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Structural Elucidation of N-glycans Originating From Ovarian Cancer Cells Using High-Vacuum MALDI Mass Spectrometry

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1. Introduction

- Glycosylation is a key post translational modification that has a profound impact on protein function and diversity. The altered glycosylation patterns in the cancer state have raised an interest in identifying tumour-specific glycosylations (glycans) that could aid diagnosis. Of special note is terminal fucosylation, which may play a role in tumour progression (Listinsky et al., 2011).
- Mass spectrometry has become the method of choice for elucidating the structure of tumour glycans because of its high sensitivity and ability to provide information-rich analyses from small quantities of material.
- Glycans from cellular lysate are varied, complicated and difficult to fully characterise. Furthermore, single-stage mass spectrometry (MS) is unable to resolve isobaric isomers.
- In this present work, a High-Vacuum Matrix Assisted Laser Desorption Ionization-Quadrupole Ion Trap-Time of Flight (MALDI-QIT-TOF) capable of tandem mass spectrometry (MS^n) was able to resolve N-glycan isomers and thus overcame the limitations of MS^2 instruments.

2. Aim

- To identify and characterize in high-detail medically relevant fucosylated glycans extracted from ovarian cancer cells (Fig. 1).

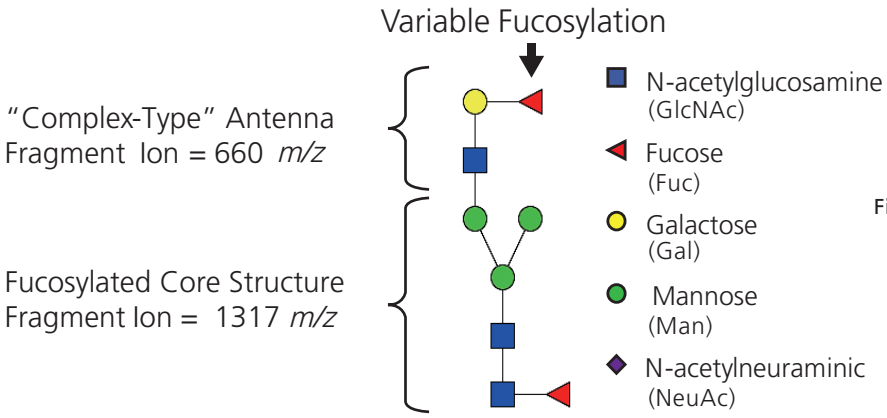

Fig. 1 An example of the "complex" type glycan. Each colored shape represents one monosaccharide, and the lines are glycosidic bonds. The Man3GlcNAc2Fuc1 core can be decorated with one to four antennae. The simplest antenna consists of an Nacetyllactosamine unit (Gal-GlcNAc), and can be modified on either the GlcNAc or Gal by fucoses (here, a Fuc is shown linked to the Gal).

3. Materials & Methods

- Glycoproteins in total lysate from 10^6 Ovarian Cancer cells were reduced with dithiothreitol and carboxymethylated with iodoacetic acid. N-glycans were enzymatically released using PNGaseF, stabilized by permethylation, and purified using C_{18} reversed-phase chromatography before being spotted onto the MALDI target with 3,4-diaminobenzophenone (DABP) matrix.
- Oligosaccharide standards (LewisX/WT Mouse Kidney, LewisA/LNFP2, H Type 1/LNFP1) were identically permethylated, purified and spotted.
- The *Axima Resonance* MALDI-QIT-TOF-MS was used for glycan analysis. 2,5-dihydroxybenzoic acid (DHB) or DABP was used as the matrix in reflectron positive mode. Glycan ions were generated under high vacuum (approx. 10^{-6} mbar) and then selected using the ion trap working with fast pressure transients – which simultaneously provided an intermediate pressure environment for thermalization and improved the trapping efficiency. Fragmentation was achieved by Collision Induced Dissociation (CID). The instrument used in this study is commercially available (Shimadzu, Manchester, UK) and the acquisition parameters used for our fragmentation experiments were set by the software controlling the instrument (LaunchPad V2.9.3).
- All the spectra were interpreted and annotated manually, aided by GlycoWorkbench (Ceroni et al., 2008) and mMass (Strohalm et al., 2010).

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MS

4. Results : MS

- The N-glycan mixture from the Ovarian Cancer cell line was analysed with MS.
- Sodiated N-glycan ions were assigned based on compositional analysis and knowledge of the human N-glycan biosynthetic pathway.
- Low abundant N-glycans were detected, demonstrating the high sensitivity of the instrument (Fig. 2).
- N-glycans with terminal fucosylation were identified and were chosen for MS^2 analysis to confirm their general structure.

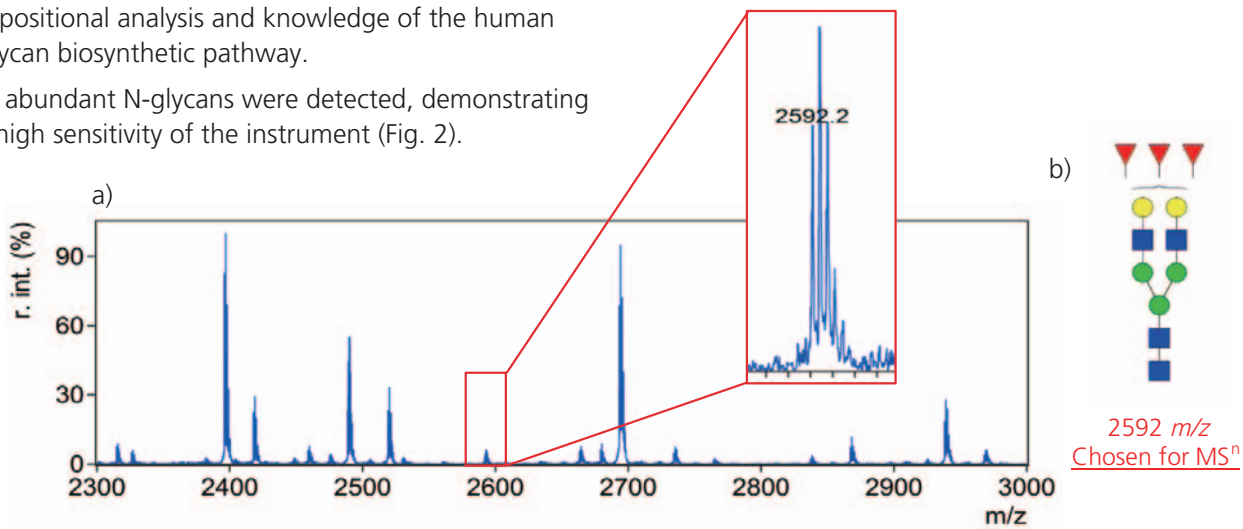
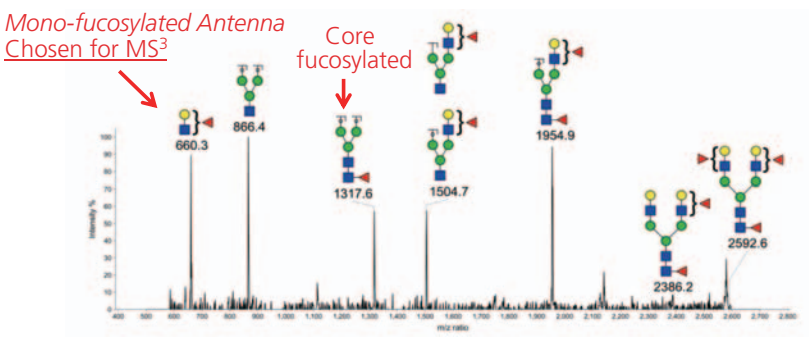


Fig. 2 a) Partial mass spectrum (2300-3000 *m/z*) of permethylated N-glycans isolated from an ovarian cancer cell line. The Axima Resonance has high-resolution and excellent sensitivity, able to identify even the low abundance triply fucosylated N-glycan at 2592 *m/z* b) The triply fucosylated Nglycan at 2592 *m/z*.

MS^2

5. Results : MS^2

- In MS^2, antenna were fragmented from the core structure of the 2592 *m/z* glycan (Fig. 3).
- A 660 *m/z* fragment corresponding to a mono-fucosylated antenna was observed.
- Fragments corresponding to a loss of one and then both mono-fucosylated antennae from the parent ion corroborated this observation.
- Therefore, the 2592 *m/z* N-glycan had two antennae, each modified with a single fucose.
- Four positional isomers of the mono-fucosylated antenna exist in humans: LewisA, LewisX, H Type 1 and H Type 2 (Fig. 4).
- To determine the position of fucose, the 660 *m/z* ion was subjected to further fragmentation (MS^3).



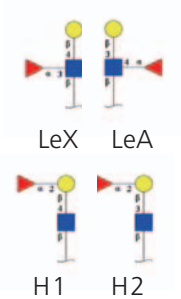


Fig. 4 Fucosyl LacNAc Isomers

Fig. 3 MS^2 fragments of N-glycan 2592 *m/z* showing that it was core fucosylated, and had two monofucosylated antennae. Brackets indicate an ambiguous linkage of terminal fucose. The exact linkage of fucose could not be determined by MS^2, therefore the 660 *m/z* fragment was chosen for MS^3.

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6. Results : MS³

- A spectral fingerprint for each isomer (H Type 1, Lewis A, Lewis X) was generated by fragmenting the 660 *m/z* ions derived from the standards (Fig. 5a-c, bottom spectra in green, orange and red respectively).
- Spectral fingerprints were compared to the fragmentation pattern of the 660 *m/z* fragment derived from the 2592 *m/z* N-glycan (Fig. 5a-c top spectra in blue colour).
- The 329 *m/z* ion in the Ovarian Cancer samples is the cross-ring cleavage $^{3,5}A_{GlcNAc}$, a theoretically unique fragment for Lewis X (Fig. 5c).
- Ions at 472 and 454 *m/z* represented the loss of Fucose from the 660 *m/z* ion and an additional loss of water.
- The ratio of intensities: 472/454 is an indicator of glycan linkage because Fuc (1-3) GlcNAc tends to lose Fuc-H_2O when the Fuc dissociates (in the case of LeA).
- Fuc (1-4) GlcNAc does not lose H_2O when the Fuc is leaved (in the case of LeX).
  - Ratios of ion intensities of 472/454 were:
  - Lewis A = 18.6
  - Lewis X = 3.6
  - Sample = 2.4
- Therefore, the sample's 660 *m/z* spectral fingerprint matched Lewis X the best.

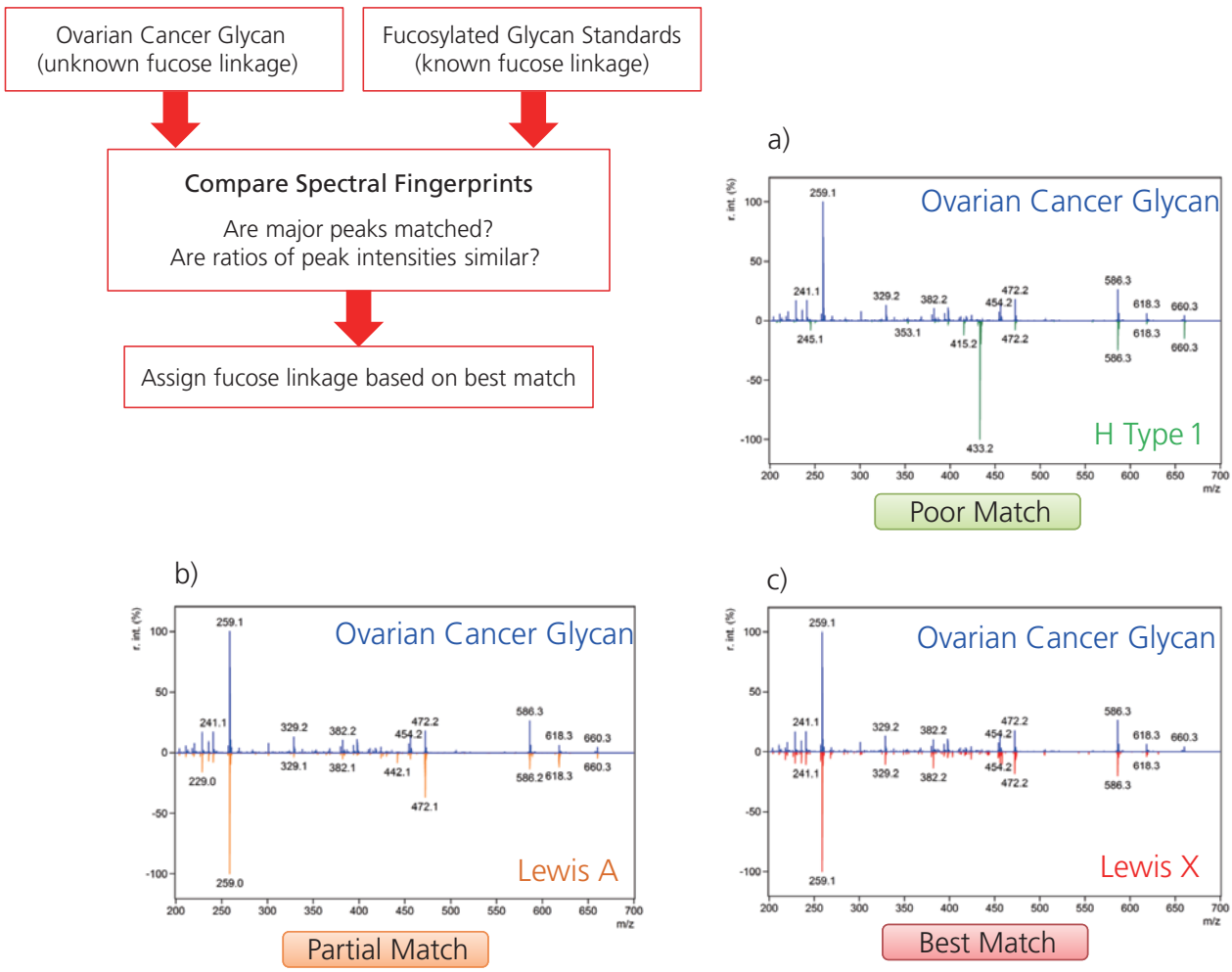

Fig. 5 Comparison between the spectral fingerprints of the Ovarian Cancer 660 *m/z* ion and the glycan standards. (c) Lewis X had the best match based on the unique 329 *m/z* major peak and the ratio of intensities of 472/454 *m/z*, while (b) Lewis A had a partial match due to structural similarities with Lewis X. (a) H Type 1 was a poor match. Therefore the unknown N-glycan on Ovarian Cancer was identified as having the Lewis X antennae.

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7. Discussion

- The MS^2 and MS^3 fragmentation of the sample's N-glycan at 2592 *m/z* was matched to a complex biantennary structure with Lewis X antennae.
- Lewis X has been implicated in breast cancer (reviewed by Listinsky et al., 2011), and therefore may potentially act as a biomarker for Ovarian Cancer.
- A biomarker could be important for diagnosis or therapy, but needs to be evaluated for clinical significance.

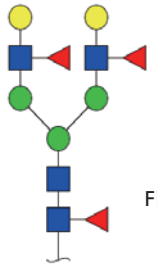

Fig. 6 The confirmed structure of the 2592 *m/z* glycan consisting of twoLewis X antenna and core fucosylation, characterized by MS^n.

8. Conclusions

- Even with a complex biological sample, MALDI-QIT-TOF was able to systematically characterize individual N-glycans from cancer.
- Library of spectral fingerprints from standards allows rapid assignment of N-glycan antenna fucosylation without the need for fragmentation pathway analysis.
- Complements traditional enzymatic/lectin methods because fucose linkages can be rapidly determined and glycans are directly observed.
- MS^n has the potential to speed up cancer glycan biomarker discovery and offer more concrete proof of the presence of certain glycan structures.

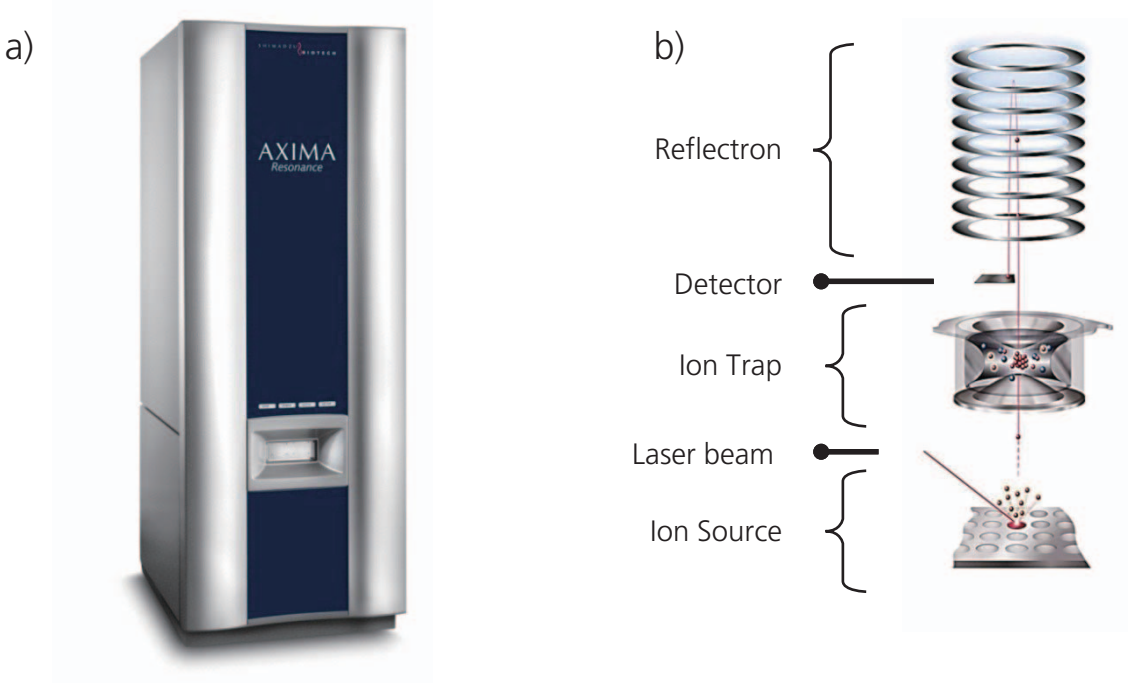

Fig. 7 (a) The Axima Resonance MALDI-QIT-TOF instrument, and (b) its internal configuration consisting of a MALDI Ion Source, Ion Trap, Reflectron and Detector.

9. Acknowledgements & References

- Anna Parry, for generously donating the Lewis X standards.
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