Analysis of post-translational modifications using fast electron-activated dissociation (EAD)

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INTRODUCTION

Characterization of human cellular proteomes enables identification of disease biomarkers and new therapeutic targets. Mass spectrometry-based techniques, such as bottom-up proteomics, are widely used to analyze cellular proteomes. For analysis of protein digests for protein identification, data-dependent acquisition (DDA or IDA) is often used. The ZenoTOF 7600 system, a QTOF platform, uses the Zeno trap to increase duty cycle to ≥90% across the entire fragment ion mass range, resulting in signal gains of 4- to 25fold in MS/MS mode.¹ These gains enable up to a 40% improvements in proteins identified using DDA workflows.²

For proteomics experiments, there are two fragmentation options available on the ZenoTOF 7600 system: collision induced dissociation (CID) and electron activated dissociation (EAD). Unlike CID, which relies on the collision of ions with nitrogen gas to fragment molecules at their weakest bonds, EAD involves the capture of electrons by molecular ions to form a radical state that then fragments. CID and EAD result in different fragments for the same peptide, with CID yielding y and b ions and EAD yielding additional c' and z• ions. EAD fragmentation provides complimentary sequence information to CID and often preserves posttranslational modifications (PTMs) that undergo neutral loss in a CID experiment.^{3,4} This work focuses on DDA method development and analysis of HeLa digest using EAD, with an emphasis on site-specific localization of phosphorylation sites. In addition to phosphorylated peptides, glycated human peptides were also investigated using EAD.

MATERIALS AND METHODS

Sample preparation: A 100 µg sample of digested HeLa cell lysate was fractionated into 44 samples using high pH RP-HPLC, as previously described.⁵ Then, 10% of each fraction was injected for LC-MS/MS analysis. Human plasma was used as the source of human serum albumin (HSA) for glycation modification analysis.

Chromatography: HeLa peptide fractions were separated using a Waters ACQUITY UPLC M-class system with a flow rate of 6 µL/min in direct inject mode. A Phenomenex Kinetex 2.6 µm XB-C18 LC column (100 Å, 150 x 0.3 mm) was used with a rapid linear gradient from 5 – 30% B over 21 minutes to interrogate the fractions. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

Mass spectrometry: A SCIEX ZenoTOF 7600 system equipped with the OptiFlow Turbo V ion source⁶ using a low microflow probe and electrode was used for all data acquisition. DDA parameters that were implemented for all experiments included a TOF MS accumulation time of 250 ms and an exclusion time of 6 seconds. Only precursors with charge states in the range 2-5 with intensities greater than 100 cps were selected for fragmentation. For CID acquisition, the maximum number of candidate ions per cycle was 45 and the accumulation time was 20 ms. For EAD analysis of HeLa digest, the maximum number of candidate ions was 10 per cycle and an accumulation time of 50 ms was used. The reaction time for EAD was 20 ms was used, with an electron beam current of 3000 nA with a KE of 0 eV. For analysis of glycated peptides, 20 candidate ions were used per cycle with a 20 ms reaction time and 25 ms total acquisition time.

Data processing: MASCOT software was used for all data processing. HeLa digest files were searched against the Swissprot_Human database using the SCIEX EAD algorithm for EAD and the ESI-QUAD-TOF algorithm for CID. For both phosphorylated and glycated peptides, high quality figures were generated using Bio Tool Kit in SCIEX OS software.⁷



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Proteomic coverage of HeLa digest obtained using EAD

In order to select the acquisition parameters for Zeno EAD DDA analysis of HeLa digest, a variety of parameters were tested, including electron beam current, electron kinetic energy, reaction time and accumulation time. For peptides, it was determined that using an electron KE of 0 eV and a beam current of 3000 nA promoted fragmentation and yielded high fragment ion intensities. To evaluate the performance of EAD for protein identification, the same fractionated HeLa cell lysate was analyzed with data-dependent analysis using both CID and EAD fragmentation for MS/MS. The analysis identified 52,905 peptides at 1% FDR (Figure 1) using EAD. Fewer total numbers of peptides were identified using EAD due to the slightly slower acquisition speed (2.5x slower). However, the orthogonal fragmentation mode provided identifications of peptides not found using CID, yielding 11% more peptides. This highlights that while slower, the use of EAD is easily fast enough to be compatible with data-dependent acquisition. An example peptide is shown in which nearly complete c and z• (z+1) ion series were detected, with CID fragmentation yielding only partial y and b ion series (Figure 2).







Figure 2. Comparing CID and EAD fragmentation of a non-phosphorylated peptide. (Top) CID fragmentation yielded incomplete y and b ion series for the LHILASGADVTSANAKAK peptide. (Bottom) Nearly complete z• (z+1 in table) and c series were detected for the same peptide when fragmented by EAD. Detected target fragment ion masses (+1 charge state) are shown in bold red in the tables below each associated spectrum.

Peptide Identifications @ 1% FDR

Figure 1. Comparison of peptide identifications at 1% FDR as a function of fragmentation mode on the ZenoTOF 7600 system. Due to the higher frequency of sampling with CID, peptide identifications were approximately 2-fold higher than with the EAD fragmentation mode, but EAD provided over 10,000 new peptide identifications.

Phosphorylation site localization using Zeno EAD MS/MS spectra

Figure 3. Localization of phosphorylation site in a peptide with multiple serines. An EAD spectrum for peptide sequence ISSSS[Pho]FSR is shown (right) with a nearly complete z• (z+1) ion series. The associated fragment ion masses are shown in the table below the spectrum for reference and detected +1 fragment ions are shown in bold red.



	Y	163.06333	2	263.13902
	T[Pho]	181.01401	3	444.15303
	Н	137.05891	4	581.21194
	E	129.04259	5	710.25454
	V	99.06841	6	809.32295
	V	99.06841	7	908.39136
	Т	101.04768	8	1009.4390
	L	113.08406	9	1122.5231
	W	186.07931	10	1308.6024
	Y	163.06333	11	1471.6657
	R	156.10111	12	1627.7668



state are tabulated in bold red.



Overall, 52,905 peptides were identified at 1% FDR from the EAD data, including 158 phosphopeptides. The sample preparation for the digest was not optimized for preservation of modifications and no enrichment step was performed. EAD spectra from phosphopeptides were used to identify the sequence and site of phosphorylation in peptides, including peptides with multiple serines (Figure 3).

Other challenging phosphorylation sites were located with EAD spectra, including on a threonine residue near the peptide N-terminus (Figure 4) and on a peptide with 17 amino acids and a molecular weight of 1889.88 Da (Figure 5). The associated c and z+1 ion series clearly detailed the location of the phosphorylation sites on the peptides. EAD fragmentation provided a means to identify challenging phosphorylation sites otherwise unattainable with CID.



۲	1	113.08406	1	114.09134	131.11789	950.39791	933.37136	934.37918	935.38701	8
	S	87.03203	2	201.12337	218.14992	837.31384	820.28729	821,29512	822.30294	7
	S	87.03203	3	288.15540	305.18195	750.28181	733.25526	734.26309	735.27091	6
	S	87.03203	4	375.18743	392.21398	663.24978	646.22324	647.23106	648.23889	5
	S[Pho]	166.99836	5	542.18579	559.21234	576.21776	559.19121	560.19903	561.20686	4
	F	147.06841	6	689.25420	706.28075	409.21939	392.19285	393.20067	394.20850	3
	S	87.03203	7	776.28623	793.31278	262.15098	245.12443	246.13226	247.14008	2
	R	156.10111	8	932.38734		175.11895	158.09240	159.10023	160.10805	1

1367.62696 1383.64568 1366.61913 1367.62696 1368.63478 598.23849 1202.63167 1185.60512 1186.61295 1187.62077 727.28108 1065.57276 1048.54621 1049.55403 1050.56186 826.34950 936.53016 919.50362 920.51144 921.5192 925.41791 837.46175 820.43520 821.44303 822.45085 **1026.46559 738.39334** 721.36679 **722.37461 723.38244** 1139.54965 637.34566 620.31911 621.32693 622.334 1325.62897 524.26159 507.23504 508.24287 509.25069 **1488.69230 338.18228** 321.15573 **322.16356** 323.17138 175.11895 158.09240 159.10023 160.10805

Figure 4. Localization of phosphorylation site near N-terminus of peptide with tyrosine and threonine. The c fragment ion series shown (left) enabled localization of a phosphorylated threonine adjacent to a tyrosine. Both c and z• (z+1) ion series are highlighted in the table below the EAD spectrum for +1 charge state and detected fragments are highlighted in bold red.

		Symbol	Res. Mass	# (N)	ь	с	У	z	z + 1	z + 2	# (C
	Þ	R 1	156.10111	1	157.10839	174.13494	1890.88918	1873.86263	1874.87045	1875.87828	17
		A	71.03711	2	228.14550	245.17205	1734.78807	1717.76152	1718.76934	1719.77717	16
		Р	97.05276	3	325.19827	342.22481	1663.75095	1646.72440	1647.73223	1648.74005	15
		S[Pho]	166.99836	4	492.19663	509.22318	1566.69819	1549.67164	1550.67946	1551.68729	14
		V	99.06841	5	591.26504	608.29159	1399.69983	1382.67328	1383.68110	1384.68893	13
		A	71.03711	6	662.30215	679.32870	1300.63141	1283.60486	1284.61269	1285.62051	12
		N	114.04293	7	776.34508	793.37163	1229.59430	1212.56775	1213.57558	1214.58340	11
		V	99.06841	8	875.41350	892.44004	1115.55137	1098.52482	1099.53265	1100.54047	10
		G	57.02146	9	932.43496	949.46151	1016.48296	999.45641	1000.46423	1001.47206	9
		S	87.03203	10	1019.46699	1036.49354	959.46149	942.43494	943.44277	944.45059	8
		Н	137.05891	11	1156.52590	1173.55245	872.42947	855.40292	856.41074	857.41857	7
		C[CAM]	160.03065	12	1316.55655	1333.58310	735.37055	718.34400	719.35183	720.35965	6
		D	115.02694	13	1431.58349	1448.61004	575.33990	558.31335	559.32118	560.32901	5
$1 z_{12} + 1 z_{14} + 1$		L	113.08406	14	1544.66756	1561.69411	460.31296	443.28641	444.29424	445.30206	4
-13 -14 -		S	87.03203	15	1631.69958	1648.72613	347.22890	330.20235	331.21017	332.21800	3
12 ⁺¹ Z ₁₆ +1		L	113.08406	16	1744.78365	1761.81020	260.19687	243.17032	244.17814	245.18597	2
առանվորնել նիւս եւսվոր վեր էլ բերվանությունը է լ		К	128.09496	17	1872.87861		147.11280	130.08626	131.09408	132.10191	1

Mass/Charge, Da

Figure 5. Identification of phosphorylation site in a 17-residue peptide. c and z• (z+1) fragment ions enabled identification of a phosphorylated serine in the sequence of an 1889.88 Da peptide. Detected fragment ions for +1 charge

Glycation site localization using Zeno EAD MS/MS

A panel of human peptides was analyzed to evaluate hexose modifications using Zeno EAD DDA. While a hexose modification could not be localized using CID, EAD enabled its unambiguous localization on a lysine residue of a long, 24-amino acid peptide (Figure 6).



Figure 6. EAD fragmentation of a glycated peptide from human serum albumin (HSA) protein. A hexose modification was localized on a lysine using a nearly complete c ion series. Detected fragment ion masses are shown in bold red in the table to the right of the EAD spectrum for +1 charge state, with fragments matching to a different charge state shown in red italics.

CONCLUSIONS

The Zeno EAD DDA workflow developed in this work enabled robust identification of thousands of unique peptides in HeLa digest. High pH fractionation of HeLa digest followed by LC-MS/MS analysis with the ZenoTOF 7600 system generated a large-scale Zeno EAD DDA data set for interrogation. The Zeno trap was activated to further improve MS/MS sensitivity and yield high fragment ion intensities. EAD provided an alternative fragmentation option for the study of proteomic samples and enabled better characterization of some types of peptides vs. CID, such as long tryptic or mis-cleaved peptides and post-translational modified peptides, especially those modified with labile PTMs. In EAD spectra evaluated here, intact z• and c ion series were readily detectable for sequencing and localization of challenging phosphorylation and glycation sites.

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	Symbol		Res. Mass	# (N)	b	с	У	z	z + 1	z + 2	# (C)
	Þ	L	113.08406	1	114.09134	131.11789	3019.34417	3002.31762	3003.32544	3004.33327	24
		V	99.06841	2	213.15975	230.18630	2906.26010	2889.23355	2890.24138	2891.24920	23
		Т	101.04768	3	314.20743	331,23398	2807.19169	2790.16514	2791.17297	2792.18079	22
		D	115.02694	4	429.23438	446.26093	2706.14401	2689.11746	2690.12529	2691.13311	21
		L	113.08406	5	542.31844	559.34499	2591.11707	2574.09052	2575.09834	2576.10617	20
		Т	101.04768	6	643.36612	660.39267	2478.03300	2461.00645	2462.01428	2463.02210	19
		K[Hex]	290.14779	7	933.51391	950.54045	2376.98533	2359.95878	2360.96660	2361.97443	18
		V	99.06841	8	1032.58232	1049.60887	2086.83754	2069.81099	2070.81881	2071.82664	17
		н	137.05891	9	1169.64123	1186.66778	1987.76912	1970.74258	1971.75040	1972.75823	16
		Т	101.04768	10	1270.68891	1287.71546	1850.71021	1833.68366	1834.69149	1835.69931	15
		E	129.04259	11	1399.73150	1416.75805	1749.66253	1732.63598	1733.64381	1734.65164	14
		C[CAM]	160.03065	12	1559.76215	1576.78870	1620.61994	1603.59339	1604.60122	1605.60904	13
		C[CAM]	160.03065	13	1719.79280	1736.81935	1460.58929	1443.56274	1444.57057	1445.57839	12
		Н	137.05891	14	1856.85171	1873.87826	1300.55864	1283.53209	1284.53992	1285.54774	11
		G	57.02146	15	1913.87318	1930.89973	1163.49973	1146.47318	1147.48101	1148.48883	10
		D	115.02694	16	2028.90012	2045.92667	1106.47827	1089.45172	1090.45954	1091.46737	9
		L	113.08406	17	2141.98419	2159.01073	991.45132	974.42477	975.43260	976.44042	8
		L	113.08406	18	2255.06825	2272.09480	878.36726	861.34071	862.34854	863.35636	7
		E	129.04259	19	2384.11084	2401.13739	765.28320	748.25665	749.26447	750.27230	6
		C[CAM]	160.03065	20	2544.14149	2561.16804	636.24060	619.21405	620.22188	621.22970	5
		Α	71.03711	21	2615.17861	2632.20515	476.20995	459.18340	460.19123	461.19905	4
		D	115.02694	22	2730.20555	2747.23210	405.17284	388.14629	389.15411	390.16194	3
hikilde del legele de legele de legele		D	115.02694	23	2845.23249	2862.25904	290.14590	273.11935	274.12717	275.13500	2
kilailigi olehai aliiqida alalad alala ja taina ara Mayada migla ka dalladin Jadaliinin ka merena para a		R	156.10111	24	3001.33360		175.11895	158.09240	159.10023	160.10805	1
1000 1100 1200 1300 1400 1500 1600											
/Charge Da											

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