BEILSTEIN INSTITUT

Discovering the Subtleties of Sugars
June 10th – 14th, 2013, Potsdam, Germany

Tools to MSn Sequence and Document the Structures of Glycan Epitopes

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Received: 6th May 2014 / Published: 22nd December 2014

Abstract

Sequential disassembly (MSn) has been applied to fully characterise and document native samples containing glycan epitopes with their synthetic analogues. Both sample types were prepared by methylation, solvent phase extracted, directly infused and spatially resolved. Product ions of all samples were compiled and contrasted using management tools prepared for the fragment ion library. Each of the epitopes was further disassembled to confirm the multiple structural isomers probable within component substructures of linkage and branching. All native samples tested proved to be matched with their synthetic analogues and reasonably identical on either linear or cylindrical ion traps. Not surprisingly, spectra of mixed epitopes fragment independently, being uninfluenced by similarities. The approach has been coupled with computational tools for data handling and presentation.

Introduction

Glycan epitopes: components of function

Accumulating evidence indicates that carbohydrate glycans are participants in numerous functional roles as a consequence of their interactions with inter- and intracellular ligands. It is also clear that much of this activity is attributed to a small set of residues, often referred

This article is part of the Proceedings of the Beilstein Glyco-Bioinformatics Symposium 2013.
www.proceedings.beilstein-symposia.org

to as epitopes or glycotopes. Despite the functional importance of these epitopes, the current methods used leave numerous deficiencies. The protocols usually include lectin trapping, antibody and enzymatic assays, and multiple forms of chromatography in conjunction with MS and/or MS/MS for structural understanding. Epitopes (glycotopes) are frequently distal oligosaccharide substructures that mediate biological function through their interactions with various inter- and intracellular ligands [1]. Such glycotopes are involved in cell adhesion, blocking, signalling and increasingly are considered as disease biomarkers [2 – 6]. Examples of these include the Lewis (Le) series: Le^a, Le^x, and their sialylated analogues (SLe^a, SLe^x). These latter structures have been also considered to play pivotal roles in tumour metastasis [7]. Many recombinant biopharmaceutical drugs have glycosylation motifs that originate with host cell lines. Such products may be incompatible (antigenic) with human tissues. As one example, the *N*-glycan containing a Galα1 – 3Gal sequence (known as the α-Gal epitope) is commonly detected in murine-derived glycoproteins. Such α-Gal epitopes are highly antigenic to humans [8], and have even been observed in Chinese Hamster Ovary (CHO) cell lines [9]. Therefore, sensitive and reliable structural characterisation of these samples has significant implications in biopharmaceutical QA/QC applications.

Bringing glycomic analysis to comprehension

Unlike proteins, glycans are not direct gene products but are synthesised through a series of step-wise additions catalysed by glycosyltransferases and often modulated by the proximal environment [10 – 12]. Although it has been attempted [13], glycan structures cannot be predicted by following gene expression data. Instead, elucidation requires direct analysis of the sample itself. Moreover, glycans are often branched with numerous structural and stereo isomers. The stereo-chemical variations in each monomer coupled with the multiple sites of oligomer linkage results in an astonishing number of possible structures [14]. These intrinsic properties and problems make comprehensive oligosaccharide sequencing a fundamental as well as a challenging task.

Considerable effort has been exerted in the development of glycan characterisation methodologies. Different from DNA/RNA, glycan samples cannot be amplified. Therefore, sequencing technologies need to operate with minute quantities, often eliminating Nuclear Magnetic Resonance (NMR) as a solution. Structural analysis may be augmented by the use of specific glycosidases [15], but here specificity is frequently local, missing larger topological details of antennae. Additionally, troublesome are factors such as cost, availability, and impurities which all introduce challenges [16]. Currently, the presence of a specific glycoconjugate epitope is determined by lectin, antibody-binding, or enzymatic assay [17, 18]. While these methods can shed light on the underlying structures, it is not uncommon to find that the results from different methods may contradict each other and inevitably the resulting conclusions can be misleading. Importantly, many glycotopes have isomeric analogues; for example, the Le^a and Le^x antigens are structural isomers of galactose, fucose, and *N*-acetyl glucosamine (GlcNAc), with the linkage positions of fucose

and galactose reversed on GlcNAc. Often, separation techniques cannot resolve these structural isomers effectively. While liquid chromatography in conjunction with MS and MS/MS, plus glycosidase treatment, is common. Such described data often includes considerable inference and intuition of know biological systems, failing to provide a clear and complete picture [19]. In summary, the current strategies for glycan characterisations are found suboptimal [20], and MS and MS/MS applications are incomplete.

Sequential mass spectrometry (MSn): a comprehensive approach

Repetitive steps of disassembly (MSn) in an ion trap mass spectrometer provide ion compositions and spectral products that match the stereo and structural isomers of standards. Such products, along with metal binding, and know fragment pathways associated with activation provide insight for reassembly [21 – 23]. This approach has been used successfully to characterise unique and unusual structural features [24], as well as understanding to isomeric mixtures [25 – 28]. Evidence has shown that MSn spectra of glycotopes are highly reproducible and spectra of standards have been used for documentation. Library matching and *de novo* bioinformatics tools have been developed to handle these data sets [29 – 32].

Methods and Results

Analytical methods: sample preparation and mass spectrometry

In this study various standard biologicals and synthetic epitopes were prepared and processed providing fragment ion spectral products for library documentation. The workup requires methylation, solid phase extraction, and MSn analysis. Precursor ions of synthetic and natural epitopes were isolated, and each disassembled and contrasted for identity. The resulting spectra and pathways of disassembly provided opportunities to document exacting and comprehensive structural detail from the searchable fragment library.

The samples studied include: (1) *N*-linked glycans from human plasma; (2) *O*-linked glycans from human colon tissue; (3) *N*-linked glycans from human breast cancer cell line MCF-7; (4) Lacto-*N*-difucohexaose I (LNDFH I; purified from human milk), a standard containing Lewis B; (5) CFG Te118 (Fucα1-2Galβ1-4[Fucα1-3]GlcNAcβ-CH2CH2N3), a synthetic standard containing Lewis Y; (6) CFG Te140 (Neu5Acα2-3(Galβ1-4[Fucα1-3]GlcNAcβ1-3)2-CH2CH2N3), a synthetic standard containing Sialyl-Lewis X.

For human plasma samples, blood was collected in a Becton Dickinson vacutainer (East Rutherford, NJ, USA) containing sodium citrate as an anticoagulant. Plasma was separated by centrifugation and passed through a Protein A/G column ThermoFisher/Pierce (Rockford, IL, USA) to obtain IgG-depleted plasma and IgG fractions. *N*-linked glycans from IgG-depleted human plasma were released with *N*-glycanase (Prozyme).

All *N*-linked glycans were derivatised with 2-AA via reductive amination. These samples were separated by HPLC using a HILIC phase. Collected fractions were dried and permethylated. *O*-linked glycans were released via β-elimination using alkaline borohydride [33]. Samples were purified via cation exchange and porous graphitised carbon solid phase extraction prior to permethylation.

Permethylation was carried out in spin columns Harvard Apparatus (Holliston, MA, USA) as described [34]. Sodium hydroxide beads and iodomethane were purchased from Sigma. Purification of permethylated oligosaccharides was performed by liquid–liquid extraction with dichloromethane and 0.5 M aqueous sodium chloride.

LNDFH I (GKAD-02010) was acquired from Prozyme (Hayward, CA, USA). Other synthetic glycan standards were graciously supplied by the Consortium for Functional Glycomics (CFG). All standard materials were permethylated, extracted and used directly.

The methylated oligosaccharides were dissolved in 1:1 methanol/water. The samples were loaded onto a Triversa Nanomate Advion (Ithaca, NY, USA) mounted onto an ion trap mass spectrometer (LTQ, ThermoFisher). Activation Q and activation time were left at the default values, 0.250 and 30 ms, respectively. Collision energy was set to 35% for all CID spectra. The scan rate was set to ''Enhanced''. Data were acquired and are displayed in profile mode. MSn peaks were selected manually. In general, the precursor ion mass window was set to capture the full isotopic envelope. This was done to obtain complete isotope clusters of fragment ions at higher stages of MSn, and to simplify fragment ion charge state determination. For each data file, at least one scan was obtained of the isolated precursor ion with the collision energy set to 0, to record the precursor isotope envelope. The signal was averaged for a variable number of scans, with the times indicated in each spectrum. The microscan count, AGC target value, and maximum injection times were varied, depending on the signal intensity. All ions are sodium adducts.

Bioinformatics tools: spectral data handling and scoring

The MSn spectral matching based glycotope identification strategy introduces a unique component to data processing. For instance, the MSn spectra acquired are in a proprietary native binary file format, which cannot be accessed and processed directly by the existing mass spectral searching engines. Although some peak list extraction utility tools do exist, these tools are usually designed for proteomics data: only MS and MS/MS data are supported; and the tools cannot average multiple scans/microscans contained in MSn spectra. To improve spectra quality, the MSn based approach often requires multiple microscans to be collected and averaged for each target precursor. An automated MSn experiment on the Thermo LTQ can easily result in hundreds of microscans from multiple precursors. None of the existing tools are designed for this type of data handling. The desired software tool must be able to automatically (1) extract MSn spectra peak lists from native data files;

(2) preserve the MSn precursor relationships; (3) organise spectra by precursors; and (4) average microscans for each MSn spectrum. We have developed a bioinformatics package FragLib Tool Kit to fulfil these requirements.

FragLib Tool Kit is a collection of software tools designed for building MSn spectra libraries and facilitating data handling and management tasks. Through the Application Programming Interface (API) provided by Thermo Xcalibur Development Kit (XDK) COM library, FragLib Tool Kit is capable of accessing native Thermo Xcalibur raw spectral files directly. The tool can automate the tasks of MSn spectra archiving. Instead of simply compiling peak lists, FragLib Tool Kit can capture the essential information of raw data files, and convert the data into NIST MS tool which is compatible with mass spectral libraries, which have much smaller disk footprint. For instance, one recent library archive contains ~90,000 MSn spectra. The original data files require ~100 GB disk space and cannot be searched easily. The size of the searchable archive library generated by FragLib Tool Kit is only ~30 MB, which can be carried easily with a USB flash drive. FragLib Tool Kit is developed in C#. Thermo Xcalibur XDK and Microsoft .Net framework are required to install and run the tools properly. The highlights of functionalities offered by FragLib Tool Kit include: (1) Archive Xcalibur raw spectral files directly (in both interactive and daemon modes): The predefined regular archiving tasks can be automated under the daemon mode using the tool built-in archiving scheduler; (2) Search/Filter spectral archive records using multiple query constrains; (3) Support attaching structural annotation to archive records; (4) Convert archives into NIST MSP and other popular MS library formats; (5) Build mass spectral libraries in NIST format; (6) Compute spectrum matching scores to quantify the similarity between given input spectra.

Spectrum matching scores were generated using the following formula:

$$\text{Similarity score} = \cos\theta = \frac{\mathrm{u}\cdot\mathrm{v}}{\sqrt{\mathrm{u}\cdot\mathrm{u}}\sqrt{\mathrm{v}\cdot\mathrm{v}}}$$

Where "·" indicates the dot product, u and v are the aligned mass spectral intensity vectors of the two input spectra, with the intensity values ranging from 0 to 100. Using u to denote the vector of the observed spectrum and v to denote the vector of the standard, the most abundant peak has an intensity of 100 and all other peaks are normalised accordingly. The resulting similarity score is a numeric measurement of the spectral similarity, between 0 and 1, where 1 indicates that the spectra are identical and 0 indicates that no similarity exists. For our purposes, a similarity score greater than 0.850 is strongly indicative of a match using the described scoring algorithm.

A similarity score of two given mass spectra can be visualised as the cosine of the angle between the intensity vectors of the two spectra. Figure 1 depicts the visualisation of similarity score (Figure 1c) using two simplified input spectra: spectrum A (Figure 1a) and spectrum B (Figure 1b). When the two spectra match perfectly, the two intensity vectors

overlap: the angle between the two vectors is 0, so the similarity score is 1 indicating a perfect spectra match. On the other hand, while the difference between two spectra increases, the angle between the vectors increases, and the similarity score decreases indicating a greater difference between the two spectra.

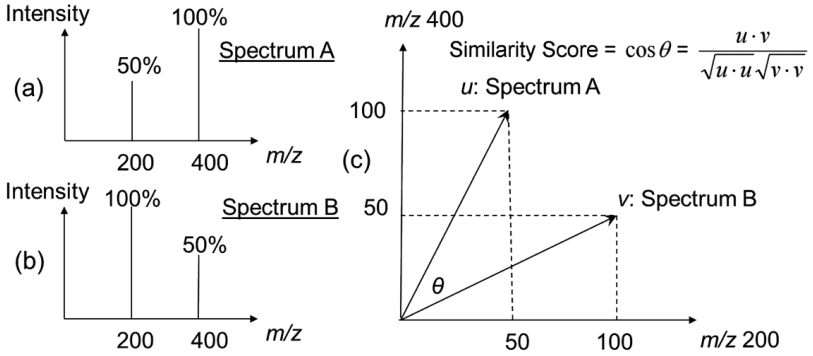

Figure 1. Visualisation of Similarity Score **(c)** using two simplified mass spectra: Spectrum A **(a)** and Spectrum B **(b)**. A Similarity Score of two given mass spectra can be visualised as the cosine of the angle between the intensity vectors of the two input mass spectra.

Application example 1: Sialylated Lewis X, B-Type Tetrasaccharide (m/z 1021) and B/Y-Type Trisaccharide (m/z 646)

Figure 2 shows a comparison of Sialyl-Lewis X substructure fragments isolated from the synthetic standard, CFG Te140, and one human plasma *N*-linked glycan. Typically, at the MS/MS stage, both *m*/*z* 646 and 1021 fragments are detectable; sometimes the *m*/*z* 1021 fragment ion is very weak or not detectable. CID of the *m*/*z* 1021 ion produces the spectra shown in Figure 2a, b, and c, isolated from CFG Te140 (Figure 2a), a human plasma *N*-glycan (Figure 2b), and a normal human colon tissue *O*-glycan (Figure 2c), respectively. These spectra are relatively simple because of the liability of the sialic acid linkage favouring formation of the *m*/*z* 646 fragment. Further, CID of the *m*/*z* 646 ion provides a much more informative spectrum (Figures 2 d, 2e, and 2f). All three samples exhibit very similar spectra, both in terms of fragment masses and the overall intensity pattern, despite the differences in sample and precursor ion structures. Figure 3 shows putative fragment assignments for this structure. As with Lewis X, the 3,5A-type cleavage across the *N*-acetyl-glucosamine residue positions the galactose residue at the 4-position rather than the 3-position. This provides additional *de novo* evidence of the SLe^x structure rather than SLe^a. Further comparison could be made between these same mass fragments generated from a sialylated Lewis A standard. Reliable mass spectrometric distinction of SLe^x and SLe^a would have significant potential utility [33]. Unfortunately, we are not aware of any suitable standard that is available. The SLe^a tetrasaccharide is commercially available, but is

unsuitable for this analysis, as it would not produce the correct B-type fragment. One could use the tetrasaccharide to prepare a glycoside (other than methyl) prior to permethylation. In our prior experience, although such structures produce the same mass fragments, they produce different intensity patterns than is obtained from cleavage of glycosidic bonds. The reasons behind this are not well understood; however, a suitable standard typically must have at least two monosaccharide residues between the epitope of interest and any aglycone functional group to be used in this way.

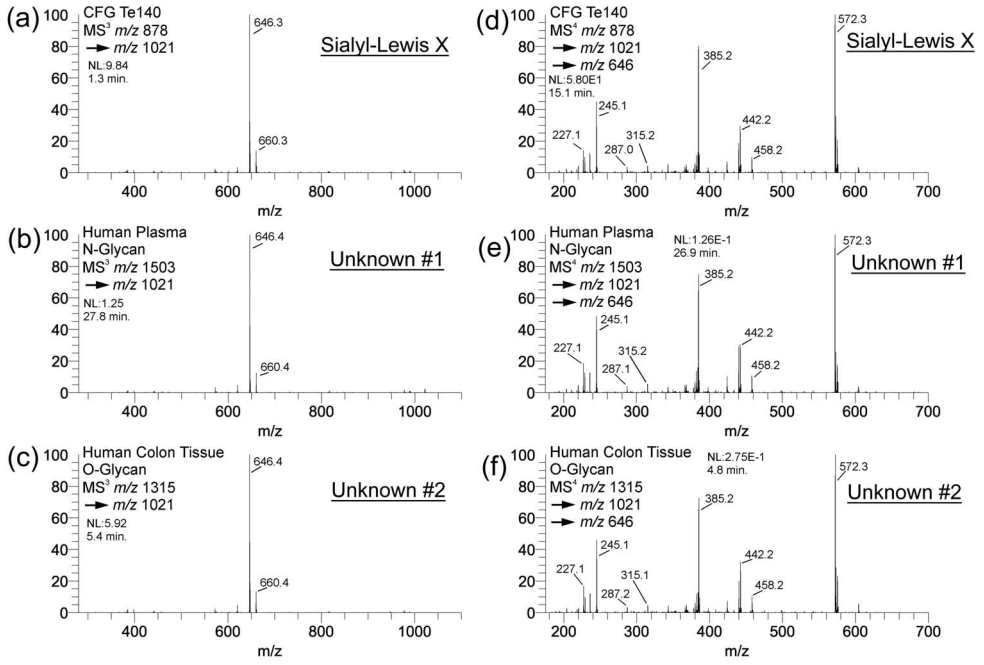

Figure 2. Comparison of B-type sialyl-Lewis X tetrasaccharide ion and B/Y-type trisaccharide ion spectra. Spectra **(a)**, **(b)**, and **(c)** show the tetrasaccharide spectra of CFG Te140 (sialyl-Lewis X standard), human plasma *N*-glycan, and normal human colon tissue *O*-glycan, respectively. Spectra **(d)**, **(e)**, and **(f)** show the trisaccharide spectra of CFG Te140 (sialyl-Lewis X standard), human plasma *N*-glycan, and normal human colon tissue, respectively. At the tetrasaccharide level, the spectra are dominated by NeuAc loss; at the trisaccharide level (*m*/*z* 646), the fragmentation is much more informative.

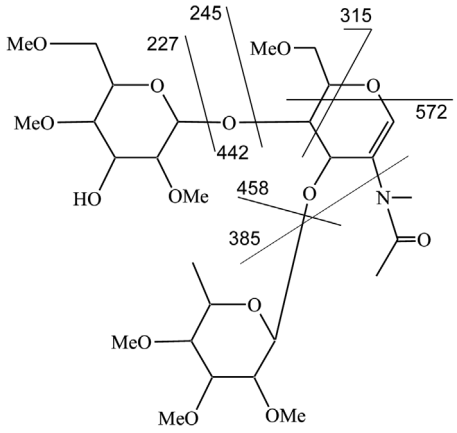

Figure 3. Structure and fragmentation of sialyl-Lewis X fragment ion m/z 646.

Application example 2: Lewis Y, Lewis B, B-Type Tetrasaccharide (m/z 834), and B/Y Trisaccharide (m/z 646)

Lewis Y is a tumour-associated carbohydrate antigen [35 – 37]. Studies have suggested that it plays a role in cell cycle perturbation [37], and it has been selected as the target of vaccine development [38]. Lewis B is also a difucosylated Hex-HexNAc, but built upon a type 1 lactosamine. Figure 4a and Figure 4 d show the MS 3 and MS 4 spectra of the tetrasaccharide B ion *m*/*z* 834 and trisaccharide B/Y ion *m*/*z* 646, respectively, for a Lewis B standard (LNDFH I). Figure 4b and Figure 4e show the same spectra isolated from a Lewis Y standard (CFG Te118). At the MS 3 level (Figure 4a and Figure 4b), the spectra show subtle intensity pattern differences, but they do not clearly distinguish these two structures. At the trisaccharide level (Figure 4 d and Figure 4e), however, the differences are much more pronounced and easily serve to distinguish the two structures. Figure 4c and Figure 4f show the fragmentation spectra with the same *m*/*z* precursor obtained from an *N*-glycan antenna (precursor *m*/*z* 16913+, composition Hex10HexNAc9dHex4) isolated from MCF-7 cultured cells. The MS 3 spectra are virtually identical to those of the Lewis Y standard (similarity score 0.952) despite the nearly three orders of magnitude greater intensity for the standard spectra (NL 1.03E4 vs. NL 4.41E1); the MS 4 spectra shown are also identical (similarity score 0.996), despite four orders of magnitude difference in intensity (NL 9.24E2 vs. NL 6.74E-2).

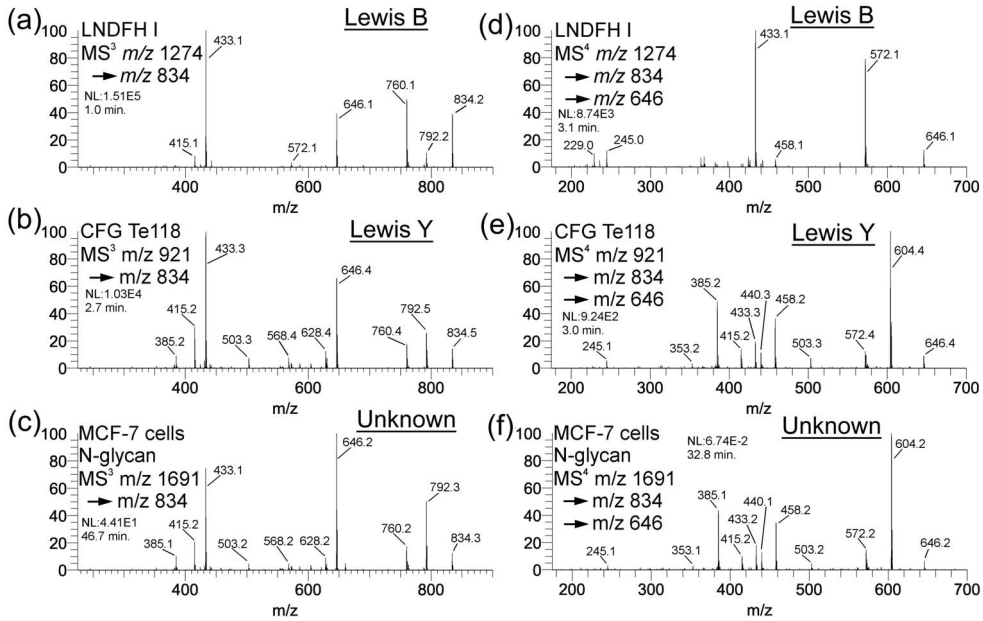

Figure 4. Comparison of spectra of B-type Lewis B and Lewis Y tetrasaccharide and B/Y-type trisaccharide ions. Standard spectra **(a) - (d)** and **(b) – (e)** were obtained from lacto-*N*-difucohexaose I (Lewis B) and CFG Te118 (Lewis Y), respectively. Spectra **(c) - (f)** represent the analogous spectra isolated from an *N*-glycan (Hex10HexNAc9dHex4, triply-charged *m/z* 16913+) of MCF-7 cultured cells.

Conclusion

Antibodies and lectins are widely accepted for performing specific analytical roles in glycomic studies; however, mass spectrometry offers a broader range of supplemental information, often with extending insight; a neutral loss, positive/negative ion extraction, alternative adducting ions and their compositions. Such considerations all contribute component understanding at minimal cost and sample loss. In addition, MSn can resolve mixtures even in the presence of multiple isomers [24, 25, 27]. While *de novo* structural assignments rely on fragment composition masses as the primary information, spectral intensity can be most specific when resolving subtle structural differences and here, synthetic standards are excellent ways of confirmation. Sample impurities can be worked with by considering alternative derivatisation strategies coupled with selective solvent phase extraction. If ionisation of the parent or precursor can be achieved MS 2 ion abundances are not influenced in product ion patterns. Structural problems of this type require comparison of spectra to standard materials with known structure and high purity to provide relevant benchmarks. A requirement for choosing suitable standard materials is that they must be larger than the substructure of interest to provide for fragment generation inside the ion trap

mass spectrometer. Ideally, standards are larger oligosaccharides containing the epitope of interest. Synthetic glycoconjugates with an aglycone, such as the azido linkers found on the CFG standards, are also useful.

Inevitable to glycomic methodological discussions are questions of sensitivity and required sample amounts. While typical sequential mass spectrometric analyses may require somewhat greater sample amounts than fluorescence chromatography or online LC-MS, the level of information acquired versus the level of structural detail needed to answer a particular question also should be considered. It has been frequently acknowledged that glycans offer a tremendous challenge because of their structural complexity and the potential for multiple isomeric forms, especially among larger oligosaccharides [14]. Although a multitude of methods are available that require very little sample, these also provide comparatively less structural information. While sample quantities may limit the depth of analysis, the informational limitations of the chosen methodologies should not be ignored, and structures that are assumed from mass composition only should also be acknowledged as such.

The possibility of isomerism in large complex oligosaccharides remains a frequently ignored or oversimplified analytical problem, owing to the inability of many common methods to approach this issue. In short, the comparison of methods primarily in terms of sensitivity disregards the relative amounts of information obtainable using the chosen strategies. Having mixtures of isomers in biological samples should be considered a routine occurrence, and the absence of isomers the unusual event. Having the library of epitope MSn fingerprinting spectra, of pure materials, can make mixture spectra more easily interpretable by realising that they can be considered as superpositions of the relevant standard spectra. Thus, spectra generated from biological materials can be matched against multiple standard spectra, of the same precursor mass, to ascertain the likelihood that the unknown spectrum is a superposition of pure spectra, and therefore a mixture of isomers.

The increasing availability of interesting synthetic oligosaccharides has made the generation of oligosaccharide MSn libraries a viable goal and will vastly extend the utility of this technique to any analysts with the appropriate sample preparation and instrumentation. Moreover, Thermo has made the API to an instrument control COM object library publicly accessible to provide a programming interface to allow for custom software codes to control Thermo IT instruments. Using the COM library API, one can control the instrument at a high level without needing to know low level hardware control details. By integrating our MSn library with the Thermo Instrument Control COM, we would be able to direct the instrument to perform data-dependent MSn experiments using prior knowledge accumulated in an MSn library. At present, we are exploring and evaluating this possibility for future development of our MSn bioinformatics tools.

Acknowledgements

The authors thank James M. Paulson (Scripps Research Institute) and the Consortium for Functional Glycomics for the synthetic standards, Robert Sackstein and Cristina I. Silvescu (Brigham and Women's Hospital) for the human colon tissue, Dipak K. Banerjee (University of Puerto Rico) for the MCF-7 cultured cells.

This work was supported in part by an NIH Program of Excellence in Glycosciences grant (P01 HL 107146, PI: Dr. Robert Sackstein) and Glycan Connections, LLC, Lee, NH 03861, USA.

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