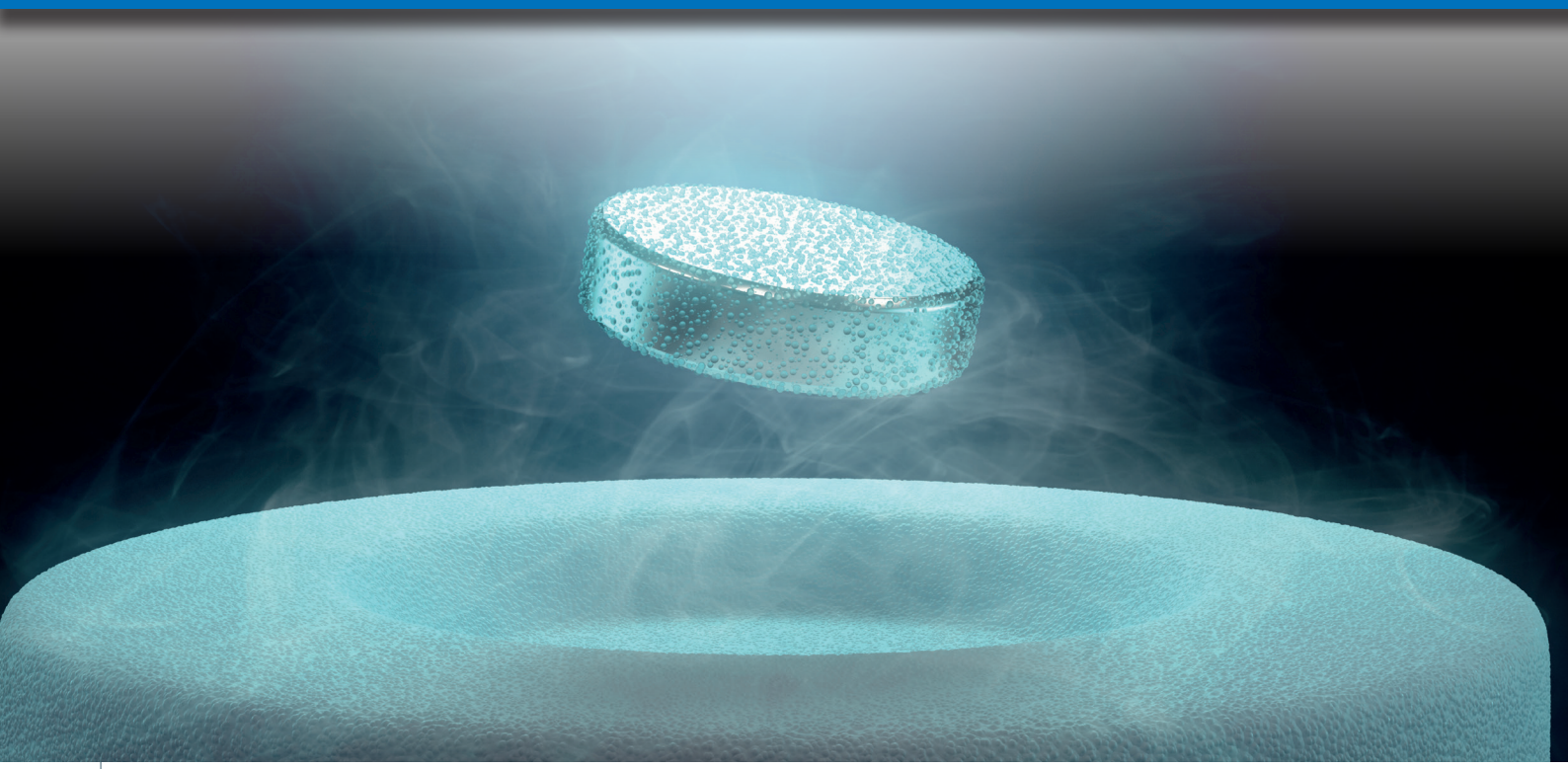


Glycan Sequencing by Electronic Excitation Dissociation Tandem Mass Spectrometry

Glycoconjugate glycans play vital roles in a wide range of physiological and pathological processes, such as embryogenesis, host-pathogen interactions, inflammation, and tumorigenesis. In the pharmaceutical industry, glycosylation is a key determinant for the solubility, stability, efficacy, and immunogenicity of recombinant monoclonal antibody therapeutics.

Introduction

Understanding the diverse functions of glycans requires determination of many structural variables, including their sequences, branching patterns, linkages, and stereochemical configurations. This presents an analytical challenge that is further exacerbated by the presence of numerous glycoforms, including isomers, in naturally occurring glycan mixtures. Unlike proteins, biosynthesis of glycans is not template-driven. There is no genome-predicted glycan structural database, and novel glycan structures can only be determined through *de novo* approaches.

Keywords: Glycan Sequencing, MRMS (magnetic resonance mass spectrometry), ExD, Electron Induced Dissociation, MS/MS, tandem MS

Authors: Yang Tang[1], Juan Wei[2], Pengyu Hong[3], Cheng Lin[2];
[1] Department of Chemistry, Boston University, Boston, MA 02215, USA; [2] Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA; [3] Department of Computer Science, Brandeis University, Waltham, MA 02454, USA

Tandem mass spectrometry (MS/MS) is a powerful structural characterization tool that has found many applications in biopolymer sequencing. For glycans, however, conventional collision-induced dissociation (CID)-based methods often fail to produce a sufficient amount of structural information for sequence and linkage elucidation. Therefore, sequential tandem mass spectrometry, or MS^n (n>2), is typically required to delineate the glycan structure, yet the MS^n approach suffers from its low throughput, lack of sensitivity, and difficulty in automation. Furthermore, MS^n analysis is usually performed on ion trap MS, and its limited mass accuracy and fragment ion co-isolation can adversely affect spectral interpretation.

A number of electron activated dissociation (ExD) methods were recently developed and applied to glycan analysis. In particular, we showed that irradiation of electrons with energy exceeding the ionization potential of the precursor ions led to a new fragmentation pathway, electronic excitation dissociation (EED), providing rich structural information in a single stage of MS/MS analysis [1-4]. Unlike MS^n, EED MS/MS may be efficiently coupled to on-line liquid chromatography (LC) or ion mobility (IM) separation for facile characterization of glycan mixtures [5-6]. A novel bioinformatics algorithm was also developed for accurate reconstruction of the glycan topology from its complex EED tandem mass spectrum [7]. In this application note, we will introduce an integrated workflow that combines EED with on-line glycan separation and automatic spectral interpretation for detailed glycan structural characterization.

Experimental

Materials

Sialyl Lewis A (SLe^A) and sialyl Lewis X (SLe^X) were acquired from Dextra Laboratories (Reading, UK). Lacto-*N*-fucopentaose V (LNFP V) was obtained from Carbosynth Limited (Berkshire, UK). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Liquid chromatography	
Instrument	Waters nanoACQUITY™ UPLC
Trapping column	Waters nanoACQUITY UPLC 2G-VM, C18, 5 µm, 180 µm ID × 20 mm
Analytical column	Waters nanoACQUITY UPLC Peptide BEH, C18, 1.7 µm, 150 µm ID × 100 mm
Mobile phase A	99% water, 1% acetonitrile, 0.1% formic acid
Mobile phase B	1% water, 99% acetonitrile, 0.1% formic acid
Gradient:	0 – 2 min 38% B
	2 – 48 min to 42% B
	48 – 50 min to 80% B
	50 – 54 min 80% B
	54 – 56 min to 2% B
Flow rate	500 µL/min
Injection volume	1 µL (approximately 2 pmol)
Column temperature	60°C

Sample preparation

Deutero reduction and permethylation were performed according to the method described previously [6]. Briefly, 2 µg of native glycans were dissolved in 200 µL of 0.1 M NH_4OH/0.25 M $NaBD_4$ solution for 2 h at room temperature, followed by addition of acetic acid (10%) until bubbling ceased. Reduced glycans were dried and resuspended in 100 µL of NaOH/DMSO mixture and vortexed for 1 h at room temperature, followed by addition of 50 µL of methyl iodide and 1 h of gentle vortexing in the dark. Additional NaOH/DMSO (100 µL) and methyl iodide (50 µL) were added followed by 1 hour of vortexing, and repeated 4 times to ensure complete methylation before the addition of 200 µL of chloroform and 200 µL of water to quench the reaction. Deutero-reduced and permethylated glycans were extracted by liquid-liquid fractionation in water and chloroform, and desalted using the Pierce PepClean C18 spin columns.

Tandem mass spectrometry analysis

All MS analyses were performed on a solariX™ Magnetic Resonance mass spectrometer (MRMS) equipped with a 12T magnet (Bruker Daltonik GmbH, Bremen, Germany). Permethylated glycans were introduced into the mass spectrometer either via static nanoelectrospray (2 µM in 50:50, v:v, methanol:water), or through LC (2 pmol per injection). Up to 2 s external ion accumulation time was used for on-line EED analysis. CID analysis was conducted with the collision energy set at 47 eV. EED analysis was performed with the cathode bias set at 16 V, and an electron irradiation time of 0.5 s. A 0.5 s transient was acquired for each mass spectrum.

Data analysis

Tandem mass spectra were processed using the DataAnalysis™ (Bruker Daltonik GmbH). Peak picking was performed with the SNAP™ algorithm using a quality factor threshold of 0.1 and S/N cutoff at 5. Peak assignment was achieved with the assistance of a custom VBA (Visual Basic for Applications) program, and ChemDraw 16.0. Automatic topology elucidation was achieved using the GlycoDeNovo software [7].

Results and Discussion

Glycans have relatively homologous elemental compositions and can generate many different types of fragments. This results in the common occurrence of isomeric and isobaric fragments that complicates tandem MS-based glycan sequencing. Peak assignment accuracies may be improved through the use of high-performance MS instruments, stable isotope labeling, and machine learning. Here, all glycans were deutero-reduced and permethylated, and analyzed in their sodiated forms. Reduction eliminates anomerism that can lead to undesirable chromatographic peak splitting; deuterium incorporation enables differentiation of non-reducing-end and reducing-end fragments; permethylation improves the glycan stability, ionization efficiency, and facilitates differentiation of internal and terminal fragment ions; and finally, sodium adduction promotes cross-ring cleavages and prevents proton-mediated gas-phase structural rearrangement.

EED provides greater structural details than CID

Figure 1 shows the CID and EED tandem mass spectra of LNFP V with their respective glycosidic cleavage maps. Although it is possible to deduce the sequence of this glycan from its CID spectrum based on the presence of the Y-ion series, no linkage configuration can be determined due to a lack of cross-ring fragments. In contrast, EED generated many linkage-diagnostic cross-ring and secondary fragments. For example, the presence of $^{1,3}A_{2\alpha}$ and $C_{2\alpha}/Z_{3\alpha}$ ions is indicative of the Gal1→3GlcNAc linkage at the non-reducing end, and the observation of $Z_{1\beta}$•-$CHDOCH_3$ ion is characteristic of the Fuc1→3Glc linkage at the reducing end. The $C_{2\alpha}/Z_{3\alpha}$ and $Z_{1\beta}$•-$CHDOCH_3$ ions likely resulted from the primary $Z_{3\alpha}$• and $Z_{1\beta}$• ions, respectively, where the location of the initial radical site dictates the type of secondary fragments that can be generated via subsequent alpha cleavages. A detailed discussion of the utility of secondary fragments in linkage elucidation can be found in a recently published article [6].

Table 1: Topology reconstruction by GlycoDeNovo. The number inside the parenthesis indicates the number of co-ranked candidates with the true topology. The SPN and IC scores of the top three ranked candidates are given, with the values for the true topology underscored.

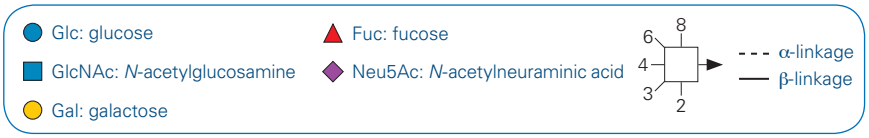
Glycan	Reducing End Modification	# Candidates	Rank by SPN	SPN of the top three candidates	Rank by IonClassifier	IC score of the top three candidates
LNFP V	D-reduced	9	1 (0)	**8**, 7, 7	1 (0)	**26**, 17, 14
SLe^A	D-reduced	19	1 (0)	**6**, 5, 5	1 (0)	**23**, 15, 14
SLe^X	D-reduced	12	1 (0)	**7**, 6, 6	1 (0)	**21**, 15, 14
SLe^A	none	14	1 (2)	**7**, 7, 7	1 (0)	**20**, 15, 15
SLe^X	none	22	1 (2)	**8**, 8, 8	1 (0)	**20**, 18, 18

Reversed-phase liquid chromatography (RPLC)-EED analysis of isomeric glycans

For glycan mixtures containing isomeric structures, isomer separation is necessary prior to tandem MS analysis. EED MS/MS can be efficiently coupled to on-line RPLC for separation and characterization of isomeric glycans. Figure 2 shows a RPLC-EED MS/MS analysis of a mixture of SLe^A and SLe^X. These two linkage isomers were baseline resolved by RPLC, as evident from the extracted ion chromatogram of the singly sodiated precursor (insets A1 and B1), where the arrows mark the retention times when the single-scan EED spectra shown in Figures 2A and 2B were acquired. EED generated complete series of glycosidic fragments for unambiguous determination of the glycan topology. Determination of the linkage configurations at the reducing-end for either isomer can be readily achieved based on the three pairs of secondary fragments labeled in red. Insets A2 and B2 show the zoomed-in regions containing the linkage-diagnostic $Z_{1\beta}$•-$CH2OCH_3$ and $Z_{1\beta}$•-$CHDOCH_3$ fragments, respectively. Scheme 1 shows the proposed mechanisms for the formation of these linkage-diagnostic secondary fragments.

Automatic topology elucidation from EED spectra

De novo glycan sequencing requires the presence of at least one glycosidic fragment, or sequence ion, at each glycosidic linkage site. Identification of all sequence ions allows the construction of an interpretation-graph for glycan topology inference. However, by chance, a non-sequence fragment can have the same mass as a combination of several monosaccharide residue masses, and may be misinterpreted as a sequence ion. Although experimental measures, such as



Abbreviations and Symbol Nomenclature for Glycans

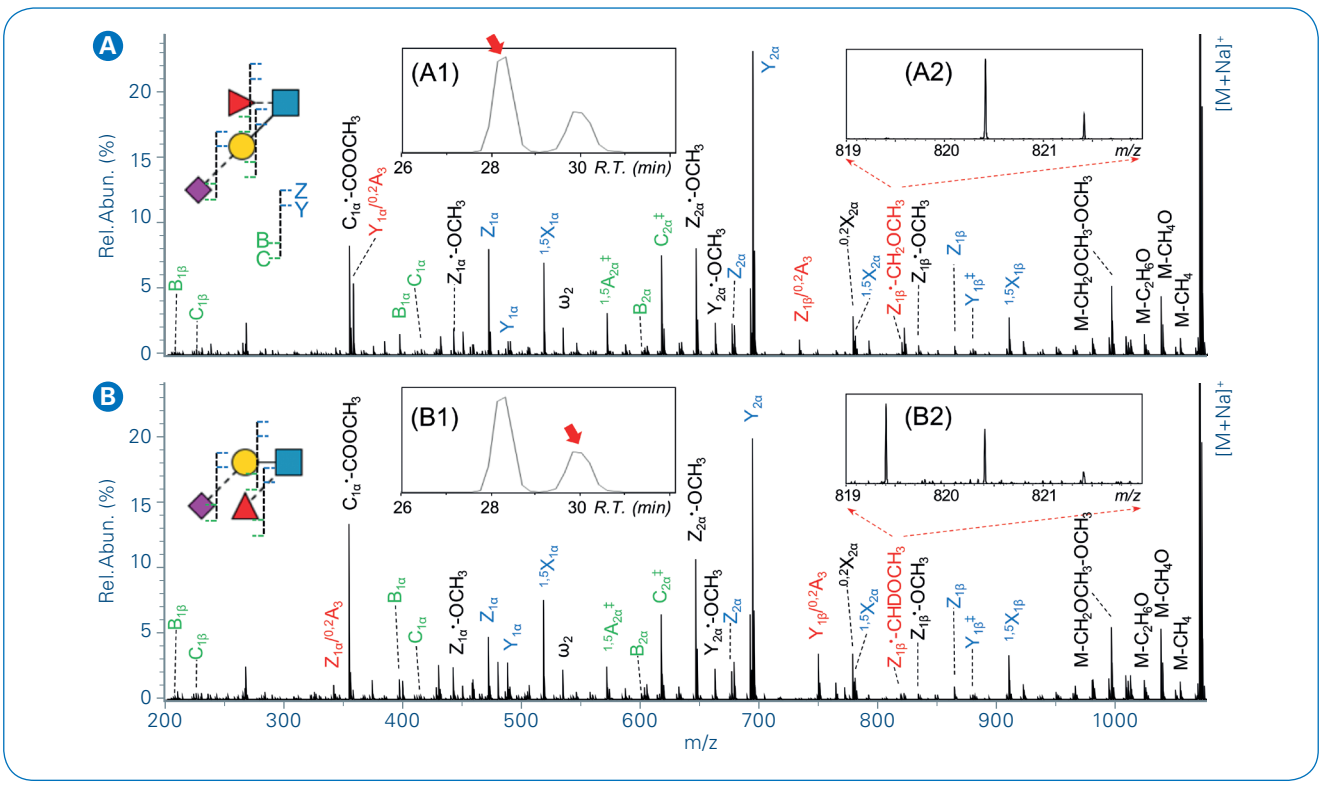


Figure 1: CID A and EED B tandem mass spectra of singly sodiated, deutero-reduced and permethylated LNFP V

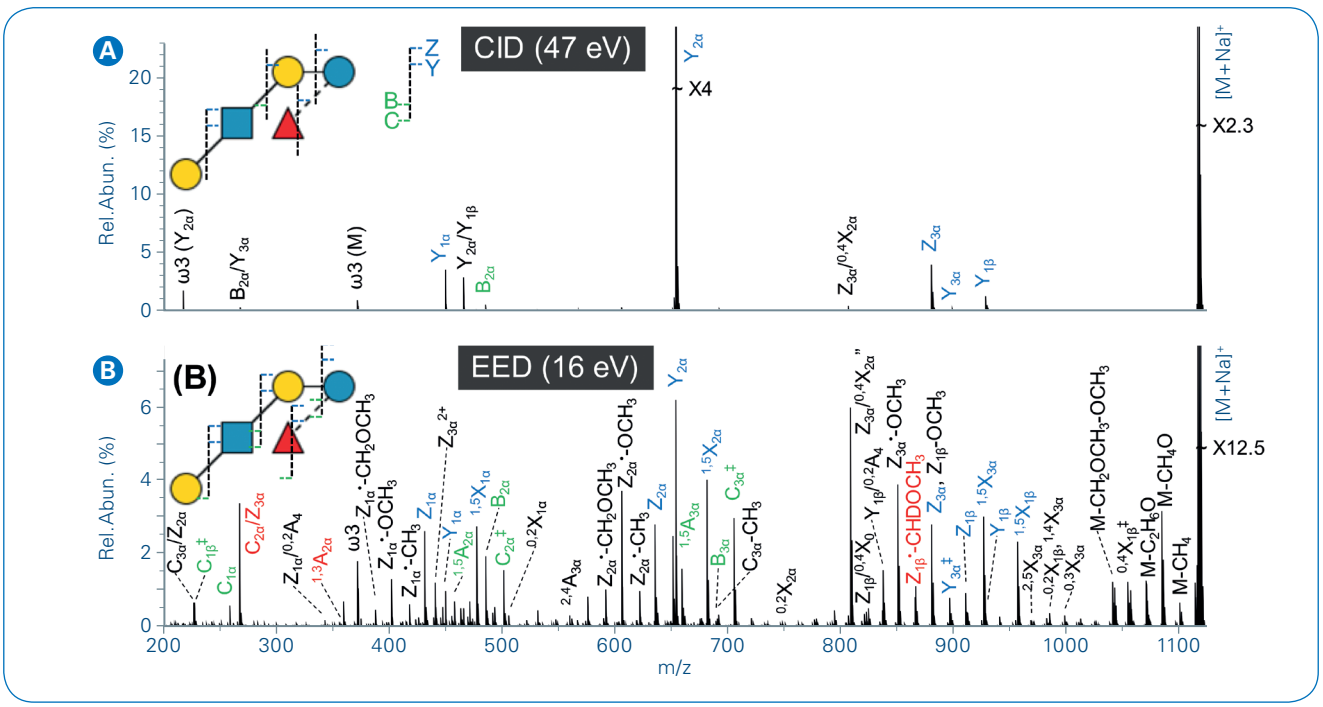
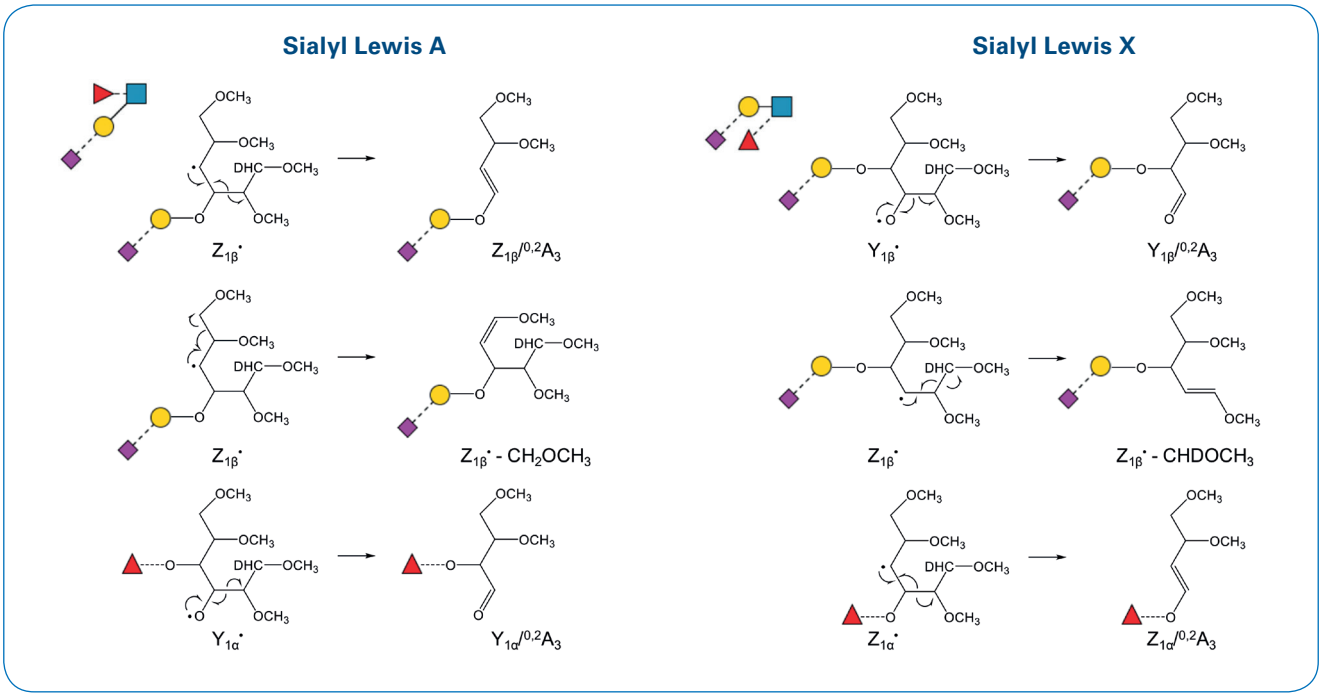


Figure 2: RPLC-EED MS/MS analysis of a mixture of deutero-reduced and permethylated sialyl Lewis A and X



Scheme 1: Proposed mechanisms for the formation of linkage-specific secondary fragments highlighted in Figure 2

permethylation and selective isotope labeling, can be taken to reduce peak assignment ambiguity, they do not eliminate misidentification due to fortuitous matches. Candidate ranking by the their supporting peak numbers (SPN) is also ineffective as revealed by a recent study [7].

We recently introduced an improved scoring algorithm in which the peak context is utilized to estimate the peak assignment accuracy. The context of a fragment is a collection of its neighboring peaks within a pre-determined mass window, expressed as mass shifts from the peak of interest. For example, a Y-ion is often accompanied by a Z-ion with a -18 Da mass shift, and a $^{1,5}X$ ion with a +28 Da mass shift (+86 Da for sialic acid residues), forming a characteristic triplet (labeled in blue in Figures 1 and 2). Given a set of EED spectra of glycan standards, it is possible to identify contextual features for each type of fragments via machine learning, allowing construction of an IonClassifier that computes the likelihood of a peak assignment being right based on its peak context. Candidate structures can then be ranked by the cumulative IonClassifier scores of all their supporting peaks. Table 1 illustrates the performance of GlycoDeNovo on topology reconstruction from the EED spectra of the glycan standards shown here. With the reducing-end isotope labeling, the true topology was correctly ranked as the top candidate in each case by either SPN or the IonClassifier. However, the IC score provides much better differentiating power than SPN. For SLe^A and SLe^X without the reducing-end modification, SPN failed to differentiate the correct topology from two co-ranked structures, whereas the IC score still ranked the true topology as the highest ranked candidate by itself. A detailed discussion of the GlycoDeNovo algorithm, the IonClassifier, and its performance on glycans with more complex structures, can be found in a recent publication [7].

Conclusions

- In this application note, we show that EED can generate far richer structural information than CID for elucidation of the glycan sequence (topology) and linkage configurations. Presently, EED of glycans has only been demonstrated on MRMS instruments, whose high mass resolving power and mass accuracy are critical for accurate peak assignments. Coupling of EED MS/MS with on-line LC separation allows characterization of glycan mixtures with isomeric structures. Finally, one can take advantage of the complexity of the glycan EED spectra to improve the confidence in fragment assignments by considering the contextual features of the peak of interest. The workflow presented here provides a powerful tool for facile and rapid characterization of complex glycan samples.

Learn More

You are looking for further Information?
Check out the link or scan the QR code for more details.

www.bruker.com/scimaX

References

[1] Yu X, Huang Y, Lin C, Costello CE (2012) *Energy-dependent electron activated dissociation of metal-adducted permethylated oligosaccharides.* Anal. Chem., **84**, 7487-7494. PMC3568493
[2] Yu X, Jiang Y, Chen Y, Huang Y, Costello CE, Lin C (2013) *Detailed glycan structural characterization by electronic excitation dissociation.* Anal. Chem., **85**, 10017-10021. PMC3851312
[3] Huang Y, Pu Y, Yu X, Costello CE, Lin C (2016) *Mechanistic study on electronic excitation dissociation of the cellobiose-Na⁺ complex.* J. Am. Soc. Mass. Spectrom., **27**, 319-328. PMC4724539
[4] Tang Y, Pu Y, Gao J, Hong P, Costello CE, Lin C (2018) De novo *glycan sequencing by electronic excitation dissociation and fixed-charge derivatization.* Anal. Chem., **90**, 3793-3801. PMID29443510
[5] Pu Y, Ridgeway ME, Glaskin RS, Park MA, Costello CE, Lin C (2016) *Separation and identification of isomeric glycans by selected accumulation-trapped ion mobility spectrometry-electron activated dissociation tandem mass spectrometry.* Anal. Chem., **88**, 3440-3443. PMC4821751
[6] Tang Y, Wei J, Costello CE, Lin C (2018) *Characterization of Isomeric Glycans by Reversed Phase Liquid Chromatography-Electronic Excitation Dissociation Tandem Mass Spectrometry.* J. Am. Soc. Mass. Spectrom., **29**, 1295-1307. PMC6004250
[7] Hong P, Sun H, Sha L, Pu Y, Khatri K, Yu X, Tang Y, Lin C (2017) *GlycoDeNovo–an Efficient Algorithm for Accurate* de novo *Glycan Topology Reconstruction from Tandem Mass Spectra.* J. Am. Soc. Mass. Spectrom., **28**, 2288-2301. PMC5647224

For Research Use Only. Not for Use in Clinical Diagnostic Procedures.

Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com