LC/MS Analysis of Monoclonal Antibody Structure Utilizing HALO® BioClass Fused-Core® Particles
Multilevel Analysis for Proteins and Glycovariants

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Summary

Efforts have been directed at optimizing high performance HALO® BioClass Fused-Core® silica materials, which exhibit favorable mass transfer properties for large molecules. Superficially porous silica packing materials have been applied to HPLC/UHPLC characterization of biomolecules including: proteins, peptides, and glycosylation variants from monoclonal antibodies. HALO BioClass Fused-Core™ materials show considerable utility for analysis of these highly complex molecules, using conditions that permit excellent separations and analysis via LC-MS. Alternatives to standard trifluoroacetic acid (TFA) and formic acid (FA)-containing mobile phases were demonstrated for intact antibody separations, subunit analyses, and for analyses of tryptic digests.

Objectives

- Optimize separation conditions of biomolecules using alternatives to TFA/FA for intact molecules, subunit analysis, and tryptic digests.
- Employ HALO® BioClass Fused-Core® silica materials to perform optimal large molecule separations and analysis by HPLC/UPLC-MS.
- Perform top down and bottom up characterization of trastuzumab (Herceptin, Genentech).

HALO BioClass Columns for mAb Characterization

Monoclonal Antibody Characteristics	Technique(s)	Applicable HALO BioClass Column	Pore Size (Å)	Particle Size
Purity, impurities, post-translational modifications, molecular weight	Reversed-phase LC-MS	HALO PROTEIN	400	3.4
Identity, purity, impurities, site-specific modifications	Reversed-phase LC-MS RPLC-UV	HALO PEPTIDE	160	2.7, 5
Glycosylation (sequence, composition, linkage, branching)	HILIC-MS HILIC-FLD	HALO GLYCAN	90	2.7

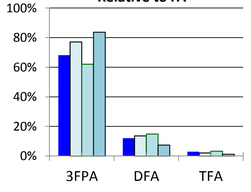
On Trastuzumab

Trastuzumab was the first monoclonal antibody targeted for a cancer-related biomarker to obtain approval by the FDA. Trastuzumab consists of two light chains, two heavy chains, and has an ensemble of N-linked glycans attached to Asn 297 of each heavy chain.

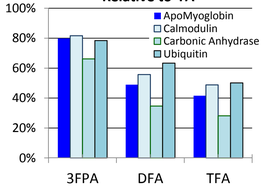
Mobile Phases for Improved Protein LC/MS

- TFA is notorious for ESI signal suppression, background problems (chemical noise), and system persistence.
- Formic and acetic acid are widely adopted for LC/MS applications, with variable performance for protein separations, but excellent ESI/MS compatibility.

Peak Area (TIC) Mobile Phase Relative to FA



$PW_{1/2}$ Mobile Phase Relative to FA



- 3FPA = 3,3,3,-trifluoropropanoic acid, DFA = 2,2-difluoroacetic acid
- Graphs compare MS and LC performance for 10 mM of each acid modifier to 10 mM FA.
- Each acid was examined at varying concentrations (2-50 mM for fluorinated acids; 20-500 mM for FA), exhibiting progressive suppression of ESI signal with concentration: plateau at 50 mM for FA, 10-20 mM for the others.

Experimental

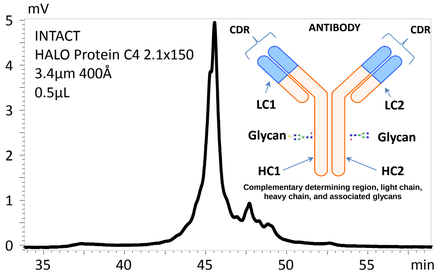
Intact Sample Prep: Herceptin stock solution (46 µL of 8.7 mg/mL) was buffer exchanged into 50 mM ammonium bicarbonate. This solution was mixed with 44 µL of aqueous 0.1% TFA, and 10 µL of acetonitrile (0.1% TFA). The resulting sample solution was comprised of 4 mg/mL Herceptin in 10/90 (v/v) ACN/H_2O with 0.1% TFA.

Tryptic Digest Prep: Herceptin (Trastuzumab, 440mg, Genentech) was solubilized in 20mL 1.1% benzyl alcohol water for injection. A 2mg/mL sample of trastuzumab was reduced in 6M Guanidine HCl, 20mM Tris pH 7.8 (Sigma), and 10mM dithiothreitol (Thermo). The solution was incubated for 1 hr at 37°C. The reduced trastuzumab was alkylated in 20mM iodoacetamide (Thermo) and 10mM Tris HCL (Sigma). The sample was incubated at ambient temperature in darkness for 30 minutes. The reaction was quenched with 30mM DTT. The reduced and alkylated trastuzumab was buffer exchanged (4 x 20min at 4500 rpm) into 0.1%TFA using a 5k MWCO VIVASpin2 centrifugation filter (Sartorius). The samples were dried, then reconstituted in 50mM ammonium bicarbonate (4mg/mL). The sample was digested overnight in Trypsin (Promega) at 37°C, then was adjusted to 0.25% formic acid.

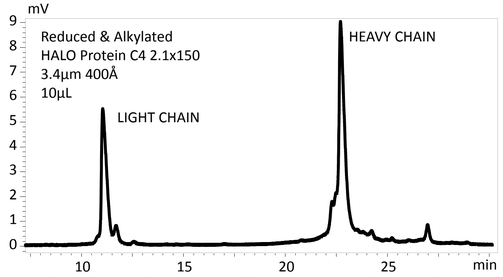
ESI-LC/MS and ESI-LC/MS/MS: Analyses were performed on a Shimadzu Nexera UFLC coupled to single quadrupole LCMS-2020 mass spectrometer. Analysis was also performed on a Dionex Ultimate3000 UHPLC coupled to a Thermo Orbitrap Velos Pro hybrid mass spectrometer. Separations were performed by RPLC using the HALO Protein C4 100 x 0.3mm 3.4µm, the HALO Peptide ES-C18 250 x 0.2mm 5µm, and the EXP stem cartridge 33µl HALO C4 (Optimize Technologies).

Data analysis: Shimadzu LabSolutions™, Thermo Xcalibur™, Thermo Protein Deconvolution™, and Thermo Proteome Discoverer™.

Intact, Reduced, and Alkylated Trastuzumab Analysis



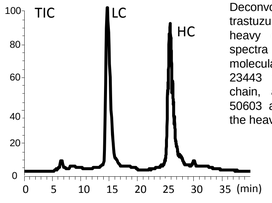
Intact Trastuzumab 2mg/mL in 0.1%TFA, 0.1mL/min, gradient 27-33%B in 80min, 80°C, 280nm. Mobile phase A: 0.1%DFA, B: 0.1%DFA in ACN



Reduced Trastuzumab 1mg/mL in 0.1%TFA, 0.2mL/min, gradient 28-34% in 40 min, 80°C, 280nm, Mobile phase A: 0.1%DFA, B: 0.1%DFA in ACN

HRMS Light & Heavy Chain Analysis

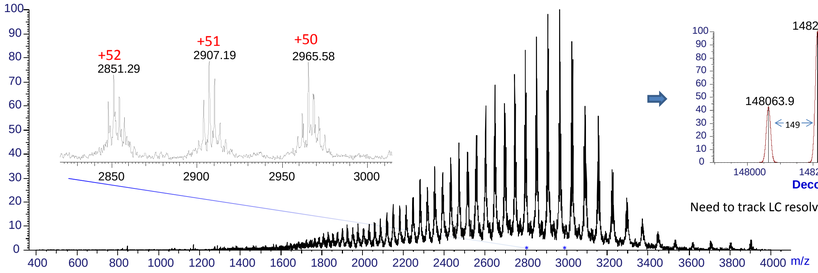
Column:	**HALO Protein C4, 0.3 x 150 mm, 3.4 µm**
Part Number:	**94316-714**
Mobile Phase A:	0.1% difluoroacetic acid (DFA) in water
Mobile Phase B:	0.1% difluoroacetic acid in acetonitrile
Flow Rate:	4 µL/min
Gradient:	29–35% B in 40 min.
Temperature:	75 °C
Injection Volume:	2.0 µL of 1 µg/µL reduced mAb in 0.1% TFA
UHPLC:	Dionex Ultimate 3000 UHPLC
MS:	Thermo Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap



Deconvolution of the trastuzumab light and heavy chain LC-MS spectra revealed a molecular weight of 23443 for the light chain, and MWs of 50603 and 50764 for the heavy chains.

HRMS Intact Analysis

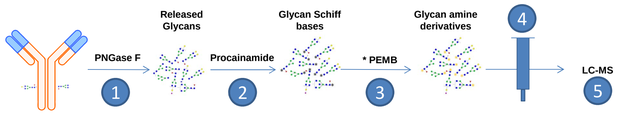
Column:	**HALO Protein C4, 0.3 x 150 mm, 3.4 µm**
Part Number:	**94316-714**
Mobile Phase A:	0.1% DFA in water
Mobile Phase B:	0.1% DFA in acetonitrile
Flow Rate:	7.5 µL/min
Gradient:	20–40% B in 20 min.
Temperature:	75 °C
Injection Volume:	10 µL of 0.2 µg/µL Herceptin in 0.1% TFA
UHPLC:	Dionex Ultimate 3000 UHPLC
MS:	Thermo Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap

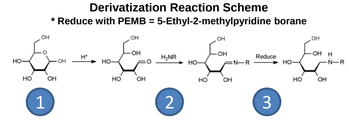


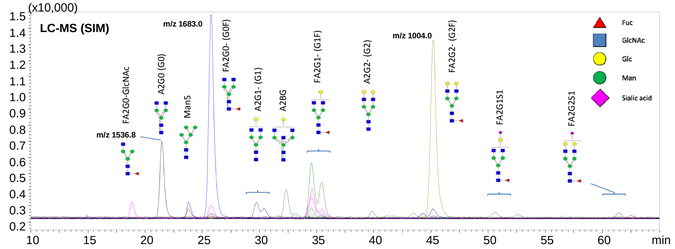
Need to track LC resolved peaks to actual structures

Glycosylation Variants of Trastuzumab

Released Glycan Sample Prep: Trastuzumab glycans were released by PNGaseF (New England Biolabs) overnight at 37°C. Utilized FASP to denature, buffer exchange, and recover glycans. PAM-glycans were cleaned up using Discovery Glycan SPE sorbent (50mg bed, Supelco).





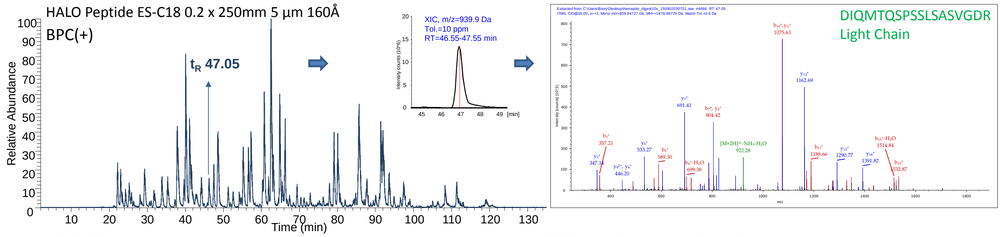


Analysis Conditions

Column:	**HALO Glycan, 2.1 x 150 mm, 2.7 µm**
Part Number:	**92922-705**
Mobile Phase A:	50 mM ammonium formate (pH 4.5)
Mobile Phase B:	acetonitrile
Flow Rate:	0.35 mL/min
Gradient:	75–68% acetonitrile in 75 min.
Temperature:	40 °C
Injection Volume:	15 µL
UHPLC:	Shimadzu Nexera
MS:	Shimadzu LCMS 2020 with selected-ion monitoring

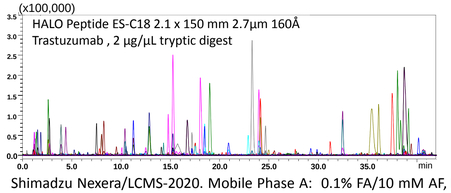
- The variety and identities of the glycans associated with a monoclonal antibody drug are critically important for its efficacy and bioavailability. However, it is not certain that glycosylation, seen by LC-MS of proteins or subunits, is accurately reflected by released glycan analysis.
- Glycoprofiling of N-linked glycans, using HILIC (NP) combined with fluorescence or absorbance detection and/or MS is becoming a standard for biopharmaceutical glycoproteins, particularly IgGs.

Analysis of Trastuzumab Tryptic Digest: UHPLC-HRMS and HPLC-MS



Two 250x0.2mm columns in series. Thermo Orbitrap Velos Pro/Dionex Ultimate 3000 UHPLC. 5µL injection of 3 µg/µL Trastuzumab tryptic digest. A = 0.1% DFA, B = 0.1% DFA in ACN. Loading Pump: 2%B @ 10µL/min. EXP stem cartridge 33µl HALO C4. NC Pump: 5-55%B in 182min @ 3µL/min, 60°C,

- LC/HRAM MS is permitting evaluation of structures that are resolved, but it is not clear yet what all of these are
- ESI signal is moderately reduced with DFA, in line with other proteins, however it's clear TFA is not REQUIRED for high-res separations



Shimadzu Nexera/LCMS-2020. Mobile Phase A: 0.1% FA/10 mM AF, B: ACN with 0.1% FA, 0.4 mL/min; 5–40% B in 60 min.; 60 °C; 50 µL.

- Ammonium formate (AF) has also been shown to work in synergy with FA to provide good peak shape, recovery, selectivity, and detection capabilities.
- DFA alone or DFA/AF together may provide a reasonable alternative to TFA

Conclusions

- Bottlenecks in protein LC/MS have been improved by newer SPP materials and MS instruments.
- A wider range of useful operating conditions could take advantage of improvements in column and MS capabilities.
- Effort will be required to understand retention and resolution of larger proteins and fragments. This is already the case for common current MP additives. Understanding variant resolution will likely require a top-down, middle-down and bottom-up approach.

Acknowledgements:

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