State-of-the-art LC/MS methods applied to the characterisation of a highly-glycosylated fusion protein: Etanercept

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INTRODUCTION

A large number of pharmaceuticals are glycosylated proteins. An adequate glycosylation is critical for therapeutic proteins in terms of safety, bioactivity, solubility, stability, and pharmacokinetics and dynamics. Consequently, their glycosylation profile must be thoroughly analysed. However, these proteins are typically produced in different expression systems, whose glycosylation machineries function through sequential and competitive steps, hence creating heterogeneities of glycosylation (nature of glycans, number and location of sites). This creates a challenging analytical puzzle that requires a number of orthogonal analytical techniques, at different levels of analysis (released glycans, peptides, intact and subunits), to be solved.

Etanercept is a tumor necrosis factor-α (TNFα) antagonist, commercialised as Enbrel® for the treatment of rheumatoid arthritis, psoriasis, psoriatic arthritis, and ankylosing spondylitis. Below, we present the use of LC(/MS) methods to characterise both the N- and O-glycosylation of Etanercept in a reduced amount of time (sample preparation, data analysis), and from limited protein amounts.

COMPREHENSIVE ANALYTICAL WORKFLOW

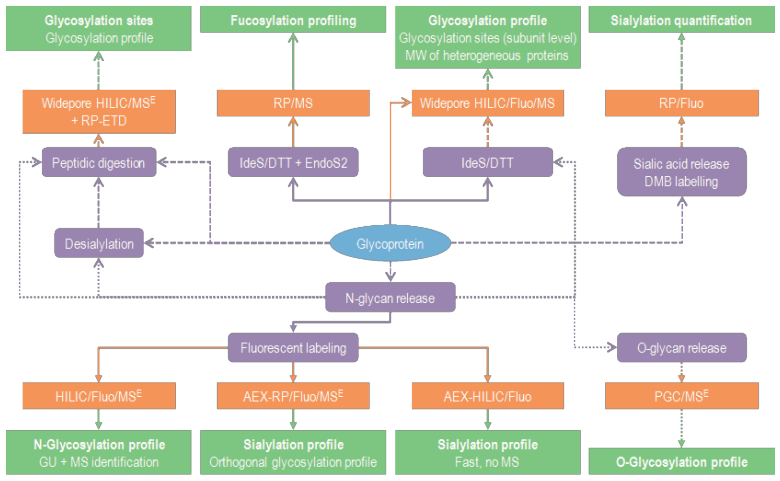

Figure 1: Analytical workflow for the characterisation of the glycosylation of Etanercept

RELEASED N-GLYCANS PROFILING

The most common way to study the N-glycosylation of proteins is to analyse the N-glycans released by an endoglycosidase, followed by labelling with a fluorescent moiety. Separation in the HILIC mode is often used.

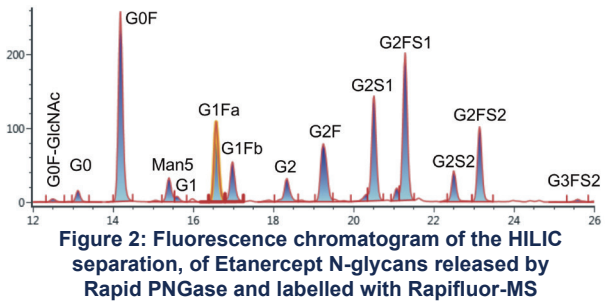

Figure 2: Fluorescence chromatogram of the HILIC separation, of Etanercept N-glycans released by Rapid PNGase and labelled with Rapifluor-MS

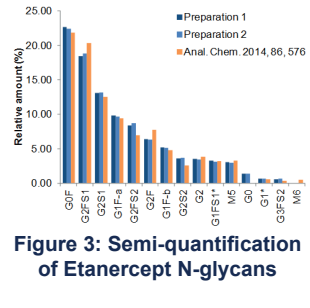

Figure 3: Semi-quantification of Etanercept N-glycans

- N-glycan release and labelling performed with RapiFluor-MS labelling kit (Waters), starting from **15 µg of protein, in less than an hour.**
- Analysis by HILIC/FLR/MS: Identification based on dextran ladder calibration coupled with glycans database.
- Identifications **semi-automated** and **confirmed by MS**, with mass errors below 3 ppm.
- Relative quantification of two independent sample preparations, on different days, yields **very little deviation** (1.5% average RSD), and is consistent with data from the literature (*Anal. Chem.* 2014, 86, 576).

N-GLYCANS SIALYLATION PROFILING

Sialylation can significantly influence **the safety and efficacy** of protein-based drugs, notably related to their **half-life** and **immunogenicity**. It is thus critical to profile the sialylation of N glycans, as it is also a **useful measure of consistency** during manufacturing.

Sialic acids bear a negative charge, which makes anion exchange (AEX) chromatography a method of choice for their separation. We developed two mixed-mode LC separations, namely **anion exchange-reverse phase (AEX-RP)** and **anion-exchange-hydrophilic interactions (AEX-HILIC)**, compatible with both 2-AB and RapiFluor-MS labelled N-glycans.

Anion-exchange-hydrophilic interactions analysis

- AEX-HILIC gradient developed to **group various sialylation levels in single peaks.**
- Allows determination of sialylation profile in **reduced amount of time**, with **straightforward peak integrations**, and no MS hyphenation.
- Presence of neutral (42%), monosialylated (41%), and disialylated (17%) glycans exclusively, which is consistent with RapiFluor-MS profile.

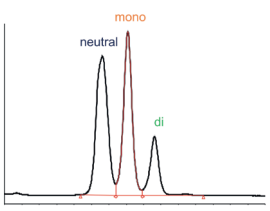

Figure 4: Fluorescence chromatogram of the AEX-HILIC separation of 2-AB labelled N-glycans

Anion-exchange-reverse phase / MS analysis

- AEX-RP developed to confirm N-glycan profile and sialylation profile.
- **Grouping by sialylation levels** obtained but with resolution of individual glycans.
- Co-elutions observed in HILIC mode can be resolved with this **orthogonal LC separation.**
- **Identification based on MS** only.
- Confirmation by **MS^E fragmentation.**
- **Sialylation profile** of N-glycans consistent with other methods.

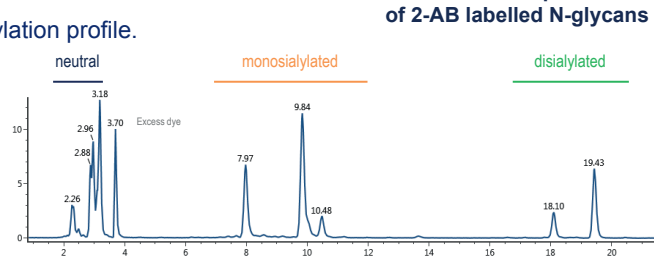

Figure 5: Fluorescence chromatogram of the AEX-RP separation of 2-AB labelled N-glycans

SITE-DEPENDENT N-GLYCANS PROFILING

Peptide mapping is the method of choice for the determination and **profiling of glycosylation sites at the amino-acid level.** There are **three concensus sites** on Etanercept, two on the TNFα receptor, and one the Fc subunit.

- Classical tryptic digestion yields 64-amino acid peptide containing two glycosylation sites. **Triple digestion** (trypsin, Glu-C and Asp-N), with complementary cleavage sites specificity yields **small peptides with no more than one site.**
- **Glycosylated peptides completely separated** from non-glycosylated ones on **widepore HILIC column**, facilitating their discovery.
- Peptides **identified by MS**. Presence and identity of glycans confirmed by high-energy MS^E data.
- Typical glycosylation found for IgG1 Fc subunit (Asn317; G0, G0F, G1F, G2F, and M5). **All sialylated species found on two sites of TNFα receptor subunit** (Asn149 and 171).
- Overall profiling (assuming similar response factors) yielded **similar relative responses** to RapiFluor, and only two low-intensity glycans remaining undetected (G1 and G3FS2).

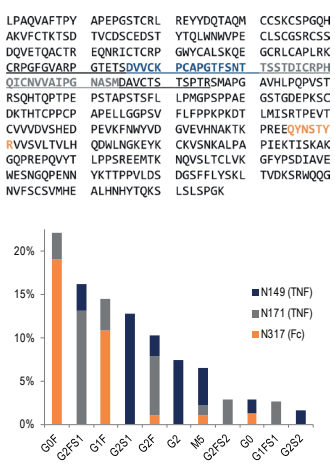

Figure 6: Profiling of the N-glycosylation sites

RELEASED O-GLYCANS PROFILING

There is no universal enzyme that cleaves all O-glycans. We opted for a chemical O-deglycosylation, where a common issue is glycans degradation by a peeling reaction. We therefore developed a **high-yielding alkaline β-elimination procedure**, using reducing conditions to prevent most of the peeling to take place by formation of a stable alditol.

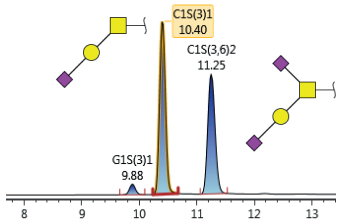

Figure 7: ESI/MS profiling of Etanercept O-glycans

- Analysis performed by **porous graphitic carbon (PGC) chromatography**, coupled to MS in negative ion mode.
- 1-mm diameter column allows profiling unlabelled O-glycans from **low protein amounts** (2.5 µg / injection).
- **Two main core 1 O-glycans** detected with mass errors below 3 ppm, and confirmed by use of automated MS/MS fragmentation.
- **Peeling product** also identified, in **much smaller amounts** than alternative methods (*Anal. Chem.* 2014, 86, 576).

O-GLYCOSYLATION SITES DETERMINATION

Each Etanercept chain carries **89 putative O-glycosylation sites.** Therefore, to reduce the glycosylation heterogeneity of Etanercept, the protein was **N-deglycosylated and desialylated,** yielding exclusively asialylated core 1 O-glycans. Peptides generation and identification were performed as described for N-glycans.

- Ten O-glycosylated peptides **fully separated in the wide pore HILIC mode** and MS identified
- Presence of glycans prevents typical b/y fragmentations of peptide backbone, and hence sites determination at amino-acid level for peptides containing multiple putative sites.
- **ETD fragmentation** targeted on glycosylated peptides carried out, yielding c/z peptide fragmentation, ultimately allowing determination of each O-glycosylation site, as well as occupancy.
- **Thirteen sites unambiguously characterised** with **average occupancy of 9.4 sites**

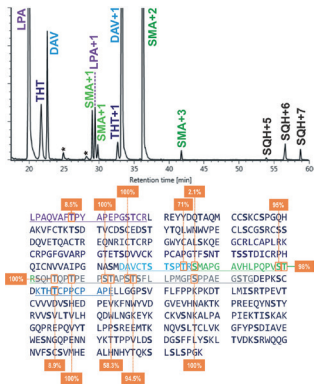

Figure 8: Identification of O-glycosylated peptides and subsequent O-glycosylation sites determination

O-GLYCOSYLATION SUBUNIT-LEVEL ANALYSIS

Subunit analysis is a **straightforward methodology** because it does not require long sample preparation steps. Etanercept was **digested with IdeZ** (Promega), cleaving the TNFα receptor and Fc moieties, then **N-deglycosylated** and **reduced *in situ.***

- Subunits separated by **HILIC chromatography**, monitored by intrinsic fluorescence and MS.
- TNFα receptor subunit displays large heterogeneity of O-glycosylation and thus yields very broad peak.
- **Automated and systematic approach identifies 31 unique species** carrying from 5 to 12 O-glycans per chain, with remarkable mean error of 9 ppm, below 5 ppm for 25 most intense hits

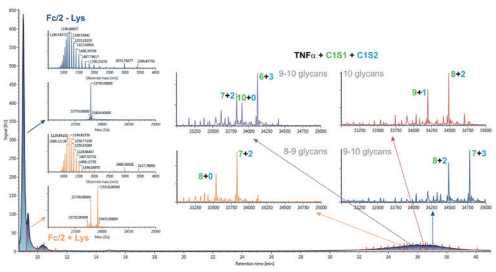

Figure 9: Fluorescence chromatogram of etanercept subunits separated on a widepore HILIC column.
Raw and deconvoluted mass spectra of the Fc/2 (left) and example of four deconvoluted mass spectra of the TNFα receptor subunit (right)

- Most abundant species conjugated by 9 or 10 glycans (35% and 47%, respectively), with **average of 9.5 O-glycans per chain**
- **Gives access to molecular weight** of Etanercept, normally challenging because of heterogeneity of glycosylation