QUANTITATIVE NON-ANTIBODY AFFINITY LC-MS/MS ANALYSIS OF GLYCOPROTEINS

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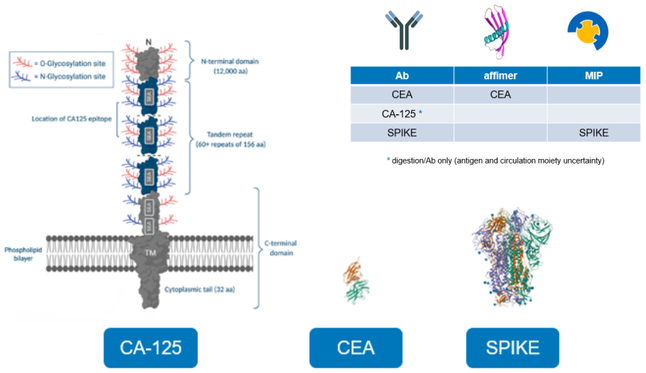
INTRODUCTION

Understanding isoform specific glycoprotein alterations and total amounts related to disease by means of bottom-up based LC-MS approaches are challenged by several parameters, including glycan composition heterogeneity, adsorption, enzymatic digestion efficiency, as well as the concentration levels that need to be reached for clinical research purposes. Approaches and methods to address the two latter parameters will be presented for the detection of example cancer and virus protein biomarkers. Proteolytic digestion efficiency was optimized off-line by means of a combined deglycosylation/digestion method using single stage on-bead amidase/protease hydrolysis. Affinity enrichment efficiency was assessed by comparing performance against traditional antibodies for generic oncology marker and virus proteins using similar automated magnetic bead-based protocols combined with LC-MS/MS.



Ab	affimer	MIP
CEA	CEA	
CA-125 *		
SPIKE		SPIKE

* digestion/Ab only (antigen and circulation moiety uncertainty)

Figure 1. Proteins and affinity technologies.

METHODS

Materials

Affinity reagents, overviewed in Figure 1, for CEA, CA-125 and Spike were conjugated to glass beads or a solid support through biotin-streptavidin interaction linkage or amine coupling. Recombinant proteins were obtained from Bio-Techne ACROBiosystems and Native Antigen Company, antibodies from Bio-Techne, AMSBIO and ACROBiosystems, Affimer® reagents from Leeds University, and Molecular Imprinted Polymers from MIP Discovery. All other affinity and digestion materials and reagents were from Thermo Fisher Scientific or Merck Group.

Digestion and LC-MS/MS

The enrichment and digestion processes are graphically summarized in Figure 2. Following affinity enrichment, the proteins were denatured, reduced and alkylated whilst still residing on the surface of the bead. PNGase F and trypsin and were added next, followed by incubation. Buffer washes and elution steps were conducted next and the supernatants collected. A Xevo™ TQ-XS MS with ESI source coupled to an ACQUITY™ UPLC™ I-Class Chromatography System was used to collect LC-MS/MS data in positive-ionisation Multiple Reaction Monitoring (MRM) mode.

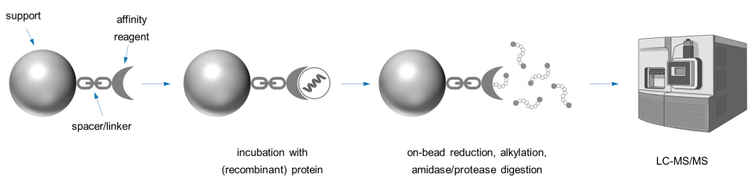


Figure 2. Generalized enrichment, digestion and detection principle.

RESULTS

Glycoprotein digestion

A-specific binding was overcome by conducting off-line experiments in the presence of Bovine Serum Albumin (BSA) or dilution of the standards into blank plasma solution. The results shown in Figure 3 illustrate a > 10^5 increase in signal when including PNGase F N-glycan removal into the digestion process and a dependency on the stage of alkylation and reduction of recombinant CEA.

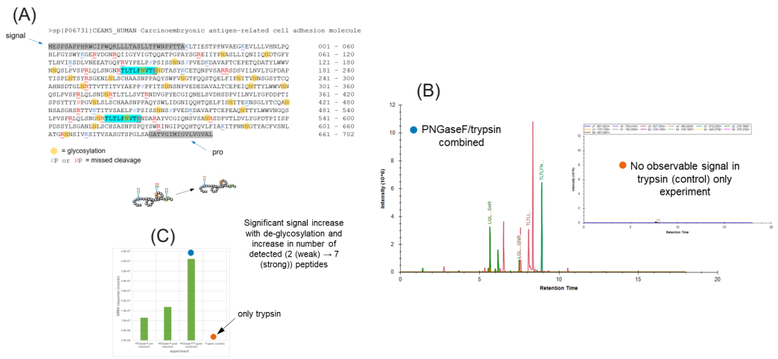


Figure 3. CEA amino acid sequence with tryptic peptide of interest highlighted in blue (A), MRM chromatograms with/without PNGase F incorporation (B), and relative signal response for sequential and combined digestion with PNGase F and trypsin (C).

A more modest increase in LC-MS signal, about ~ 10 - 50 times, was observed for CA-125 with the same digestion strategy applied, as summarized in Figure 4. Affinity enrichment was however not further explored given the uncertainty about the molecular identity of the recognized epitopes of circulating CA-125.

Figure 4. Partial amino acid sequence epigenetic region CA-125 with tryptic peptide of interest highlighted in blue (A), amounts normalized LC-MS/MS response (B), and MRM chromatograms with/without PNGase F incorporation (C).

Affimer Affinity Enrichment

Various recombinant sources of CEA, shown in Figure 5, were initially evaluated using ELISA using two Affimers and two Abs and quantification assessed by loading various amount of CEA on magnetic supports followed by SDS-PAGE and bottom-up LC-MS analysis.

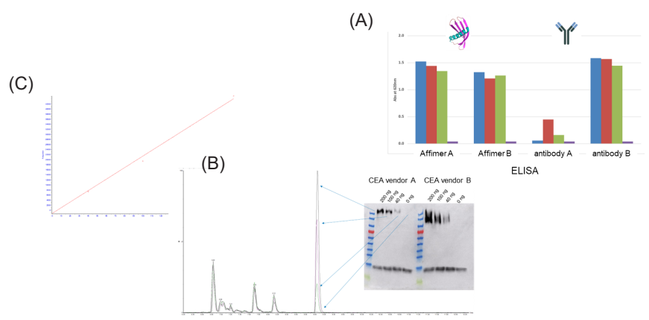


Figure 5. ELISA antibody evaluation; bar color = antibody vendor (A) and semi-quantitative SDS-PAGE (B) and LC-MS/MS assessment Affimer loadability (B) and (C).

Enrichment protocol optimization suggest that similar overall protein/affinity reagent binding efficiency and sensitivity levels can be reached for CEA using both Ab and Affimer based enrichment as illustrated in Figure 6.

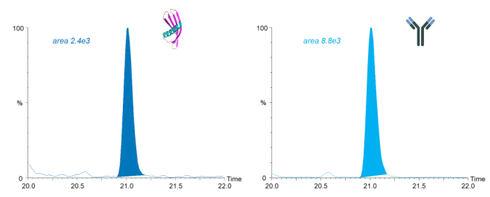


Figure 6. MRM chromatograms for Affimer (left) and Ab (right) enriched CEA in human plasma at 20 ng/mL following on-bead amidase/protease hydrolysis using trypsin/PNGase F.

MIP Affinity Enrichment

Signature Spike peptides and MRM transitions were identified through a discovery and MRM semi-automated selection/optimization driven workflow, which is shown graphically in Figure 7. As expected, including de-glycosylation of Spike into the workflow did not significantly improve either coverage or sensitivity given the minimal glycosylation of the recombinant standard. The selective and quantitative response of a Spike -selective MIP immobilised on glass is shown in Figure 7 as well.

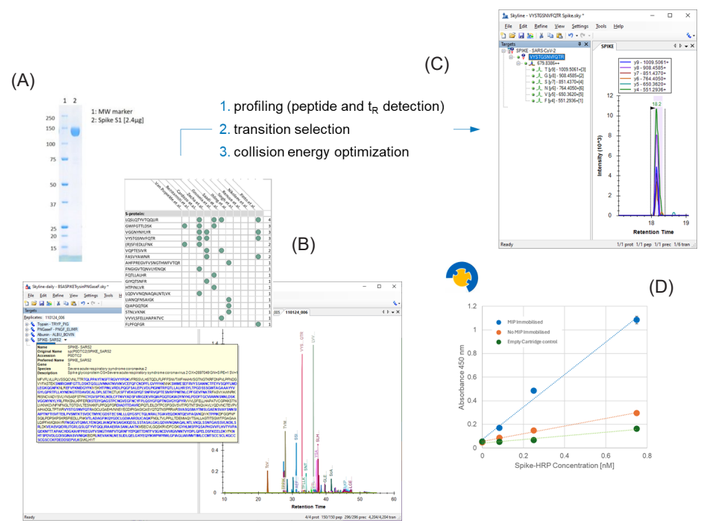


Figure 7. Coomassie-stained SDS-PAGE showing purified SARS-CoV-2 Spike S1 protein (A), literature and high-resolution MS broadband Data Independent Analysis based peptide selection (B), MRM selection/optimization (C) and MIP response vs. two negative controls (D).

CONCLUSION

- **Glycan removal can be beneficial for non-isoform specific bottom-up LC-MS based detection of glycoproteins by increasing digestion efficiency**
- **No negative effects observed as a function of introducing PNGase F deglycosylation into sample preparation processes**
- **Binding affinity was similar for the investigated reagents but independent method optimization was required; additionally, non-specific binding to the supports was noted**
- **Affinity binding was found to be quantitative within the investigated analyte concentration ranges**

References

1. Selection and characterisation of Affimers specific for CEA recognition. Shamsuddin SH, Jayne DG, Tomlinson DC, McPherson MJ, Millner PA. *Sci Rep.* **2021** Jan 12; 11(1) :744
2. Molecularly imprinted polymers for the recognition of proteins: the state of the art. Bossi A, Bonini F, Turner AP, Piletsky SA. *Biosens Bioelectron.* **2007** Jan 15; 22(6): 1131-7
3. Cov2MS: An Automated and Quantitative Matrix-Independent Assay for Mass Spectrometric Measurement of SARS-CoV-2 Nucleocapsid Protein. Van Puyvelde *et al. Anal Chem.* **2022** Dec 20; 94 (50): 17379-17387.
4. https://thenativeantigencompany.com/products/sars-cov-2-spike-glycoprotein-s1-sheep-fc-tag-hek293/

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