Improved Methods for Residue-Level Mass Spectrometry Data Acquisition and High-Resolution Hydroxyl Radical Protein Footprinting (HRPF) Data Interpretation

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Introduction

The Higher Order Structure (HOS) of a protein plays a critical role in a therapeutic's stability, safety, and biological function. Incorrect HOS or protein interactions are linked to adverse drug reactions, which can result in further sickness or death. Advanced techniques are required to robustly address the HOS of proteins. One such method is Hydroxyl Radical Protein Footprinting (HRPF).

The Fox® Protein Footprinting System is a novel HRPF method that uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH) that irreversibly modify solvent-exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes. HRPF oxidizes solvent-exposed amino acid side chains. As solvent accessibility changes, the extent of oxidation concordantly changes. With LC-MS/MS, the change in oxidation can be localized to specific residues. Using reversed-phase chromatography, oxidized residues will shift to differing extents in retention time, resulting in multiple, low abundant peaks with the same m/z. With data-dependent acquisition (DDA), many of these regions are not selected for MS2 acquisition resulting in missed information and inconsistent results across replicates. For accurate Residue-Level Analysis (RLA), MS2 scans need to be acquired throughout the full chromatographic peak of all modification isomers. Here, we describe an MS method by adding inclusion lists to improve RLA for two proteins.

Method

Ovalbumin Analysis

Three replicates of 5 µM of ovalbumin and 8.5 mM Tris (pH 7.4) were immediately mixed with 100 mM H_2O_2 before analysis. Samples were illuminated using the Fox Protein Footprinting System with a flash voltage of 800V at 2 Hz flash rate under flow conditions optimized for single illumination per volume. The Fox inline dosimeter measured Tris as an inherent buffer/radical dosimeter, monitoring a gain in absorbance at 265 nm. Samples were collected into 25 µL of quenching solution containing 0.3 mg/mL catalase and 35 mM methionine amide to quench the excess H_2O_2 and prevent secondary oxidation. Samples were digested by trypsin and analyzed in an Orbitrap Fusion Tribrid MS coupled with a Dionex Ultimate 3000 system.

Adalimumab Analysis

Three replicates of 0.2mg/mL of Adalimumab and 1 mM adenine were immediately mixed with 50 mM H_2O_2 before analysis. Samples were illuminated, quenched, and digested in the same condition as the ovalbumin experiment. Samples were analyzed in the Orbitrap Exploris 120 coupled with Vanquish Neo system.

MS Analysis

Data-dependent acquisition (DDA) MS/MS was performed first, with CID applied to each selected precursor. MS scans were carried out at high resolution, while MS/MS scans were carried out at low resolution. These DDA results were used to identify the m/z and retention time of all unmodified peptides. FoxWare® Software was then used to identify the m/z and retention time of all oxidation products of each unmodified peptide. Oxidation products were identified based on the accurate m/z, charge state, 13C isotope pattern, and retention time relative to the unmodified version of that peptide. The m/z and retention time of peptides and peptide oxidation products were noted from the initial DDA run and placed in a timed inclusion list.

(A)

Position	Peptide	Adduct	m/z	z	t start (min)	t stop (min)
1-16	GSIGAASMEFCFDVFK		883.401	2	30	32
	GSIGAASMEFCFDVFK	OH	891.398	2	28	31
20-46	VHHANENIFYCPIAIMSALAMVYLGAK		759.132	4	37	39
	VHHANENIFYCPIAIMSALAMVYLGAK	OH	763.131	4	35	39
51-58	TQINKVVR		479.297	2	17	19
	TQINKVVR	OH	487.294	2	15.5	18.5
59-84	FDKLPGFGDSIEAQCGTSVNVHSSLR		706.095	4	24.5	26.5
	FDKLPGFGDSIEAQCGTSVNVHSSLR	OH	710.094	4	23	27
85-104	DILNQITKPNDVYSFSLASR		761.064	3	27.5	29.5
	DILNQITKPNDVYSFSLASR	OH	766.395	3	27	31
105-123	LYAEERYPILPEYLQCVK		762.061	3	27.5	29
	LYAEERYPILPEYLQCVK	OH	767.392	3	26.5	29.5
127-142	GGLEPINFQTAADQAR		844.423	2	25	27
	GGLEPINFQTAADQAR	OH	852.42	2	24	27
143-158	ELINSWVESQTNGIIR		929.985	2	28.5	30.5
	ELINSWVESQTNGIIR	OH	937.982	2	26	30
159-181	NVLQPSSVDSQTAMVLVNAIVFK		1230.664	2	33	35
	NVLQPSSVDSQTAMVLVNAIVFK	OH	1238.661	2	31	33.5
182-186	GLWEK		316.673	2	21	22
	GLWEK	OH	324.67	2	19	21
187-199	AFKDEDTQAMPFR		519.245	3	22.5	24
	AFKDEDTQAMPFR	OH	524.576	3	20	22.5
191-200	DEDTQAMPFR		605.264	2	22.5	24.5
	DEDTQAMPFR	OH	613.261	2	20	22
201-219	VTEQESKPVQMMYQIGLFR		762.053	3	28.5	30.5
	VTEQESKPVQMMYQIGLFR	OH	767.384	3	25.5	28
219-226	VASMASEK		411.705	2	16	17
	VASMASEK	OH	419.702	2	15	17
229-263	ILELPFASGTMSMLVLLPDEVSGLEQLESIINFEK		1288.341	3	38	40
	ILELPFASGTMSMLVLLPDEVSGLEQLESIINFEK	OH	1293.672	3	36	39
264-276	LTEWTSSNVMEER		791.363	2	23.5	25.5
	LTEWTSSNVMEER	OH	799.36	2	21.5	25.5
280-284	VYLPR		324.197	2	19.5	21.5
	VYLPR	OH	332.194	2	18.8	22
323-339	ISQAVHAAHAEINEAGR		591.97	3	18	21
	ISQAVHAAHAEINEAGR	OH	597.302	3	17	22
340-359	EVVGSAEAGVDAASVSEEFR		1004.977	2	25.5	27.5
	EVVGSAEAGVDAASVSEEFR	OH	1012.974	2	24	28
360-369	ADHPFLFCIK		624.315	2	25.5	27.5
	ADHPFLFCIK	OH	632.312	2	25.5	29
370-381	HIATNAVLFFGR		673.371	2	25	28
	HIATNAVLFFGR	OH	689.365	2	26	29

(B)

Position	Peptide	DDA	Inclusion
1-16	GSIGAASMEFC[+57.021]FDVFK	4	19
20-46	VHHANENIFYC[+57.021]PIAIMSALAMVYLGAK	36	129
51-58	TQINKVVR	17	245
85-104	DILNQITKPNDVYSFSLASR	8	193
105-123	LYAEERYPILPEYLQCVK	15	203
127-142	GGLEPINFQTAADQAR	10	230
143-158	ELINSWVESQTNGIIR	76	506
159-181	NVLQPSSVDSQTAMVLVNAIVFK	54	195
182-186	GLWEK	7	129
191-200	DEDTQAMPFR	0	11
201-219	VTEQESKPVQMMYQIGLFR	8	141
219-226	VASMASEK	6	91
264-276	LTEWTSSNVMEER	17	462
280-284	VYLPR	2	97
323-339	ISQAVHAAHAEINEAGR	11	284
360-370	ADHPFLFC[+57.021]IK	30	207

Figure 1: Inclusion list MS method preparation and result analysis.

(A) the inclusion list for protein ovalbumin. After the DDA MS method was performed, the peptide sequence coverage and retention time results were analyzed. The inclusion list was calculated based on the interested peptide masses.

(B) The MS2 scan number comparison between DDA and inclusion list methods. The result showed that the MS2 scan numbers were much higher in the inclusion list method than in the DDA method, which provided more confident MS2 data analysis.

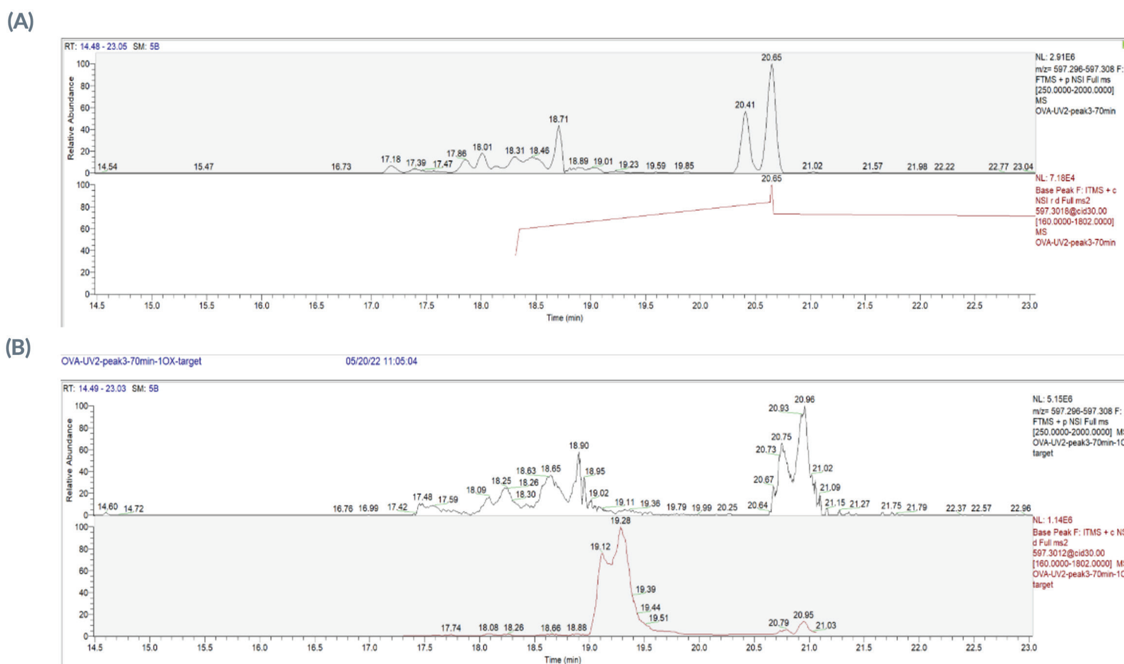

Figure 2: MS2 scan chromatogram comparison between DDA and inclusion list results for OVA.

For both chromatograms, the upper panel represents peptide 323-339 chromatogram; the bottom panel represents the MS2 scan identification based on peptide precursor mass. (A) DDA result for a peptide 323-339, only 11 MS2 scan was identified from the DDA method. Peak information from retention time 17-18 min was missed. (B) Inclusion list results for peptide 323-339, 284 MS2 scan were identified from the inclusion list, which included peaks information from retention time range from 17.5-21 min.

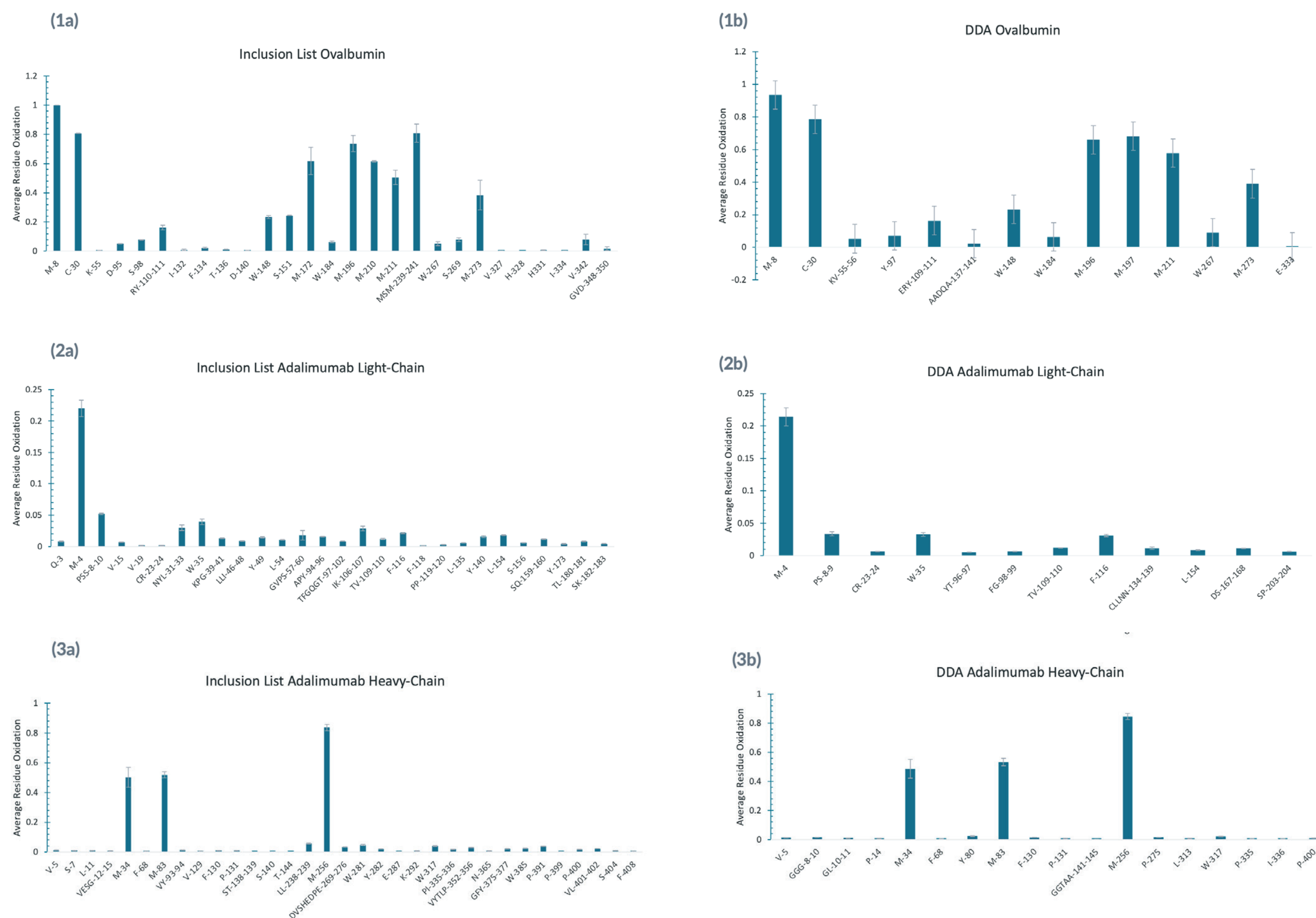

Figure 3: Comparison of ovalbumin and adalimumab DDA and inclusion list average residue oxidation result.

1a and 1b: Non-phosphorylated ovalbumin; 27 residues or small fragments regions were identified for oxidation from the inclusion list MS method, while 14 residues or small fragments were found from the DDA method. 2a and 2b: adalimumab light chain; 3a and 3b: adalimumab heavy chain. The inclusion list identified 28 and 33 regions from the adalimumab heavy and light chains, respectively. While the DDA method identified 12 and 18 regions from the adalimumab heavy and light chains, respectively.

Conclusions

- The inclusion list approach increased the number of desired mass percussor and improved the MS identification compared to the DDA method alone.
- The inclusion list approach improved the identification of low-intensity modified residues or small fragments compared to the DDA method alone.
- The identification of precursor mass was up to 25 times greater during the inclusion list MS method.
- The total number of high-confidence matches improved 30 times compared to the inclusion list result.

GenNext has pioneered a superior, compact, cost-effective, and safe means of performing advanced FPOP HRPF analysis.
By replacing expensive, complicated, and hazardous lasers with our proprietary Flash Oxidation System, you can easily perform HRPF with a convenient benchtop instrument.

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