A novel and rapid strategy for labelling *N*-glycans and glycopeptides for qualitative analysis using UHPLC-ESI-MS

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Introduction

Glycosylation can have significant effects on the clinical safety and efficacy of biopharmaceuticals[1,2]. As a result, both innovator drugs' and biosimilars' glycan profiles need to be fully characterized during the product lifecycle, from development to commercialization including batch-to-batch consistency and release to the market[2]. Additionally, a vast amount of research has been performed on the identification of disease biomarkers based on the glycosylation of biological samples[3,4,5]. Due to the high demand and institutional pressure for biopharmaceutical characterization[6] and the large amount of samples at a clinical setting that must be processed[7], there is a need for the development of a rapid, sensitive and selective method for glycan and glycopeptide analysis, reducing protocols from days to hours. Glycosylation analysis both at the *N*-glycan and glycopeptide level provide complementary information. The *N*-glycan analysis gives specific information about the glycan structure and linkages by exoglycosidases analysis while glycopeptide analysis is a more favourable approach for the characterization of site-specific glycan compositions.

This study aims to develop a new label for both *N*-glycan and glycopeptide analysis that allows the development of a rapid analytical method to reduce time and cost. This label's structure is based on 3 main principles:

a. US008124792B2 patent, Baginski's technology based on compounds and methods for rapid fluorescent labelling of *N*-glycans within 1 day.

b. LudgerTag™ V-Tag (Ludger) based on 2-ANSA, which is used to analyse and relatively quantify glycopeptides and N-glycan species (after trypsin or PNGase F release, respectively) within 1 day (Fig. 1b). This method has been validated according to ICH Q2 (R1) guidelines and is currently in the market.

c. I-tag, imidazolium label with a permanent positive charge (Fig. 2, compound 3), with properties of an ionic liquid with greater spectral peak intensities and lower limits of detection[8].

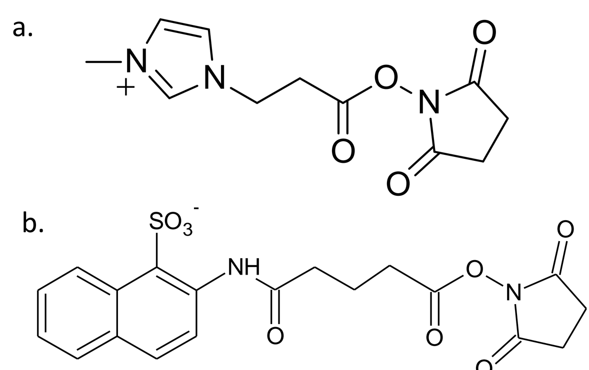


Figure 1. a. Novel label for rapid labelling N-glycans and glycopeptides for qualitative analysis using UHPLC-ESI-MS, b. V-Tag.

Therefore, this new label, 1-{3-[(2,5-dioxopyrrolidin-1-yl)oxy]-3-oxopropyl}-3-methyl-1H-imidazol-3-ium (Fig. 1a), has an N-hydroxysuccinimide group as a good leaving group and an imidazolium group for improved MS signal.

Method

Chemical synthesis of novel label

The synthesis of this new label is achieved in two steps (Fig. 2). The first step consists of a nucleophilic substitution by an amine (**2**) followed by an esterification reaction between I-tag (**3**) and N-hydroxysuccinimide (NHS). The purification of compound 4 was not performed to avoid loss of material, therefore labelling experiments were performed with the crude reaction mixture.

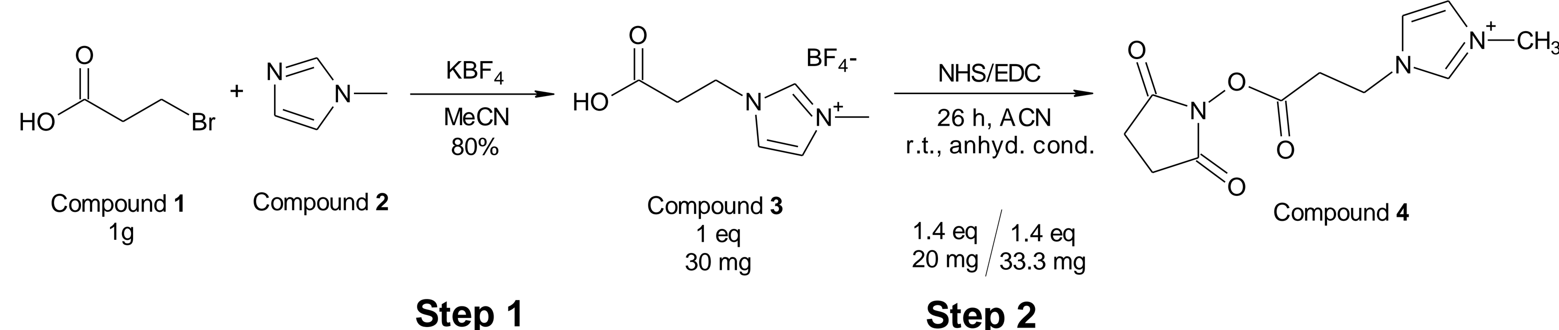
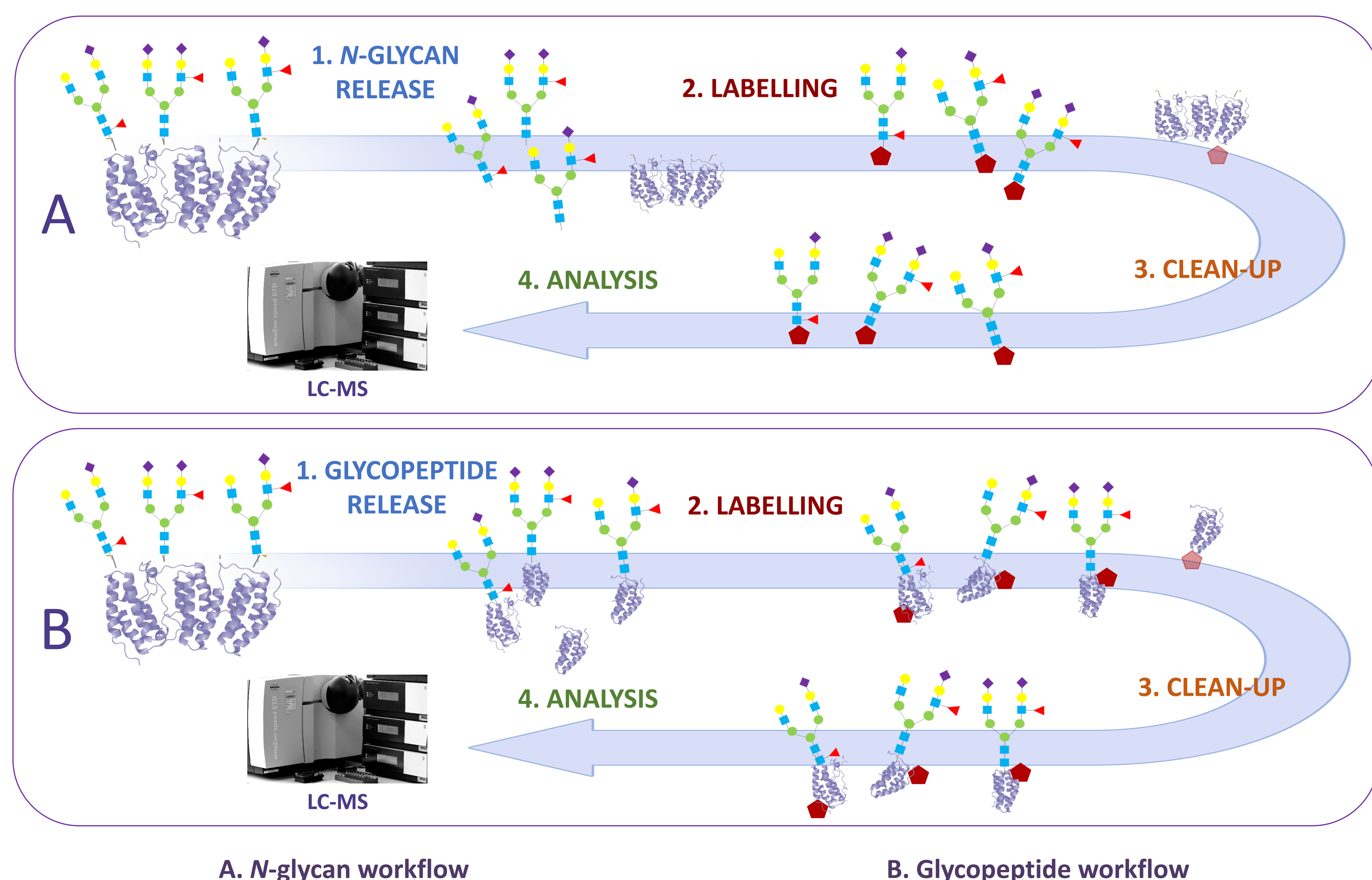


Figure 2. Chemical reaction scheme for novel label synthesis

Glycosylation analysis workflow

N-glycan analysis with this novel label was performed on γ-globulin from human blood (G4386, Sigma). This glycoprotein has a well characterised glycan profile and is representative of most biopharmaceuticals which are immunoglobulin-based compounds. *N*-glycan profile was compared with the one obtained with procainamide labelling, a current technology based on reductive amination protocol that provides good fluorescent and ESI-MS signal[9]. Glycopeptide labelling was performed on IgG1 Kappa from human myeloma plasma (I5154, Sigma) and on a glycopeptide standard containing A2G2S2 glycan attached to a short amino acid KVANKT chain (BQ-GPEP-A2G2S2-10U, Ludger).

Workflows for *N*-glycan and glycopeptide analysis are described below:



A. *N*-glycan workflow

1. *N*-GLYCAN RELEASE – 1 to 3 hours
Glycans are released from glycoproteins as glycosylamines by PNGase F digestion (LZ-rPNGaseF-kit, Ludger).

2. LABELLING – 30 min
Released glycans are conjugated to the new label.

3. CLEAN-UP – 1 to 2 hours
Labelled glycans are purified using LC-VSPE cartridges (LC-VSPE-30, Ludger).

4. ANALYSIS – 35 to 70 min
Glycans are analysed by HILIC-UHPLC-ESI-CID in positive ion mode using an ACQUITY UPLC BEH Glycan column [1.7 mm, 2.1 x 150 mm] (Waters) attached to a Bruker Amazon Speed ETD (Bruker Daltonics).

TOTAL TIME – 3 to 7 hours

B. Glycopeptide workflow

1. GLYCOPEPTIDE RELEASE – 2 to 3 hours
Glycoproteins are digested by trypsin gold (V5280, Promega) to release glycopeptides.

2. LABELLING – 1 hour
Released glycopeptides are conjugated to the new label.

3. CLEAN-UP – 1 to 2 hour
Labelled glycopeptides are purified using LC-A cartridges (LC-A-24, Ludger).

4. ANALYSIS – 35 to 70 min
Glycopeptides are analysed by HILIC-UHPLC-ESI-CID in positive ion mode using an ACQUITY UPLC BEH Glycan column [1.7 mm, 2.1 x 150 mm] (Waters) attached to a Bruker Amazon Speed ETD (Bruker Daltonics).

TOTAL TIME – 4 to 7 hours

Figure 3. Release and novel labelling of N-glycans (A) and glycopeptides (B) from glycoproteins.

Labelling with compound 4 of released *N*-glycans takes place at the amino group that was attached to the asparagine at the reducing end of the *N*-glycans, whereas glycopeptides labelling takes place at the N-terminal group of the peptide backbone, by a reaction with the NHS ester label (Fig. 7).

On both *N*-glycan and glycopeptides methods, a clean-up step using HILIC cartridges for removing excess dye was included before LC-MS analysis to avoid interferences, improper separation and promote the longevity of the LC columns.

Total time for these protocols is 3 to 7 hours. It consists of a one pot reaction – no clean-up step after PNGase F or trypsin digestion – and there are no dry down steps involved.

Results and discussion

Results with IgG *N*-glycans (Fig. 4) demonstrated that this label, even from the crude reaction mixture, was successfully reacting with the *N*-glycans. This was confirmed by the mass addition of +138.17 Da to each *N*-glycan, the mass attributed to the label attachment (Fig. 7). A good MS signal intensity for IgG *N*-glycans was obtained when using starting amount of 90 µg of glycoprotein. Interestingly, even small peaks and bigger structures with sialic acids could be identified from the MS trace.

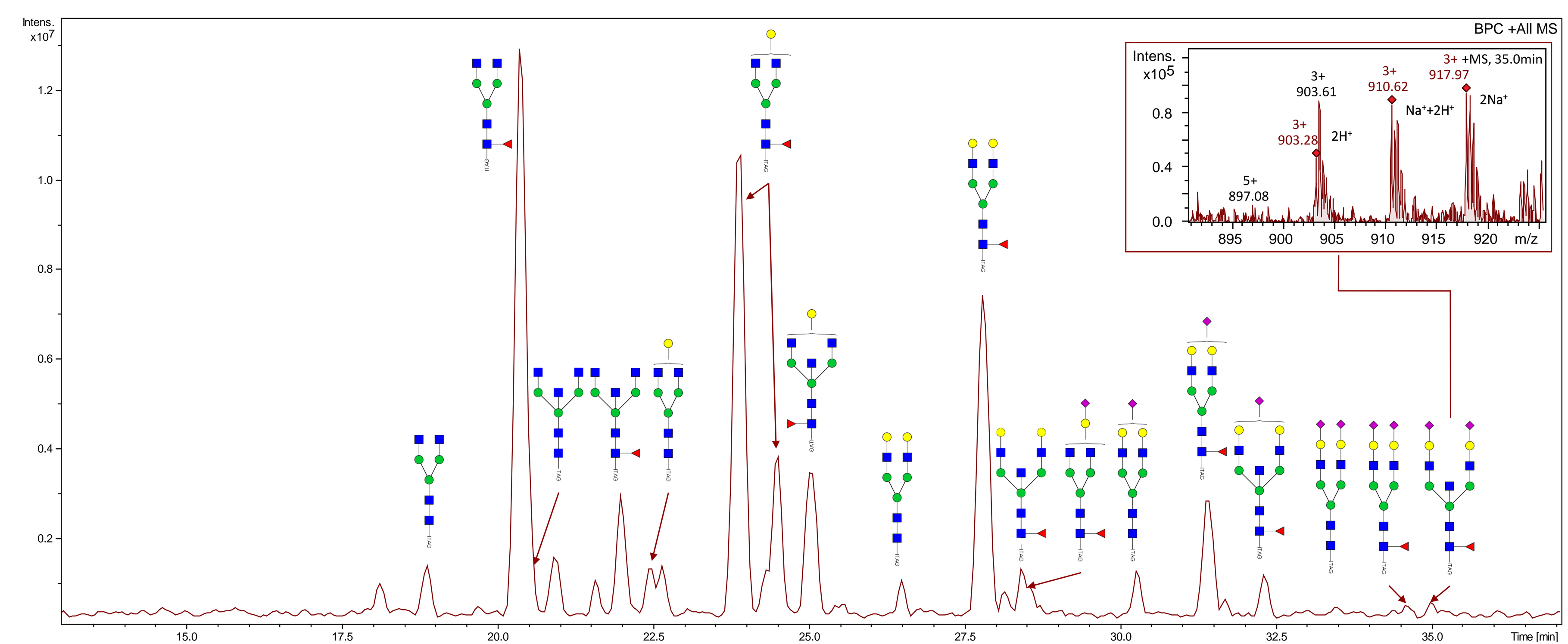


Figure 4. ESI-MS spectra showing possible structures for labelled-IgG N-glycans

Compound 4 was compared with procainamide (workflow of several days) and V-Tag (instant labelling approach) for human IgG labelling. ESI-MS results (Fig. 5b) showed that new compound provided successful identification of all expected peaks with a signal intensity of just 3.4x lower. No fluorescence signal was obtained (Fig. 5a).

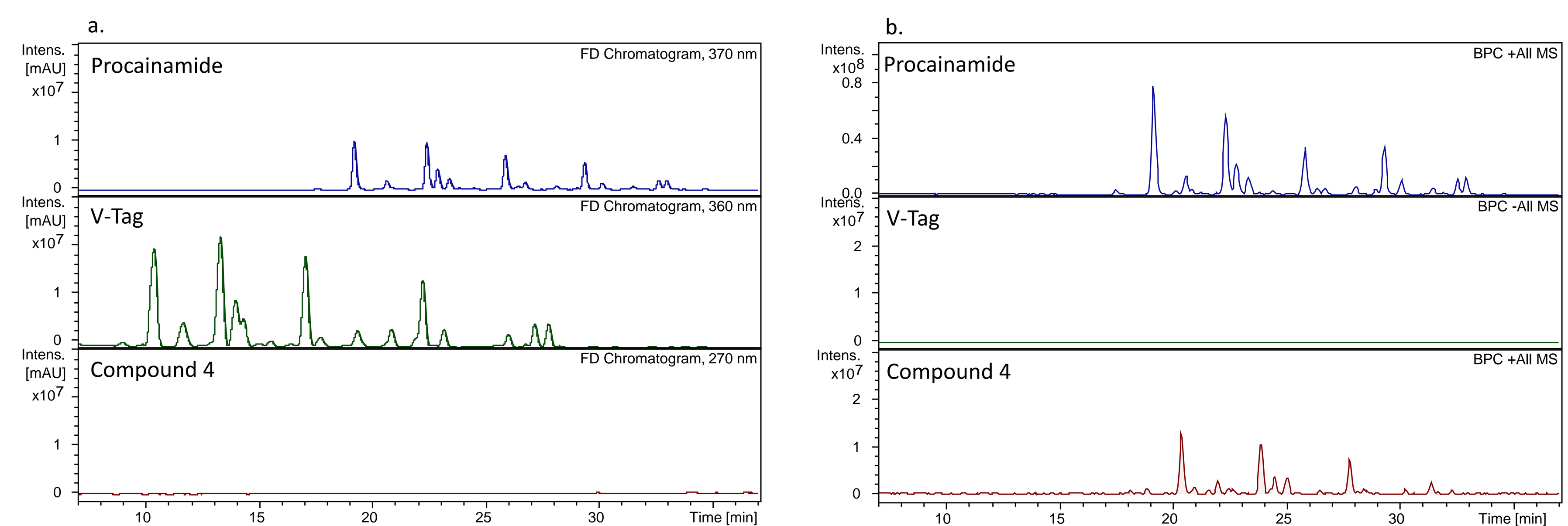


Figure 5. a. Fluorescence spectra comparison of labelled human IgG glycans with procainamide (blue), V-Tag (green) and novel label (maroon), b. ESI-MS spectra comparison of labelled human IgG glycans with procainamide (blue), V-Tag (green) and novel label (maroon)

This rapid derivatisation approach is intended to be very helpful for glycopeptides with one glycosylation site and a peptide backbone with few or no amino groups, such as monoclonal antibodies. Results with IgG1 glycopeptides (Fig. 6) demonstrated that successful labelling was achieved with a mass addition of +138.17 Da (Fig. 7), from 10 µg of glycoprotein as starting material. Nevertheless, results with labelled-A2G2S2-KVANKT glycopeptide showed further profile complexity (spectra not shown). NHS-ester labels have been reported to react with other amino groups in other parts of the peptide chain such as lysine side chains. This means that glycopeptides could present more than one labelling site, and hence various isomers of a glycopeptide with varied number of tags could be found.

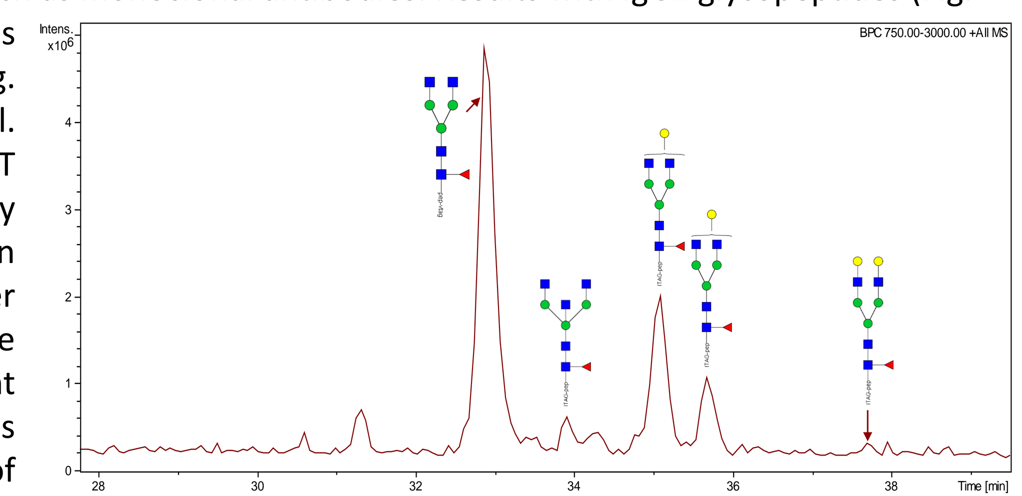


Figure 6. Possible structures for labelled-IgG1 glycopeptides

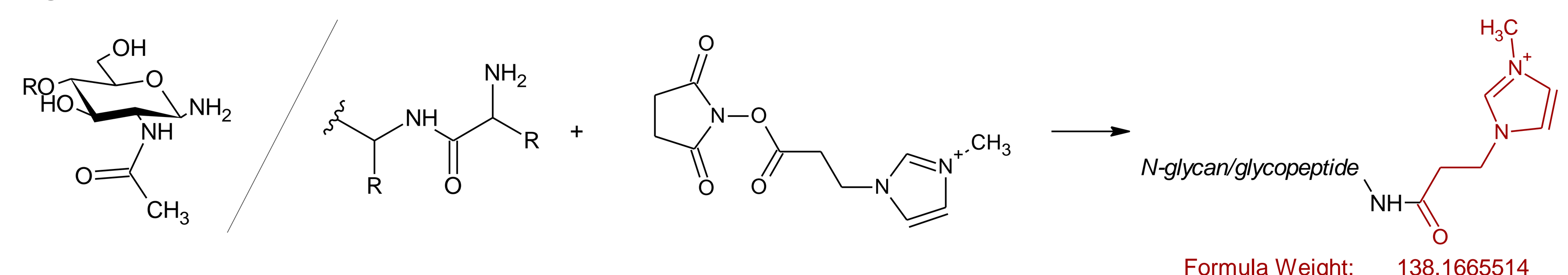


Figure 7. Labelling reaction of novel label with N-glycans and glycopeptides from glycoproteins.

Conclusion and Future Perspectives

Details of a novel chemical derivatization label have been presented. The results shown on this poster present a proof of concept to develop a rapid strategy for labelling *N*-glycans and glycopeptides for qualitative analysis using UHPLC-ESI-MS based on an imidazolium-type N-hydroxysuccinimide ester tag. This novel tag can successfully label *N*-glycans and glycopeptides and achieves good signal in ESI-MS. Glycoanalysis with this tag is quick and easy: it just requires 4 steps and no dry down is involved in the process, allowing it to be completed in as little as 3 hours. This method allows the analysis of a small amount of glycoprotein (from 10 µg for proteins with only one glycosylation site).

This work shows the first step towards a future novel label that will allow: rapid analysis, reliable and reproducible results, greater fluorescent UHPLC-ESI-MS signal and cost-effective. Optimization experiments are being planned with the purified label, as well as other label structure features are being explored. They'll be investigated for a diverse range of glycoproteins analysis.

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