High Resolution Separations for Detailed LC/MS Analysis of mAb Disulfide Variants

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Presented at ASMS 2019 Poster TP 623

Outline

- 1000Å superficially porous particles (SPPs) are a new class of HPLC materials for high resolution separations of large biomolecules, demonstrating narrow peak widths, increased retention, and high efficiency compared to sub-2µm fully porous particles.
- Large pores reduce restricted diffusion with larger molecules, evident for separations of IgG1 disulfide variants
- C4, ES-C18, and Diphenyl bonded-phase surfaces expand the range of 1000Å protein separation selectivity options.
- Fast, higher resolution LC/MS analysis of complex proteins is made possible through manipulation of the bonded-phase and mobile phase allowing for improved protein identification by MS deconvolution
- Free sulfhydryl trapping using maleimide reagents enables top-down resolution and identification of unresolved pairs of intact mAb variants, while subunit analysis of the free sulfhydryl-labeled mAb variants enables a middle down approach to structure elucidation.

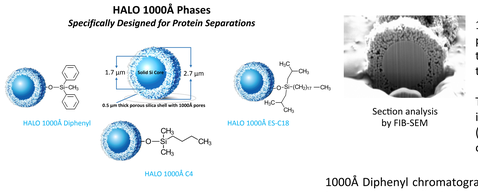
Introduction

Superficially porous particles (SPPs) demonstrate very high performance capabilities as packed columns for LC separations. Recent refinements of SPPs for separations of large biomolecules (>50,000 MW) show the advantages of the 1000Å pore size, for proteins and protein complexes using reversed-phase operation. Optimized conditions for high resolution protein LC/MS are shown for a biotherapeutic IgG1, which exhibits significant structure heterogeneity due to variable disulfide bridge formation or interconversion of bridged positions. To assess the state of such bridges we employ high resolution separations, combined with free sulfhydryl trapping, via reaction by maleimide reagents. Top down and middle down approaches are employed to understand this structure heterogeneity. Fast, high efficiency separations enable the confident assignment of protein structures and allow for improved understanding of the domain position effects on the propensity for these proteins to form free sulfhydryls.

Materials and Methods

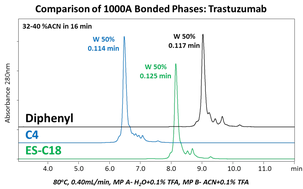
Columns of HALO 1000Å with C4, ES-C18 and Diphenyl bonded phases were produced at Advanced Materials Technology, Inc. (Wilmington, DE). SEM images were obtained using a Zeiss (Jena, Germany) Auriga 60 High Resolution Focused Ion Beam & Scanning Electron Microscope at the University of Delaware (Newark, DE). Mobile phase modifier was obtained from Synquest Laboratories (DFA). Acetonitrile (ACN) was MS grade from GFS Chemicals and n-propanol (nProp) was from Honeywell. Monoclonal antibodies were commercially obtained or generous gifts of highly purified biotherapeutic grade products. Maleimide reagents were obtained from various vendors: n-tButylmaleimide and n-cyclohexylmaleimide (Aldrich), n-ethylmaleimide (Sigma), n-(4-ethylphenyl)maleimide (Alfa Aesar), octylmaleimide (Toronto Research Chemicals). Dithiothreitol (DTT) and Iodoacetamide were obtained from Pierce. Analytical protein separations used the Shimadzu Nexera LC-30 components (40 µL or 180 µL mixer), with the SPD 20A UV or SPD-M30A PDA detector. An Orbitrap Velos Pro ETD and a QExactive HF (ThermoScientific, Inc.), with the high flow IonMax ESI interface were operated at 3.5 kV potential for electrospray. Intact protein MS spectra were recorded in the Orbitrap, using 15,000 resolution scans, an AGC setting of $1x10^6$ and 70V in-source CID dissociation for intact analysis; 60 CID for heavy chain and 0 CID for light chain analysis. Deconvolution of MS spectra used MagTran v1.02 (based on ZScore [Zhang and Marshall; JASMS 9 (1998) 225]), or Thermo Scientific Biopharma Finder 3.1. Chromatographic peak widths are reported as full width at half height ($PW_{1/2}$).

Selectivity Options for Top Down LCMS with HALO 1000Å SPPs



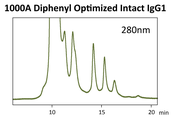
1000Å Diphenyl, C4, and ES-C18 reverse phase LC separations were compared using the biotherapeutic IgG1 monoclonal antibody, trastuzumab.

THE Diphenyl bonded phase was selected for its increased retention, decreased peak width (relative to ES-C18), and increased resolution of trastuzumab minor components.



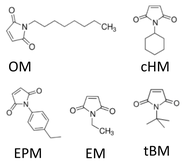
1000Å Diphenyl chromatographic separations were further optimized for temperature, gradient slope, and retention in order to obtain a high degree of resolution for the later-eluting mAb variants. The strong solvent was adjusted from acetonitrile to a binary 1:1, acetonitrile:n-propanol. The acid modifier was adjusted from TFA to DFA in order to reduce MS ion suppression while maintaining similar $PW_{1/2}$.

The chromatogram at right depicts a high resolution separation of trastuzumab using a 2.1x150mm 2.7µ column at 60°C, 0.25ml/min, 29-33% in 29'. Mobile phase A = H_2O+ 0.1% DFA; B = 1:1, ACN/n-propanol with 0.1% DFA. UHPLC: Nexera X2 with LC-30 components 180µL mixer.



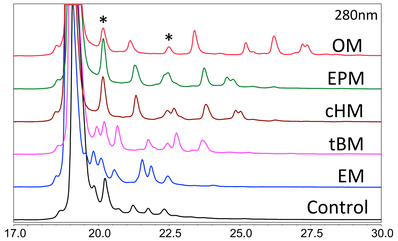
Free Sulfhydryl Trapping Via Reaction With Maleimide Reagents

Labeling Reagent, [Abbrev.]	(g/mol)
n-ethylmaleimide, [EM]	125.13
t-butylmaleimide, [tBM]	153.18
n-cyclohexylmaleimide, [CHM]	179.22
n-(4-ethylphenyl)maleimide, [EPM]	201.23
Octylmaleimide, [OM]	209.29



Antibody structure heterogeneity presents a problem for reverse phase LCMS analysis even after optimizing column chromatographic conditions. Further improvement in the chromatographic separation of unresolved IgG1 disulfide variants is possible through the use of maleimide labeling reagents, which can be employed to label mAb free sulfhydryls.

Comparing Retention of Maleimide Labeled Trastuzumab Variants

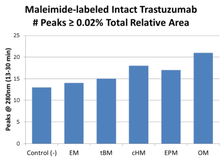


10µL of 1µg/µL in 0.1%TFA; 1000A Diphenyl 2.1x150mm 2.7µ, 27-36%B 40min 0.35mL/min 60°C 280nm; initial pressure 217 bar, A=0.1%DFA; B=0.1%DFA 1:1 ACN:n-propanol, PDA. Nexera X2 HPLC coupled with a Q Exactive HF employed ESI-MS. UV displayed in above panel. Signals are normalized.

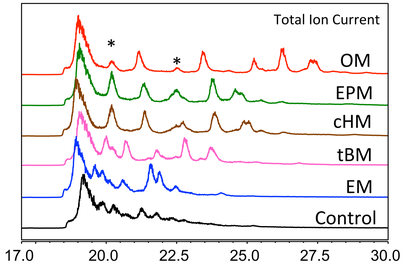
*Unlabeled mAb variants , which exhibited the same retention time for each maleimide reagent examined. Mass determined by MS spectral deconvolution.

Maleimide stock solutions were prepared in the range of 3-6 mg/mL in 50% acetonitrile for tBM, cHM, and EM; 60% acetonitrile was required for EPM and OM stock solutions. A 1:100 dilution of the stock maleimide (diluted in 0.1M acetate buffer pH 5.2/7.2M Guanidine HCl) was combined1:1 with trastuzumab (0.1M PBS pH 7). Sample was incubated at 5mg/mL @ 37°C for 30 min, then acidified with 0.5% DFA, then analyzed by LCMS.

0.1M MOPS buffer pH 7.6/7.2M Guanidine HCl yielded similar results to the acetate buffer under the same conditions.



Free Sulfhydryl Trapping Via Reaction By Maleimide Reagents



Octylmaleimide labeled trastuzumab exhibited the most retained protein variants compared to EPM, cHM, tBM and EM when using the 1000A Diphenyl 2.1x150mm column.

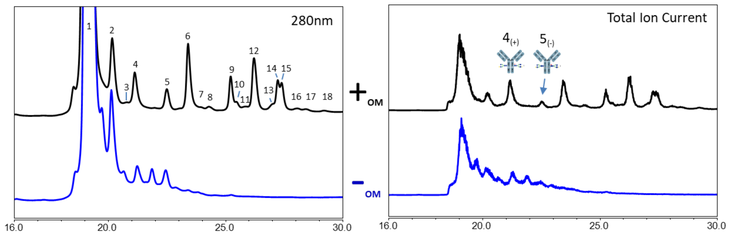
Octylmaleimide-labeled mAb variant peaks exhibited favorable electrospray ionization using RPLC-ESI/MS as well.

Fast RPLC separations (<10min) were achieved using the 2.1x150mm Diphenyl 1000A using a 30-33.5%B gradient @ 0.6ml/min, 60°C.

Above, 10µL of 1µg/µL in 0.1%TFA; 1000A Diphenyl 2.1x150mm 2.7µ, 27-36%B 40min 0.35mL/min 60°C 280nm; initial pressure 217 bar, A=0.1%DFA; B=0.1%DFA 1:1 ACN:n-propanol, PDA. Nexera X2 HPLC coupled with a Q Exactive HF employed ESI-MS; 400-5000 m/z, 0.2 trap gas, 70 CID, 60K Res., 2 micro scans, 200 IT, 35 sheath, 10 aux, 3.5kV, 275°C cap, 300°C aux, 70 S-lens. Signals normalized.

Top Down LC/MS of mAb with HALO Diphenyl 1000A

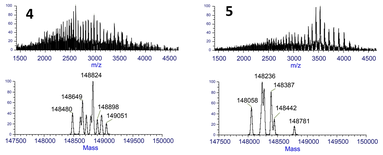
Optimized Intact Trastuzumab (+) Octylmaleimide vs. Intact Trastuzumab (-) Octylmaleimide



1000A Diphenyl 2.1x150mm 2.7µ, 27-36%B 40min 0.35mL/min 60°C 280nm; A=0.1%DFA; B=0.1%DFA 1:1 ACN:n-propanol, PDA. Nexera X2, QExactive HF. OM-trastuzumab compared to control mAb without the presence of OM. The (+) OM sample contained peaks 1, 2 and 5 which were determined to be unlabeled (-) variants by mass spectral deconvolution. Most of the peaks retained after 20 min carried at least two OM tags (+). The table below reports 18 peaks that were characterized by spectral deconvolution.

DECONVOLUTION OF INTACT (+) OM mAb sample , # OF LIGANDS			
PEAK #	(-) OM	(+) OM +(2x)	
1	X		
2	X		
3		X	
4		X	
5	X		
6		X	
7		X	
8		X	
9		X	

DECONVOLUTION OF INTACT (+/-) OM mAb, # OF LIGANDS			
PEAK #	(-) OM	(+) OM +(2x)	+(4x)
10		X	
11		X	
12		X	
13		X	
14		X	
15		X	
16		X	
17		X	X
18		X	X

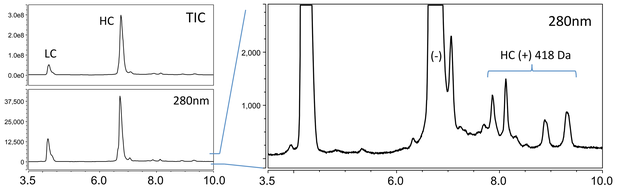


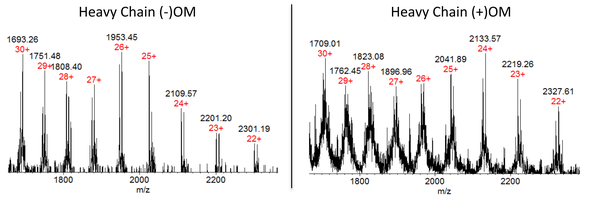
Deconvoluted Spectra for peaks (4) and (5), Octylmaleimide-labeled trastuzumab.

To examine the structures of the resolved variants, high resolution separations were conducted on intact mAb, as well as intact maleimide-labeled mAb. High quality mass spectra of the intact variants were obtained by online coupling of ESI with the Orbitrap mass spectrometer. Raw spectra used for mass deconvolution of peaks 4 and 5 are shown. Intact mass spectral deconvolution obtained the degree of maleimide load present on each variant. Later eluting (>26 min, low intensity TIC) variants exhibited possible 4x OM loads.

Middle Down LC/MS of mAb with HALO 1000A

Reduced & Alkylated [(+) Octylmaleimide-Labeled Intact Trastuzumab]





1000A Diphenyl 2.1x150mm 2.7µ, 27-36.5%B in 10min 0.6mL/min 60°C 280nm; A=0.1%DFA; B=0.1%DFA 1:1 ACN:n-propanol. Injected 5µL of 1mg/mL 0.1%DFA. The reduced sample was incubated for 45 min at 60°C.

Reduced and alkylated octylmaleimide-labeled trastuzumab exhibited (+)OM tags on the heavy chain determined by deconvolution; (+)OM was not observed on the light chain.

A mass shift from 50.6 KDa to 51.0 Kda was observed for the G0F Heavy Chain glycoform when comparing the control mass to the OM-labeled mass, indicating the presence of the OM tag on the heavy chain.

Conclusions

- High resolution reversed phase separations were demonstrated for the structural analysis of maleimide-labeled free sulfhydryl protein variants using 1000Å SPP silica packing materials.
- 1000Å SPPs exhibited superior efficiency for large protein separations. The 1000Å SPP materials showed subtle, but useful, selectivity differences between C4, ES-C18 and Diphenyl bonded-phase column packings.
- Optimized conditions including bonded phase, temperature and mobile phase modifiers were demonstrated for resolution of closely related intact IgG1 variants; optimized LC conditions were applied to the separation of maleimide-labeled counterparts. Investigation of the free sulfhydryl variants was achieved at the intact level by screening multiple reagents for favorable selectivity differences.
- Middle-up and bottom-up approaches were investigated (with limited success) to probe the domain positions of the mAb free sulfhydryl variants. Future work will employ targeted middle down and bottom up approaches to further pin-point location of the domain(s)/peptide(s) on which the maleimide tags reside. IdeS/

Acknowledgements: Tim Langlois, Stephanie Schuster, Justin Godinho, and Brian Wagner, and Matt Jackson for advice and technical assistance. This work was supported in part by National Institute of General Medical Sciences, [GM116224 to BEB]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.