QUANTITATIVE EVALUATION OF AFFIMER AND MOLECULAR IMPRINTED POLYMER AFFINITY GLYCOPROTEINS ANALYSIS BY MULTIPLE REACTION MONITORING LC-MS/MS

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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 bead-based protocols combined with LC-MS/MS.

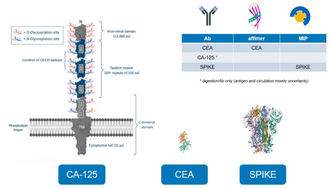


Figure 1. Investigated proteins and applied affinity technologies.

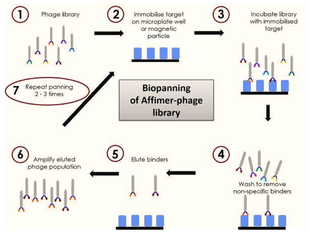


Figure 2. Development of Affimers using phage display as an in vitro selection method.

METHODS

Materials

Affinity reagents, which are overviewed in Figure 1, for CEA, CA-125 and Spike protein were conjugated to glass beads or a solid support through biotin-streptavidin interaction linkage or amine coupling.

Affimer® reagents were from Leeds University, and Molecular Imprinted Polymers from MIP Discovery. The (bio)chemical development process principles of Affimers and MIPs are graphically summarized in Figures 2 and 3, respectively.

Recombinant protein standards were obtained from Bio-Techne ACROBiosystems and Native Antigen Company, and antibodies from Bio-Techne, AMSBIO and ACROBiosystems. All other affinity and digestion materials and reagents were from Thermo Fisher Scientific or Merck Group.

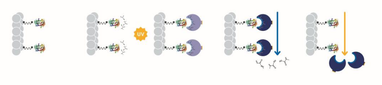


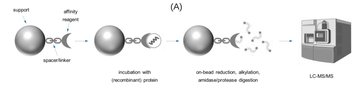
Figure 3. Chemical manufacturing process of Molecular Imprinted Polymer (MIP) affinity reagents.

Digestion and LC-MS/MS

The enrichment and digestion processes are graphically summarized in Figure 4, including an example raw time-scheduled MRM chromatogram of tryptic digested deglycosylated CEA.

Following affinity enrichment, the proteins were RapiGest™ SF denatured, dithiothreitol (DDT), reduced and iodoacetamide (IAA) alkylated whilst still residing on the surface of the bead. PNGase F and trypsin were added next, followed by overnight incubation. Buffer washes and elution steps were conducted next, and the supernatants collected.

A Xevo™ TQ-XS MS tandem quadruple mass spectrometer (MS) equipped with an electrospray ionization (ESI) source coupled to an ACQUITY™ UPLC™ I-Class PLUS Chromatography System was used to collect reversed phase (RP) peptide separation LC-MS/MS data in positive-ionisation Multiple Reaction Monitoring (MRM) mode. The LC-MS data were processed using TargetLynx™ Software or Skyline.



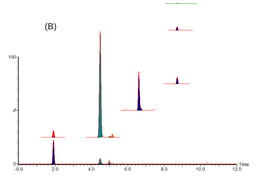


Figure 4. Generalized protein affinity enrichment, digestion and LC-MS/MS (MRM) detection principle (A) and example MRM chromatogram tryptic digest deglycosylated CEA (B).

RESULTS

Glycoprotein digestion

A-specific binding was overcome by conducting off-line experiments in the presence of Bovine Serum Albumin (BSA) or Bovine β-casein, or dilution of the standards into blank plasma solution. The results shown in Figure 5 illustrate a > $10^{\wedge}5$ 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.

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 6. Affinity enrichment was however not further explored given the uncertainty about the molecular identity of the recognized epitopes of circulating CA-125.

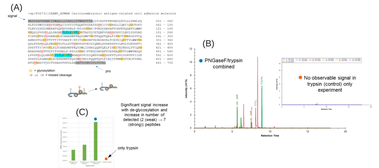


Figure 5. CEA amino acid sequence with tryptic peptide of interest highlighted in blue (A), MRM chromatograms with/without PNGase F incorporation (B), and relative signal response for sequential and combined digestion with PNGase F and trypsin (C).

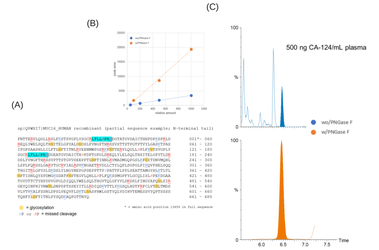


Figure 6. Partial amino acid sequence epigenetic region CA-125 with tryptic peptide of interest highlighted in blue (A), amounts normalized LC-MS/MS response (B), and MRM chromatograms with/without PNGase F incorporation (C).

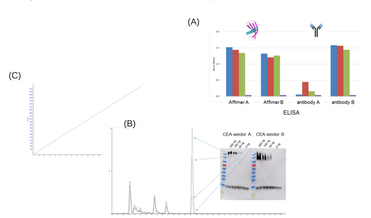


Figure 7. ELISA antibody evaluation; bar color = antibody vendor (A) and semi-quantitative SDS-PAGE (B) and LC-MS/MS assessment Affimer loadability (B) and (C).

Affimer Affinity Enrichment

Various recombinant sources of CEA, shown in Figure 7, were initially evaluated using ELISA using two Affimers and two antibodies, respectively, and quantification assessed by loading various amount of CEA on magnetic supports followed by SDS-PAGE and bottom-up LC-MS/MS MRM analysis.

Enrichment protocol optimization suggests that similar overall protein/affinity reagent binding efficiency and sensitivity levels can be reached for CEA using both antibody and Affimer based enrichment, as illustrated in Figure 8.

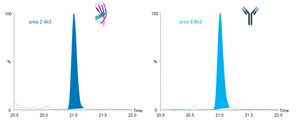


Figure 8. MRM chromatograms for Affimer (left) and Ab (right) enriched CEA in human plasma at 20 ng/mL following on-bead amidase/protease hydrolysis using trypsin/PNGase F.

MIP Affinity Enrichment

Signature Spike protein peptides and MRM transitions were identified through a discovery and semi-automated MRM selection/optimization driven workflow, which is shown graphically in Figure 9. As expected, including de-glycosylation of Spike protein 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 protein-selective MIP immobilised on glass beads is shown in Figure 9 as well.

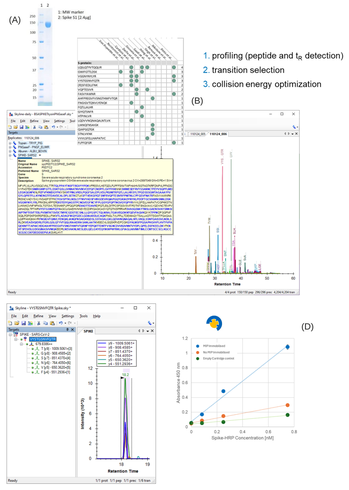


Figure 9. Coomassie-stained SDS-PAGE showing purified non-glycosylated SARS-CoV-2 Spike S1 protein (A), literature and high-resolution MS broadband Data Independent Analysis based peptide selection (B), MRM selection/optimization (C) and MIP response vs. two negative controls (D).

Compared with previously published discovery results, a relatively large number of peptide candidates were detected, which was attributed to the absence of the Spike protein carbohydrate surface moiety. Moreover, the main proteotypic tryptic target peptide VYSTGSNVFQTR did not amino acid sequence overlap with the epitope CGNSNNLDSKVGG of Spike protein.

Following the MIP development and MRM selection/optimization processes, the quantitative application of the affinity MIP was accessed through loading various amounts of HRP conjugated Spike protein and Spike protein onto glass bead immobilised Spike protein MIPs, respectively.

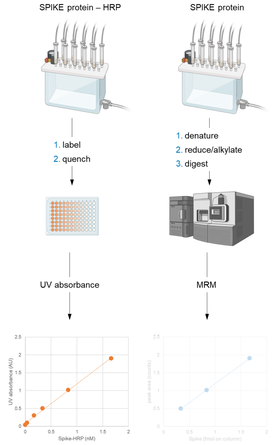


Figure 10. Off-line MIP on-bead UV absorbance (left) and on-bead digestion, MRM LC-MS/MS workflows (right, pending) and quantitative response, respectively.

The immobilised MIPs were rigorously washed and the Spike protein either labelled (HRP conjugated) or proteolytically digested (native, non-conjugated), as illustrated in Figure 10. Quantitative readout was either via UV absorbance or scheduled MRM based LC-MS/MS and linear response observed within the investigated concentration ranges.

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 were observed as a function of the addition of deglycosylation into the sample preparation processes, *that is*, the digestion of non-glycosylated proteins was not affected by the presence of PNGase F)**
- **The binding affinity was found to be similar for the investigated materials, but independent method optimization was required; additionally, non-specific binding to some of the applied supports was noted, which was/can be largely mitigated by using appropriate blocking agents or alternative supports**
- **Antibody, Affimer and MIP affinity binding were found to be quantitative and can be integrated in both offline or online LC-MS based workflows**

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/
5. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Bioinformatics. 2010 Apr 1;26(7):966-8

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OUTLOOK

Automation

The investigated materials can be applied in various formats, allowing for a higher degree of sample preparation in the form of affinity isolation or separation compared to the investigative data on this poster, providing ultimately increased throughput, precision and accuracy.

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