their vibrational spectra, using hydrogen as the tag molecule and keeping the trap temperature at 13 K. As one can see in Figure 2, the vibrational fingerprints for these different disaccharides are radically different from each other, providing a robust method for identification purposes.

Figure 2, parts a and b, compares the infrared spectra of the disaccharides GalNAcα(1–3)Gal and GalNAcβ(1–3)Gal, which are isomeric anomers with different stereochemistry of the glycosidic bond, but contain the same monosaccharide
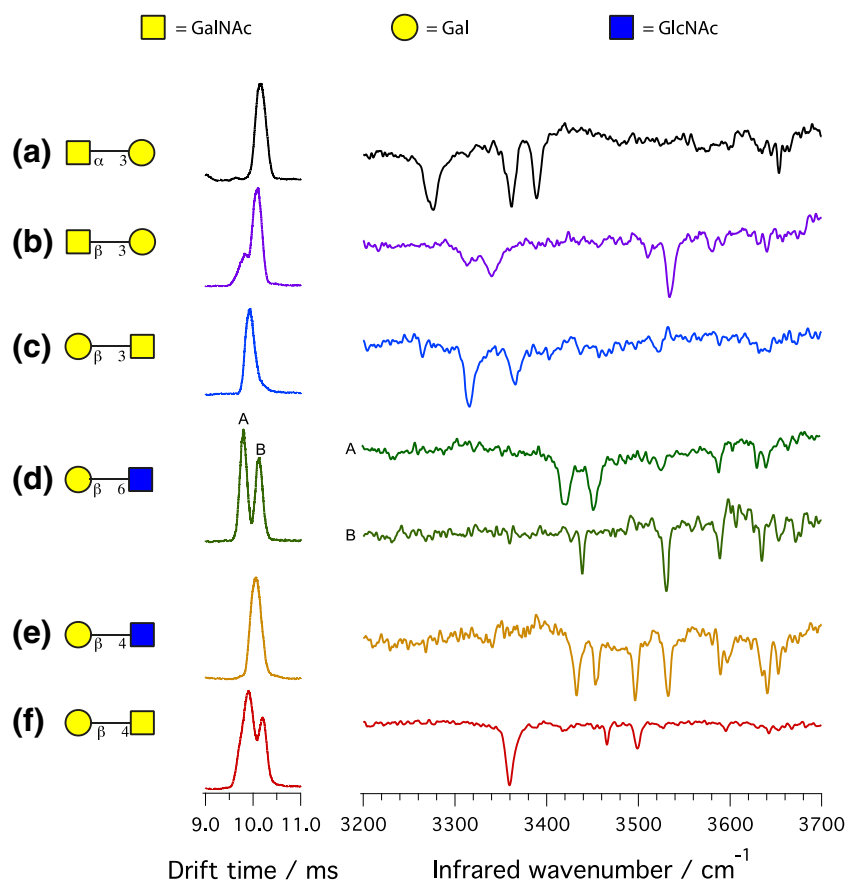

Figure 2. Drift times and infrared spectra for six isomeric disaccharides. The structure of glycans is shown with use of the University of Oxford nomenclature [34]. Each disaccharide is complexed with a sodium cation, which is not shown in this representation

content and order. Their vibrational signatures are fairly simple: GalNAcα(1–3)Gal shows major peaks at 3275, 3361, and 3388 cm^{-1}. These lines are clearly not present in the spectrum of GalNAcβ(1-3)Gal, which exhibits broader peaks at 3313 and 3339 cm^{-1}, reflecting the presence of stronger hydrogen-bonding interactions in the β anomer, and a sharp peak at 3533 cm^{-1}. This result demonstrates that infrared spectroscopy can distinguish the anomeric configuration.

Figure 2, parts d and e, shows the infrared signatures of the disaccharides Galβ(1–6)GlcNAc and Galβ(1–4)GlcNAc, which differ only by the attachment point of the glycosidic bond. One can see from the drift-time distribution that Galβ(1–6)GlcNAc exhibits two stable conformations, and these do not interconvert on annealing. This is likely due to the presence of two different complexation sites for the sodium cation. The infrared spectra of these two conformers are different: conformer A (i.e., the one with shorter drift time) shows main transitions at 3421 and 3452 cm^{-1}, whereas those of conformer B appear at 3438 and 3530 cm^{-1}. Both of these spectra are clearly different than the spectrum of Galβ(1–4)GlcNAc, which exhibits intense lines at 3432 and 3496 cm^{-1}. This observation demonstrates the ability of infrared spectroscopy to distinguish between regioisomers.

In Figure 2 parts e and f, we compare the spectrum of Galβ(1–4)GlcNAc with that of Galβ(1–4)GalNAc, molecules that differ only by the orientation of a single stereogenic center. Even in this case, the vibrational spectra of the two species are clearly different and easily identified: Galβ(1–4)GalNAc shows two features, at 3358 and 3464 cm^{-1}, that are distinct from the features of Galβ(1–4)GlcNAc. Finally, to test whether our method is able to identify disaccharides with the same monosaccharide content but different sequence order, we compare the spectrum of the disaccharide GalNAcβ(1–3)Gal and its reverse sequence Galβ(1–3)GalNAc (Figure 2, parts b and c). Once again, the vibrational fingerprints are radically different.

These experiments clearly demonstrate that the high-resolution infrared spectrum, together with mobility and mass measurements, can easily distinguish the subtle differences between isomeric disaccharides. This result would be difficult to achieve by application of MS or IMS alone, or even by application of a combination of the two.

After having demonstrated the use of our method to identify disaccharides, we now show its capability to distinguish larger isomeric species. We chose to analyze two mannose-based pentasaccharides with different shapes: 1,4-β-D-mannopentaose, which is linear, and α1-3[α1-6]α1-6[α1-3]-mannopentaose, which is branched (see Figure 3). The CCSs of the glycans complexed with sodium, reported in Figure 3, are 142 Å^2 for the linear molecule and 183 Å^2 for the branched species. The smaller CCS for the linear pentasaccharide suggests that it wraps itself around the sodium cation, forming a cluster that has a more compact shape compared with its isomeric branched partner.

We find that tagging these molecules with N$_2$ rather than H$_2$ increases the stability of the signal and hence the signal-to-noise ratio, allowing us to reduce the acquisition time and obtain better-resolved spectra. The nitrogen-tagged vibrational fingerprints for the linear 1,4-β-D-mannopentaose and the branched α1-3[α1-6]α1-6[α1-3]-mannopentaose, obtained with the ion trap at 42 K, are shown in Figure 3. As in the case of the disaccharides, the spectra are markedly different for the two species, although more congested because of the increased number of OH oscillators. Despite this spectral congestion, the spectroscopic fingerprints of the linear and branched glycans, together with their measured CCSs, allow us to distinguish between them. The peaks at 3321, 3378, and 3351 cm^{-1} for the linear species do not appear in the branched species, which exhibits unique features at 3396, 3461, and 3579 cm^{-1}. Moreover, the overall intensity pattern of the spectra is clearly different.

This experiment led us to tag all molecules with nitrogen rather than hydrogen, as N$_2$ binds more strongly to the charged molecule because of its higher polarizability and allows the ion trap to be operated at near liquid nitrogen temperature. The
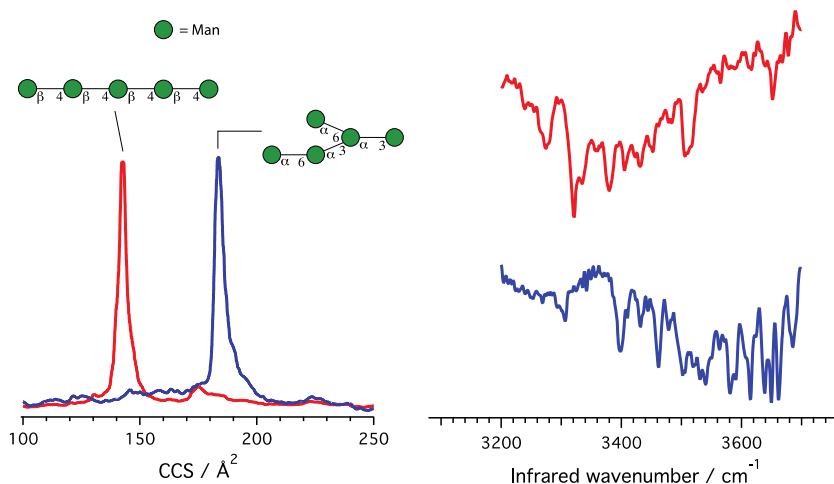

Figure 3. Collision cross section (*CCS*) and infrared spectroscopic fingerprint for a linear and a branched isomeric pentasaccharide. In this case, N$_2$ was used as the messenger tag. The structure of glycans is shown with use of the University of Oxford nomenclature [34]. Both pentasaccharides are complexed with a sodium cation, which is not shown in this representation
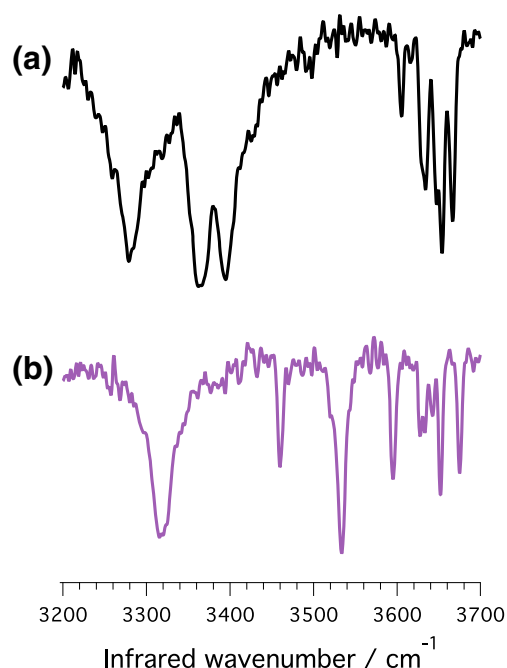
Figure 4. Infrared signatures for the α (a) and β (b) isomers of GalNAc(1–3)Gal recorded at a temperature of 69 K with N_2 as a messenger tag. The difference in the spectral fingerprint of these two molecules is clearly maintained at near liquid nitrogen temperature

only question is whether tagging with N_2 at higher temperature will give us similarly well-resolved spectra. Figure 4 shows the vibrational spectra of GalNAcα(1–3)Gal and GalNAcβ(1–3)Gal obtained when tagging with nitrogen at 69 K. For both species, the spectra exhibit similarly sharp bands with small differences in peak positions as compared with the corresponding H_2 tagging spectra (Figure 2, parts a and b, respectively), indicating that nitrogen works equally well as a tagging gas, and that the ion trap could be cooled by liquid nitrogen. This result should allow the simplification of instruments able to combine IMS with infrared tagging spectroscopy, making this technique more widely accessible.

Conclusion

In this work we have shown that the addition of cryogenic vibrational spectroscopy, using a tabletop laser, to high-resolution IMS and MS provides a powerful method to identify glycans. These proof-of-principle experiments demonstrated that we could successfully identify six isomeric disaccharides, proving that this technique is sensitive enough to distinguish compositional isomers, which is a difficult challenge for most MS- and IMS-based techniques [10]. We have also shown that this method can be applied to larger glycans. This will allow us to produce a database of vibrational fingerprints, CCSs, and masses for direct glycan identification without the need for chemical derivatization. Moreover, the ability to perform such experiments close to liquid nitrogen temperature should make this technique more broadly accessible. Finally, when

combined with selective enzymatic hydrolysis schemes [35], our approach should allow the structural characterization of unknown glycans and their incorporation into our database.

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