Sequencing of Polyethylene Glycol Conjugation Site on a Protein by High-Resolution Mass Spectrometry

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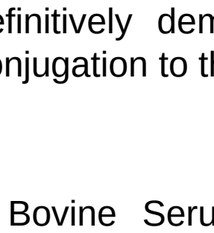
Introduction

Proteins and peptides are highly effective therapeutic agents in the treatment of many diseases. However, their efficacy is reliant on their half-lives and stability following administration. PEG-protein conjugates have prolonged half-lives and improved efficacy due to the ability of the PEG moiety to reduce renal clearance, improve stability to proteases, and reduce immunogenicity. Site-specific PEGylation is essential to produce a homogenous biotherapeutic agent(1), and analysis of the site of PEGylation on a protein is critical to drug product characterization.

Historically, peptide mapping has been used to determine modified peptide(s)(2). However, direct confirmation of the site-specific PEG conjugated amino acid in a peptide has been challenging. Here, we present a new methodology using high-resolution mass spectrometry (HRMS) to sequence a PEGylated tryptic peptide, containing a cysteine conjugated to PEG-maleimide. This method utilizes HRMS analysis of the PEG conjugates by in-source collision-induced dissociation (SID) and higher-energy collisional dissociation (HCD) MS^2. In this approach, the method allows sequencing of the tryptic peptide definitively demonstrating the location of PEG conjugation to the protein.

Methods

- Bovine Serum Albumin was conjugated on Cys-58 to M-Maleimide 20 kDa PEG (Figure 1).
- The PEG standard, intact PEG-Protein conjugate, and PEGylated peptide were analyzed by direct spotting with alpha-cyano-4-hydroxycinnamic acid onto a MALDI target.
- PEG-protein conjugate was diluted in guanidine HCl / EDTA, reduced and alkylated with dithiothreitol and iodoacetamide, respectively. Following reduction and alkylation, the buffer was exchanged to Tris-HCl (pH 8) and the protein was enzymatically digested with trypsin for 4 hours at 37ºC.
- The digested sample was analyzed by liquid chromatography and high-resolution mass spectrometry using a dual fragmentation approach of SID and HCD MS^2.



R: Cys-58
R': Linker-20kDa PEG

Figure 1: *Maleimide moiety schematic (left). Protein sequence with the targeted cysteine denoted by red (below). Residues 1-18 are the signal sequence (underlined) and 19-24 are pro-protein (blue).*

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  1 MKWVTFISLL  LLFSSAYSRG  VFRRDTHKSE  IAHRFKDLGE  EHFKGLVLIA
 51 FSQYLQQCPF  DEHVKLVNEL  TEFAKTCVAD  ESHAGCEKSL  HTLFGDELCK
101 VASLRETYGD  MADCCEKQEP  ERNECFLSHK  DDSPDLPKLK  PDPNTLCDEF
151 KADEKKFWGK  YLYEIARRHP  YFYAPELLYY  ANKYNGVFQE  CCQAEDKGAC
201 LLPKIETMRE  KVLASSARQR  LRCASIQKFG  ERALKAWSVA  RLSQKFPKAE
251 FVEVTKLVTD  LTKVHKECCH  GDLLECADDR  ADLAKYICDN  QDTISSKLKE
301 CCDKPLLEKS  HCIAEVEKDA  IPENLPPLTA  DFAEDKDVCK  NYQEAKDAFL
351 GSFLYEYSRR  HPEYAVSVLL  RLAKEYEATL  EECCAKDDPH  ACYSTVFDKL
401 KHLVDEPQNL  IKQNCDQFEK  LGEYGFQNAL  IVRYTRKVPQ  VSTPTLVEVS
451 RSLGKVGTRC  CTKPESERMP  CTEDYLSLIL  NRLCVLHEKT  PVSEKVTKCC
501 TESLVNRRPC  FSALTPDETY  VPKAFDEKLF  TFHADICTLP  DTEKQIKKQT
551 ALVELLKHKP  KATEEQLKTV  MENFVAFVDK  CCAADDKEAC  FAVEGPKLVV
601 STQTALA
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Results

MALDI-MS

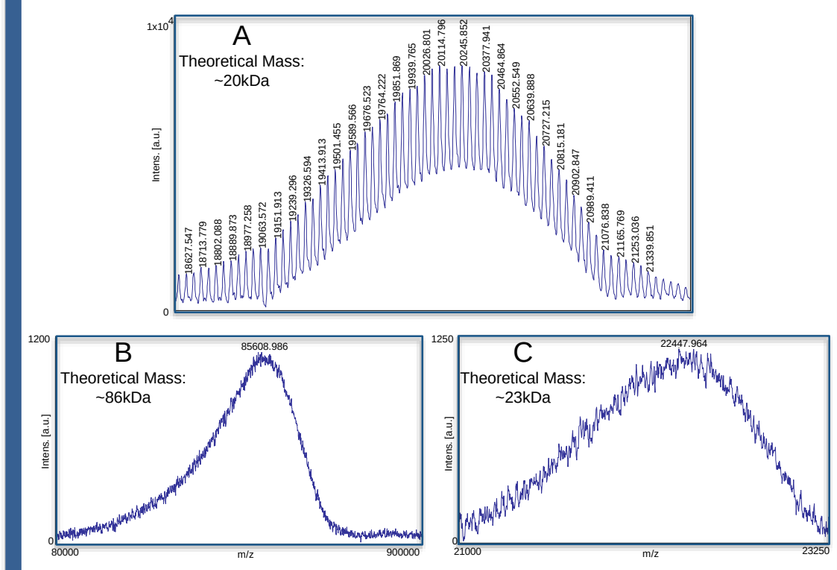


Figure 2: *Observed mass of PEG standard (A), PEG-Protein conjugate (B) and enzymatically digested PEGylated peptide (C) by MALDI-MS. Polydispersity of PEG is clearly observed in the PEG standard.*

SID Fragmentation by LC-MS of the PEGylated Peptide Resulting from Tryptic Digest

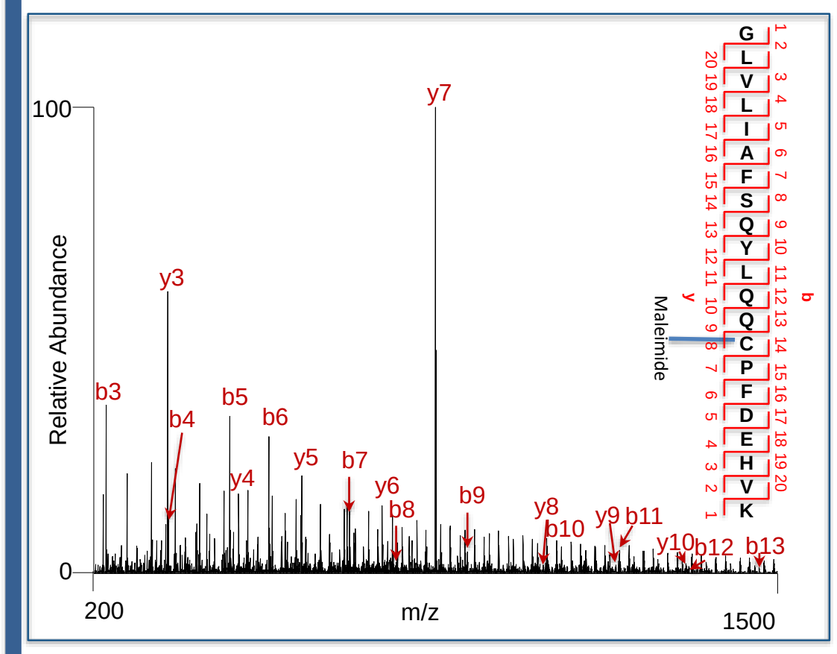


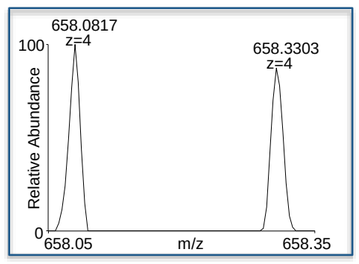
Figure 3: *Averaged spectra acquired from the PEGylated peptide when high level of SID is applied during LC-MS. Observed fragment ions are denoted in red and a theoretical schematic is illustrated on the right.*

Mass Accuracy (ppm)	b (observed)	b (theoretical) Maleimide on C				y (theoretical) Maleimide on C	y (observed)	Mass Accuracy (ppm)
			1	G	21			
		171.1128	2	L	20	2475.2377		
2.22	270.1818	270.1812	3	V	19	2362.1537		
2.35	383.2662	383.2653	4	L	18	2263.0852		
3.02	496.3508	496.3493	5	I	17	2150.0012		
3.70	567.3886	567.3865	6	A	16	2036.9171		
4.34	714.4580	714.4549	7	F	15	1965.8800		
2.62	801.4890	801.4869	8	S	14	1818.8116		
0.22	929.5453	929.5455	9	Q	13	1731.7796		
3.29	1092.6124	1092.6088	10	Y	12	1603.7210		
1.00	1205.6941	1205.6929	11	L	11	1440.6576		
2.10	1333.7542	1333.7514	12	Q	10	1327.5736	1327.5753	1.28
3.97	1461.8158	1461.8100	13	Q	9	1199.5150	1199.5141	0.75
		1661.8356	14	C	8	1071.4564	1071.4613	4.57
		1758.8884	15	P	7	871.4308	871.4341	3.79
		1905.9568	16	F	6	774.3781	774.3806	3.23
		2020.9837	17	D	5	627.3097	627.3120	3.67
		2150.0263	18	E	4	512.2827	512.2845	3.51
		2287.0852	19	H	3	383.2401	383.2414	3.39
		2386.1537	20	V	2	246.1812		
			21	K	1	147.1128		

Table 1: *Theoretical fragment ions compared to the observed fragment ions produced from SID. All observed fragment ions had mass accuracy of 5 ppm or less. The y7 ion is seen with no mass shift, whereas, the y8 ion is seen with a mass shift corresponding to the addition of the cysteine as well as the maleimide. This indicated the maleimide moiety is located specifically on the cysteine residue.*

HCD Fragmentation by LC-MS of the PEGylated Peptide Resulting from Tryptic Digest

GLVLIAFSQYLQQCPFDEHVK + Maleimide/Linker



Theoretical (M+4H): 658.0834 m/z
Observed (M+4H): 658.0817 m/z

$\frac{(658.0817-658.0834)}{658.0834} \times 1E^6 =$ **2.6 ppm**

Figure 4: *Observed mass of the multiply charged precursor ion of the tryptic peptide with the maleimide-linker moiety.*

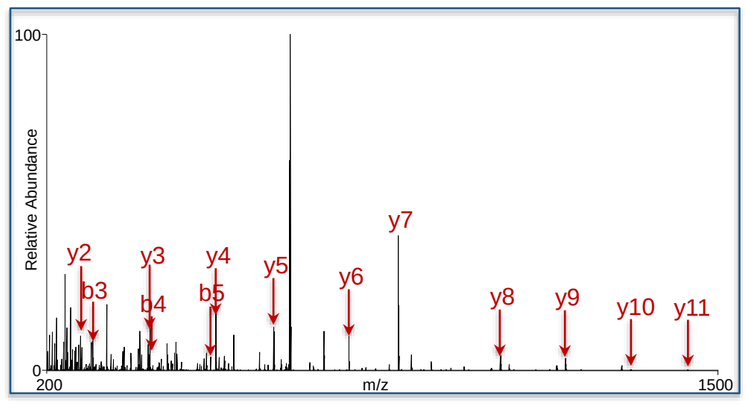


Figure 5: *Averaged MS^2 spectra acquired from the HCD fragmentation of the tryptic peptide with the maleimide-linker moiety.*

Mass Accuracy (ppm)	b (observed)	b (theoretical) Maleimide on C				y (theoretical) Maleimide on C	y (observed)	Mass Accuracy (ppm)
1.48	270.1808	270.1812	3	V	19	2362.1537		
3.91	383.2638	383.2653	4	L	18	2263.0852		
1.61	496.3485	496.3493	5	I	17	2150.0012		
		1205.6929	11	L	11	1440.6576	1440.6540	2.50
		1333.7514	12	Q	10	1327.5736	1327.5763	2.03
		1461.8100	13	Q	9	1199.5150	1199.5100	4.17
		1661.8356	14	C	8	1071.4564	1071.4522	3.92
		1758.8884	15	P	7	871.4308	871.4283	2.87
		1905.9568	16	F	6	774.3781	774.3762	2.45
		2020.9837	17	D	5	627.3097	627.3081	2.55
		2150.0263	18	E	4	512.2827	512.2825	0.39
		2287.0852	19	H	3	383.2401	383.2399	0.52
		2386.1537	20	V	2	246.1812	246.1809	1.22

Table 2: *Theoretical fragment ions compared to the observed fragment ions produced from HCD MS^2 fragmentation. All observed fragment ions had mass accuracy of 5 ppm or less.*

Conclusions

- MALDI confirmed the MW of the mono-PEGylated protein and the tryptically digested PEGylated peptide (i.e. peptide conjugated with polydisperse PEG) was isolated.
- Using SID on the tryptic digested PEG-Protein conjugate, the PEG was selectively dissociated leaving the maleimide/linker moiety still intact.
- The resulting precursor ion observed in the full scan MS was a multiply charged state of the peptide + linker with a mass error of 2.8 ppm.
- The observed b and y fragment ions from both SID and HCD MS^2 fragmentation were used to sequence the peptide.
- The site-specific location of the PEG moiety was confirmed by monitoring the mass shift caused by the maleimide moiety on the y fragment ions.
- All assigned fragment ions were observed with a mass error of 5 ppm or less.

References

(1) "PEGylation, successful approach to drug delivery" Veronese FM, et al., Drug Discov Today., 2005, 10(21), 1451-4158.
(2) "Proteomic analysis of post-translational modifications" M. Mann et al., Nature Biotechnology, 2003, 21, 255-261.