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PO-CON1240E

An MS^n platform for detailed characterisation of both the peptide and the glycan moieties and the peptide/glycan linkage in glycoproteins.

IMSC 2012 PTu-006

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Introduction

The analysis of glycoproteins has become essential in light of the recent developments in the biosimilars /follow-on biologics market. As increasing number of pharmaceutical companies have begun research in the field of therapeutic proteins and peptides, interest has significantly increased in terms of creating and manufacturing recombinant versions of biologically active proteins using *E. coli*, yeast, human or CHO cell expression models. Therapeutic proteins are much more complex than lower molecular weight drug compounds. The different expression models are able to produce diverse modifications (e.g glycosylation) that may differ from those naturally found in the original innovator biopharmaceutical. The current EU and FDA regulation regarding what may be classified as a biosimilar is very strict and requires extensive characterisation of the recombinant protein, in particular the glycosylation sites, the glycan structure and the protein sequence.
The technique described here, enables the accurate determination of the glycan linkage site, the sequence of the peptide it is attached to and the precise isomeric structure of the glycan moiety. This is achieved using a combination of MS^n analysis and novel software solutions.

Experimental

Human apo-transferrin was reduced and alkylated prior to tryptic digestion and glycopeptide enrichment. The enriched sample was analysed by MS^2 and MS^3 using a MALDI-QIT-TOF mass spectrometer (Axima *Resonance*, Shimadzu, UK). These data were investigated using in-house SIMSE software (Shimadzu Corp., Japan) for glycopeptide characterisation (glycan composition, peptide sequence and identification of the peptide glycan linkage).

In parallel, the digested sample was treated with PNGase F and the resulting glycans isolated using a combination of C18 and graphite materials and labelled with amino-pyridine. These purified glycans were analysed using Accurate Glycan Analyser - AGA (Shimadzu Corp, Japan) software-directed multi-stage MALDI mass spectrometry combining upto MS^4 spectra to unambiguously identify the specific glycan isomeric structure.

Software solutions

CID of N-linked glycopeptides exhibits a characteristic cleavage pattern (triplet of peaks) that corresponds to a single HexNAc molecule bound to the peptide (peptide + 203 Da), the peptide linked to a ring cleaved $HexNAc(^{0,2}X)$ (peptide + 83Da) and the peptide with no glycan.

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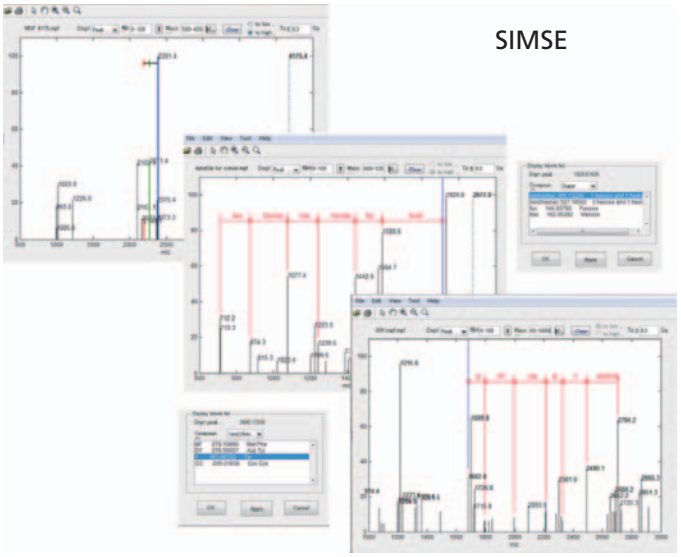
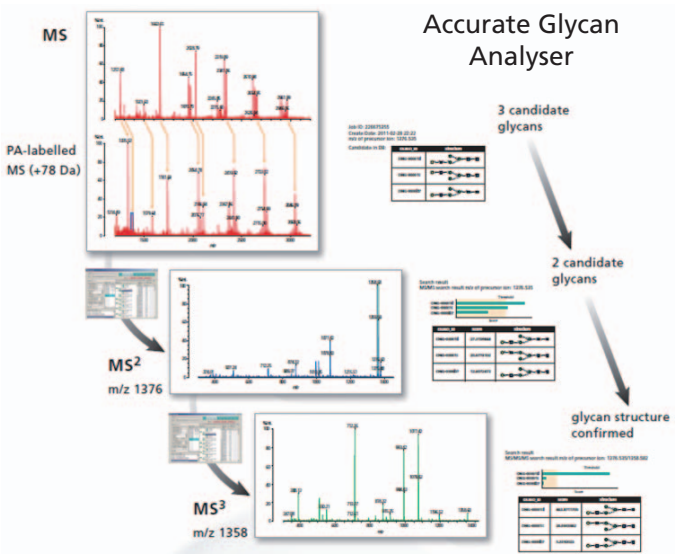
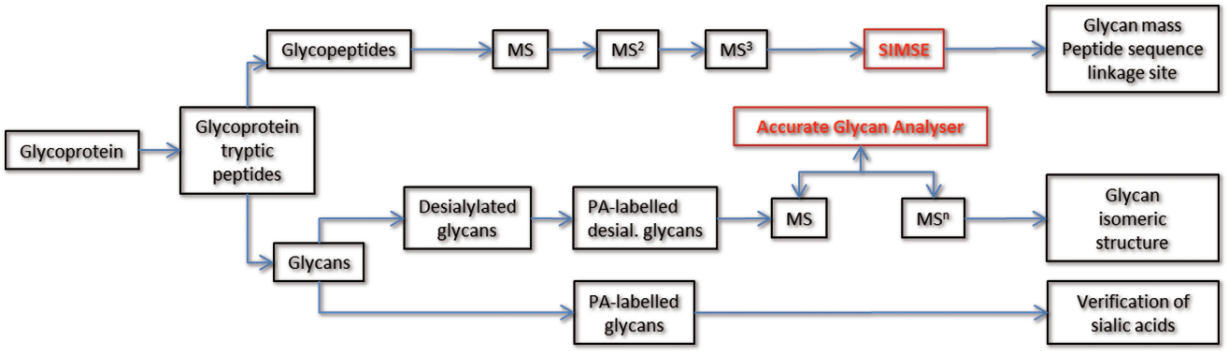




This triplet of peaks is used by SIMSE to determine the respective molecular weights of the glycan and the peptie moieties. Furthermore software guided *De-Novo* sequencing within SIMSE provides the peptide sequence. However, SIMSE can only provide the general composition of the glycan – mass, number of Hexose, HexNAc, etc and linkage – but not the precise isomeric structure.

For structural isomer characterisation of the glycan moiety, AGA software uses MS to MS^4 analyses and a populated database of real MS and MS^n glycan spectra. Determination of the correct glycan structure is achieved using a combination of the MS and MS^n data, relative intensities of the fragment ions and a probabilistic scoring scheme to differentiate isomeric glycan structures.

Workflow



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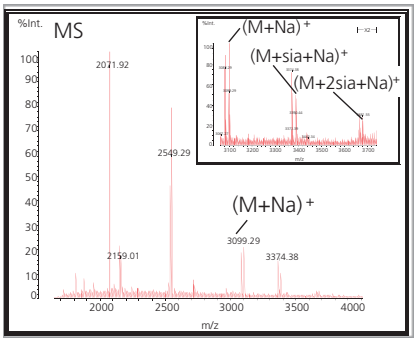
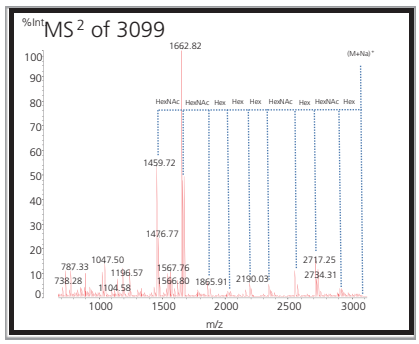
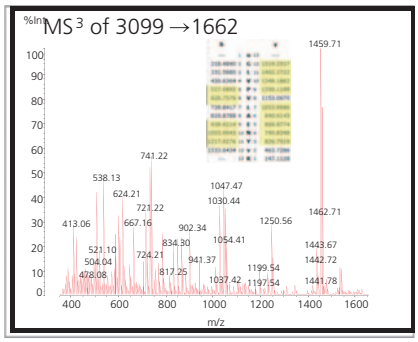
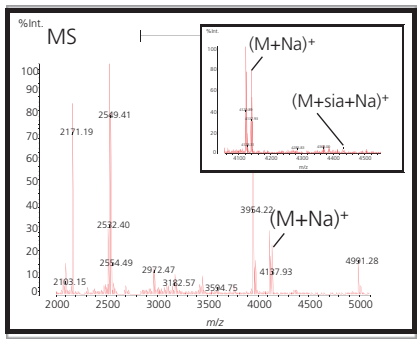
SIMSE results for transferrin GP1

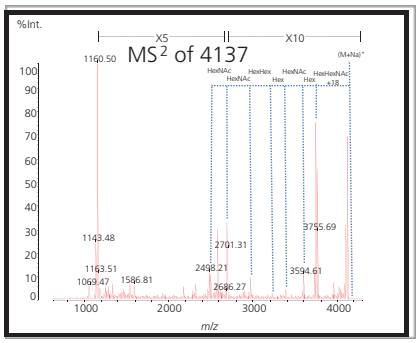


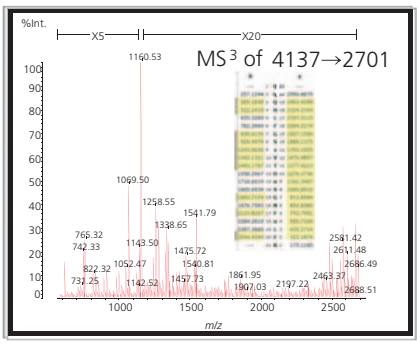




SIMSE results for transferrin GP1



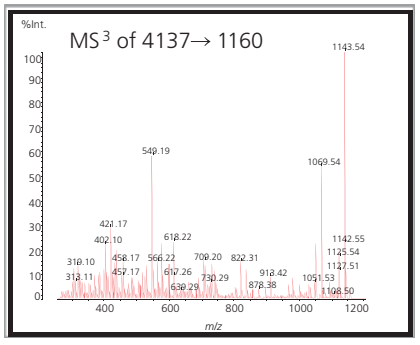




The peptide sequences of the glycopeptides GP1 (CGLVPVLAENY**N**K *m/z* 3099) and GP2 (QQQHLFGS**N**VTDCSGNFCLFR; *m/z* 4137) were characterised by combining the results from MS^2 and MS^3 analyses.

In the MS^2 spectra of GP1 and GP2, SIMSE identified the characteristic triplet of peaks indicative of the peptide/glycan bonds at Asn 432 (*m/z* 1476.77, 1559.76 & 1679.85) and Asn 630 (*m/z* 2498.18, 2581.2 & 2701.29).

The general composition of the glycan was surmised from the mass difference between glycopeptide and the lowest *m/z* peak in the triplet. The glycan mass was calculated to be 1641.52Da (mass difference + H_2O + 1 proton) and equates to a structural composition of 5 hexose residues and 4 N-acetyl-hexoseamine residues (Hex 5, HexNAC 4).



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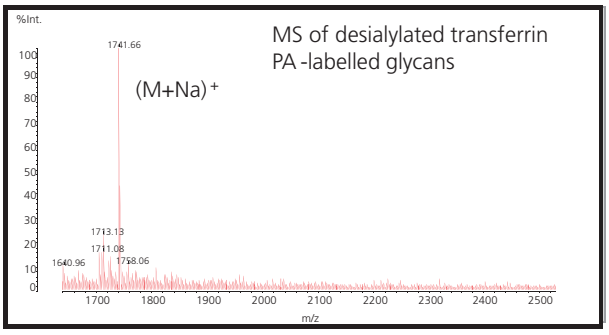
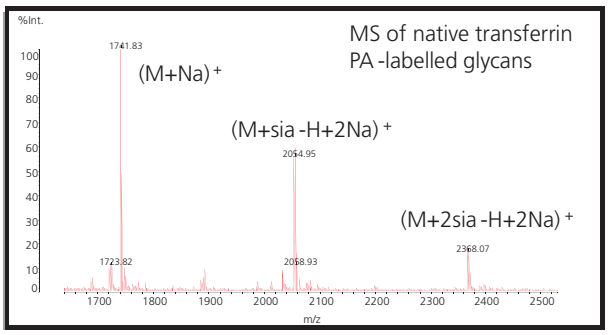
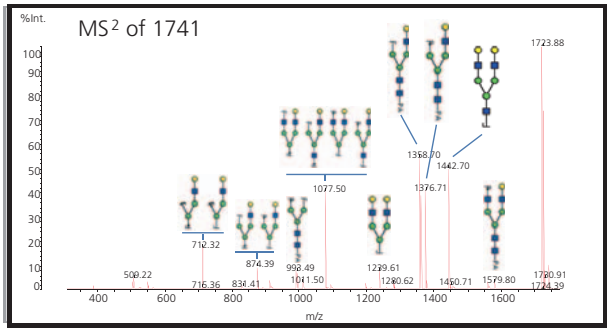
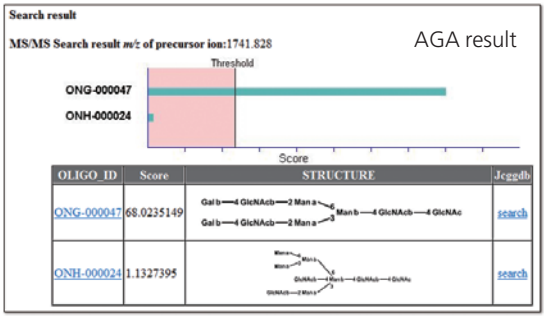
AGA results for human transferrin









MS, MS^2 and MS^3 of the PA-labelled PNGaseF cleaved glycan (*m/z* 1741.66, (M+Na)$^+$) using Accurate Glycan Analyser software provided the precise bi-antennary glycan structure for transferrin GP1 and GP2. Furthermore the comparison of the MS profile for native and desialylated PA-Labelled GP1/GP2 glycans, indicates that this glycan also appears with one (*m/z* 2054.92, (M+Sia-H+2Na)$^+$) and two sialic acids (*m/z* 2368.07, (M+2Sia-H+2Na)$^+$) attached.

Summary of SIMSE and AGA results

Glycopeptide	Sequence /linkage	Peptide mass (M+H)+	Triplet peaks	Inferred glycan mass	Glycan Structure	Glycopeptide mass (M+H)+	No. of sialic acids
GP I	CGLVPVLAENYNK	1476.77	1476.77 1559.76 1679.85	1622.53		3099.34	0
						3390.44	1
						3681.55	2
GP II	QQQHLFGSNVTDCSGNFCLFR	2515.18	2498.18* 2581.22* 2701.29*	1622.62		4137.93	0
						4428.76	1
			*(loss water)		Not observed	-	-

Conclution

The combination of the Axima *Resonance* / *Accurate* Glycan Analyser platform and SIMSE software package provides a powerful tool for complete glycoprotein characterisation. In addition to the glycopeptide sequence, this platform delivers the glycan linkage site, the glycan composition and most importantly the glycan isomeric structure.

First Edition: September, 2012

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