Analysis of Drug Attachment Site: Implications for ADC Potency and Stability

eurofins | Lancaster Laboratories

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Abstract

Antibody Drug Conjugates (ADC) have emerged as new therapies for the treatment of certain cancers by a targeted approach. These targeted molecules are synthesized by the covalent attachment of an antibody and drug through a linker. For potency, the linker must remain stable until cellular internalization, allowing for the recognition and binding of the specific cell type. Design strategies have been devised to enhance linker stability through manipulation of the attachment chemistries, such as thiol-maleimide, disulfides, or lysine amide linkers. Interestingly, a recent study has shown that solvent accessibility at the thiol-maleimide linkage attachment site can cause opening of the ring which influences *in vivo* potency.

Analytical methodologies are presented here to discern the protein attachment site and subtle chemical changes to the peptide and cytotoxic agent that can affect efficacy. For this study, a commercial IgG1 mAb was joined to SMCC-Biotin to serve as the conjugate in this study. Peptide mapping analysis with UPLC HR mass spectrometry (LTQ-Orbitrap) was used to characterize the conjugate then demonstrate that maleimide hydrolysis can be detected and identified, as was proposed earlier. A clear understanding of this linker secondary chemistry is critical for a complete realization of ADC greater efficacy with reduced side-effects.

Waters Intact mAb Mass Check Standard

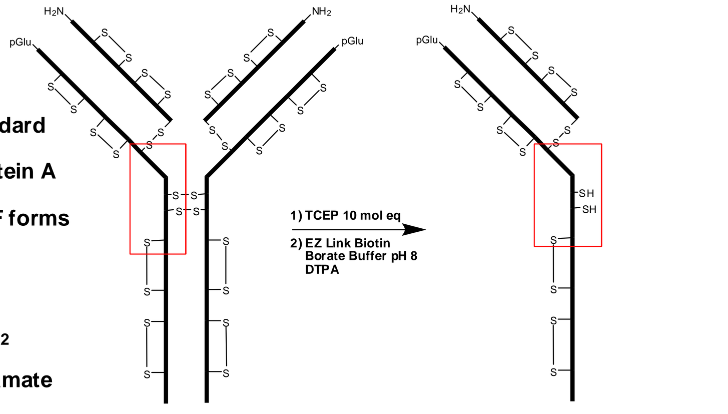
Intact Mouse protein purified by Protein A

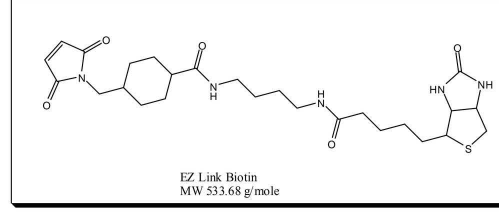
Glycosylated with G0F, G1F and G2F forms

Heavy chain $C_{2170}H_{3338}N_{562}O_{661}S_{19}$
48484.3 Da

Intact Protein $C_{6472}H_{9940}N_{1698}O_{2008}S_{52}$

Fixed Modifications on 2 x Pyroglutamate





Two TRYPSIN peptides:

Heavy Chain
TTPPSVYPLAPGSAAQTDSMVTLGCLVK

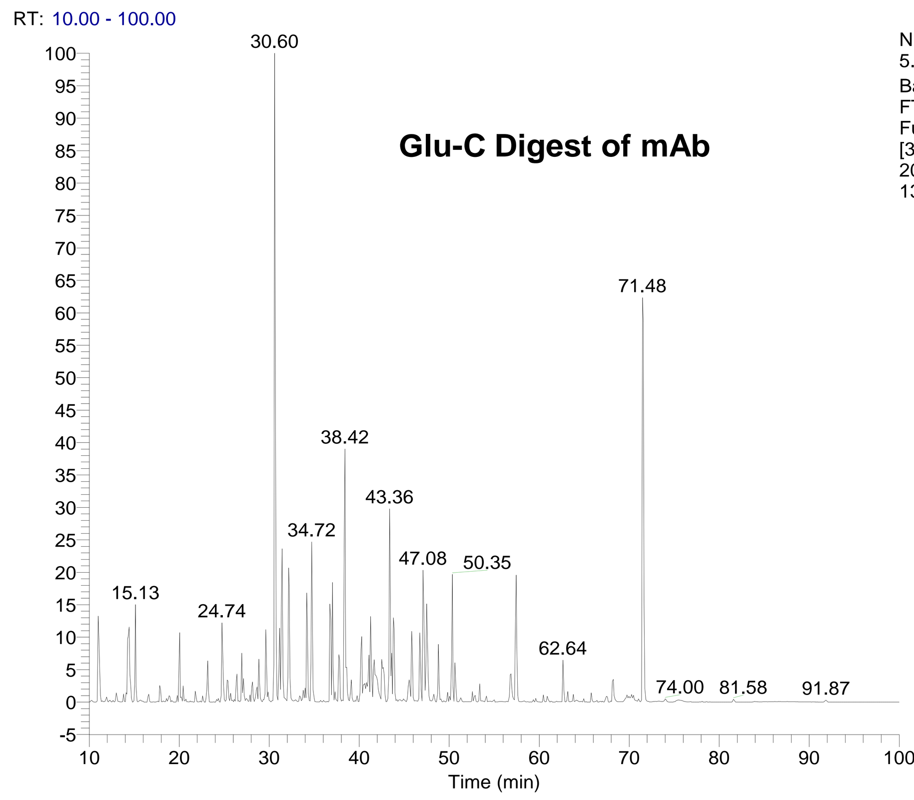
Inter-Heavy Chain Linkages
DCGCKPCICTVPEVSSVFIFPPKPK

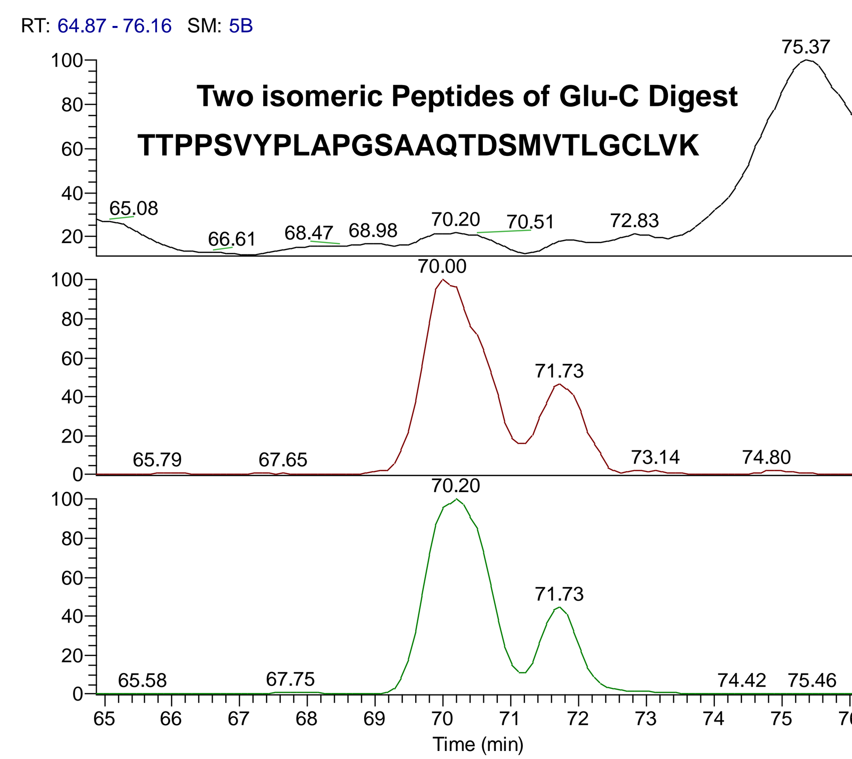
Parameter	Condition		
Model	Waters Acquity H-Class UPLC and LTQ/Orbitrap Mass Spectrometer		
Column	Waters Acquity BEH130 C18, 1.7 µm, 150 x 2.1 mm		
Flow Rate	200 µL/min		
Mobile Phase A	Water with 0.1% formic acid (v/v)		
Mobile Phase B	Acetonitrile with 0.1% formic acid		
Column Temperature	40°C		
Injection Volume (varied)	10 µL		
Autosampler Tray Temp	5°C		
Gradient (linear)	Time (min)	% A	%B
	0.0	98	2
	5.0	98	2
	110.0	55	45
	115.0	55	45
	117.0	10	90
	122.0	10	90
	125.0	98	2
	135.0	98	2
Tune Conditions	APESI Positive		
Tune File	Angiotensin_01		
TUV Detector	280 nm		
Orbitrap Mass Spectrometer	Scan FTMS 300-2000 @ 60000 Resolution		
LTQ ion trap	MS/MS Scan dependent – top 4		

References

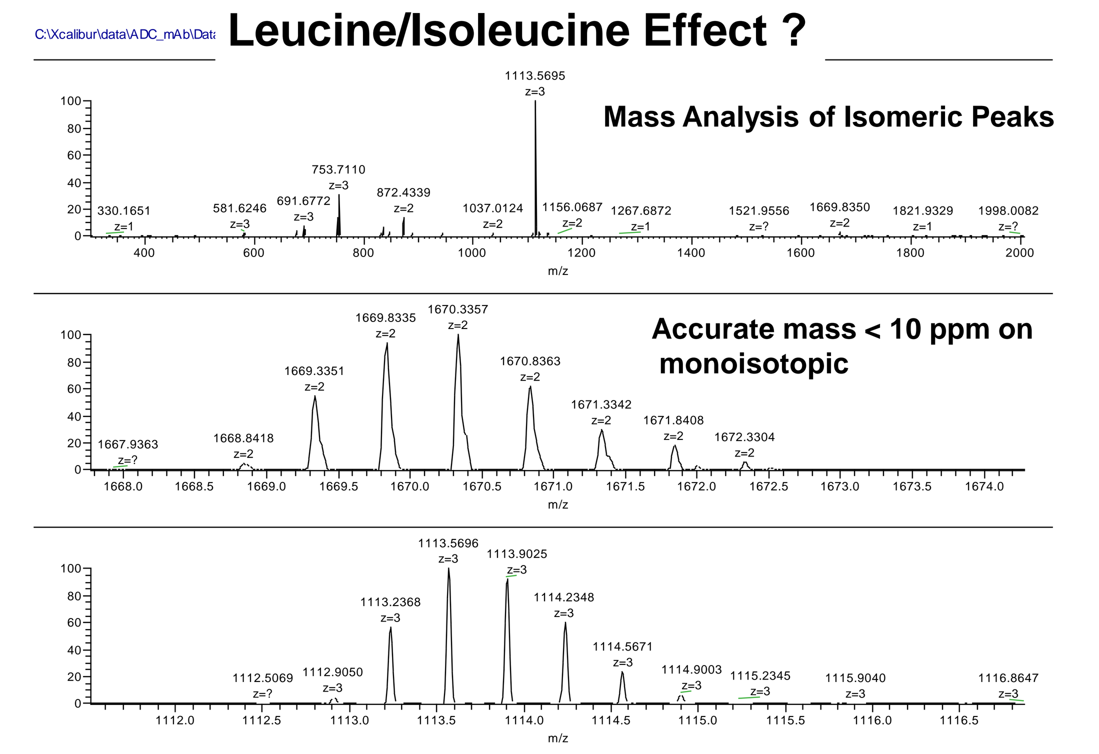
1) Shen et al. *Nature Biotechnology* **2012**, *30*, 184-189
2) Knight, P. *Biochem J.* **1979**, *179*, 191-197
3) Sun et al. Bioconjugate Chem **2005,** *16*, 1282-1290
4) Water Application Note

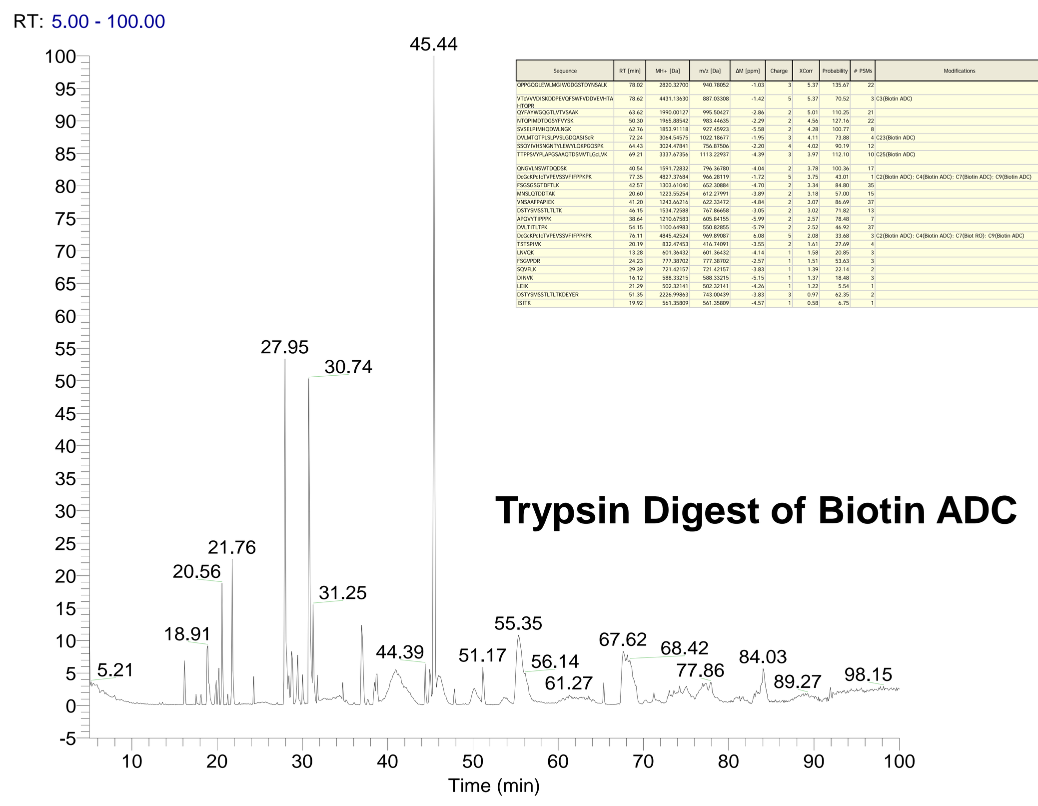
Results

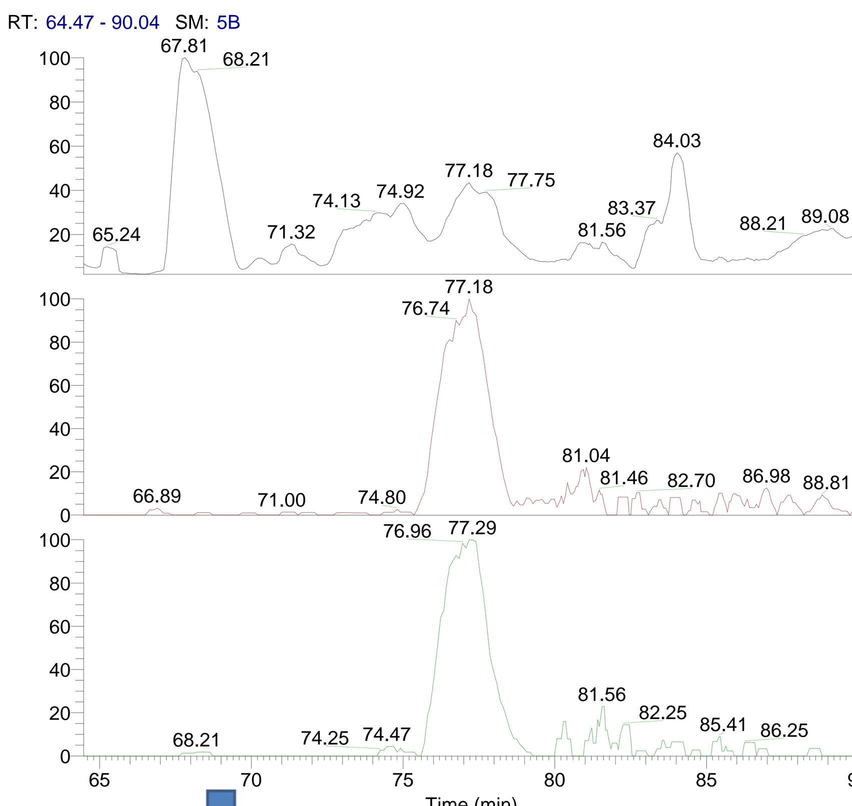


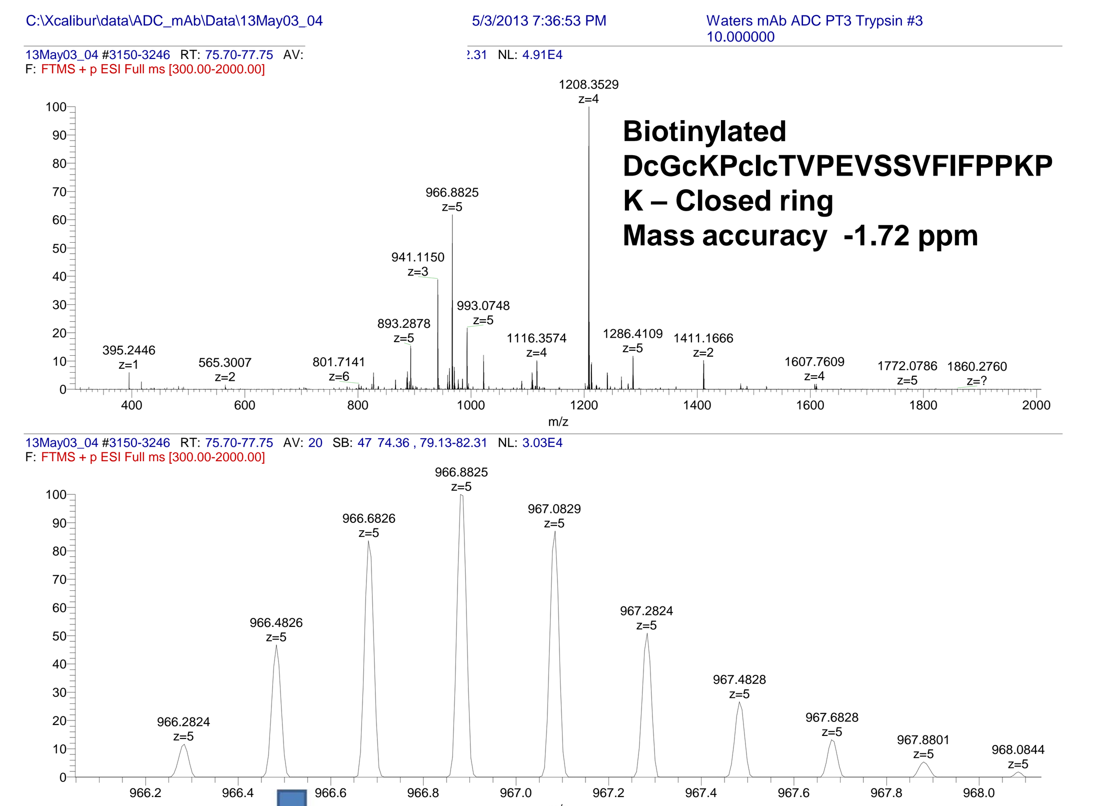


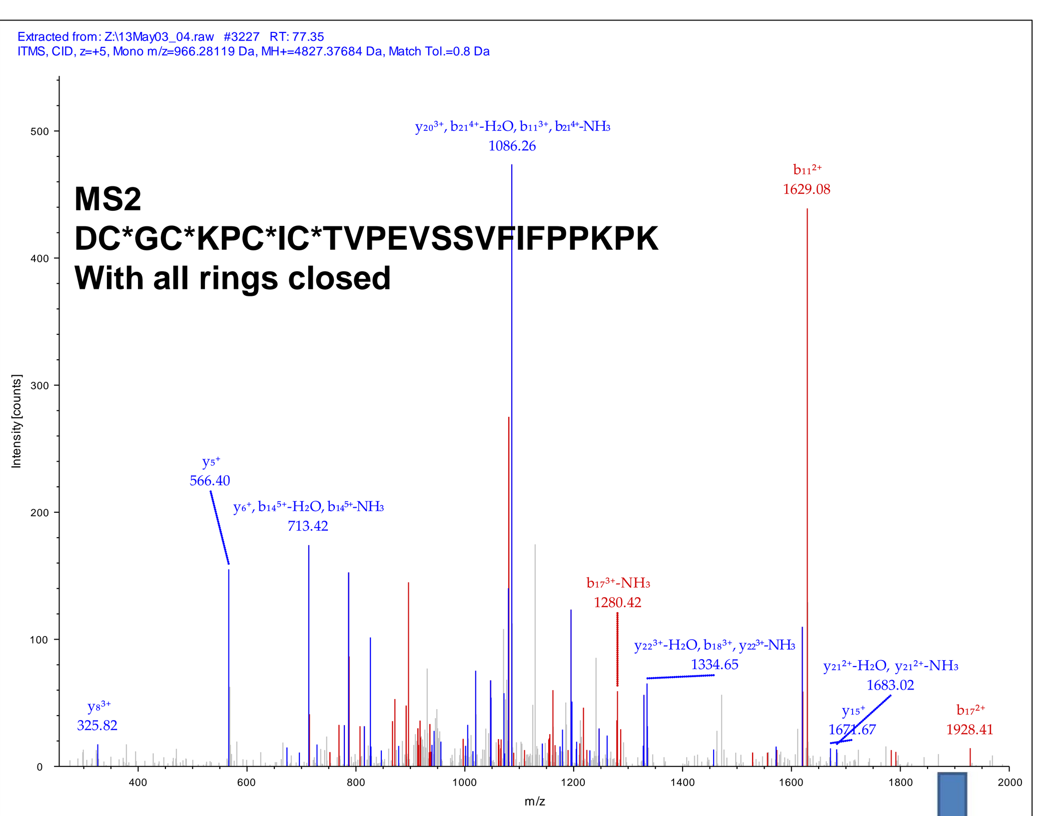
Leucine/Isoleucine Effect ?

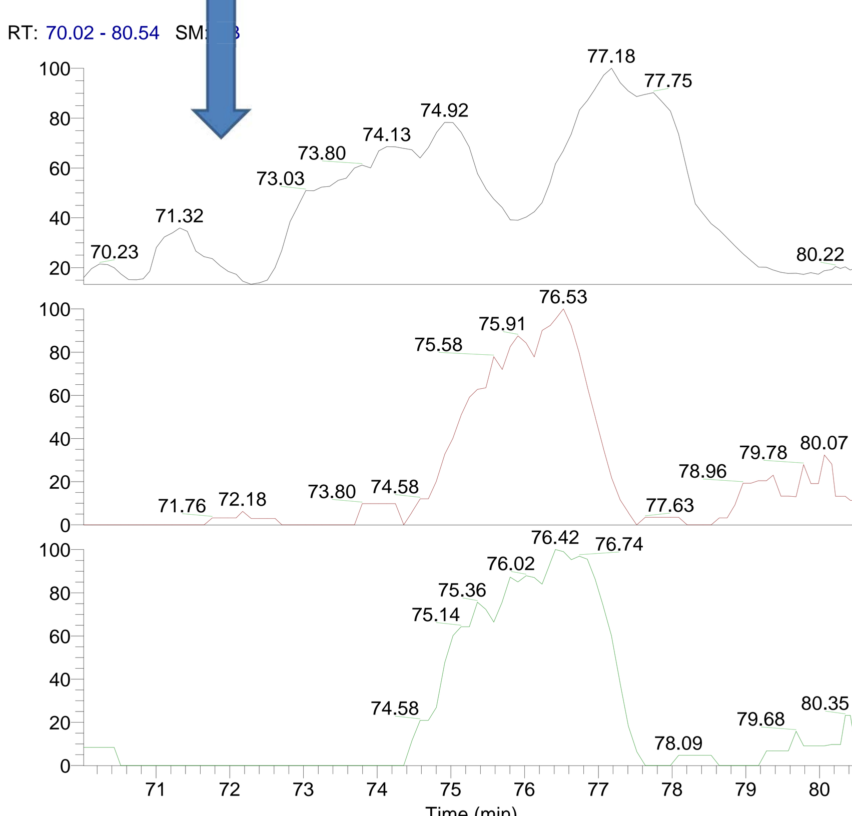


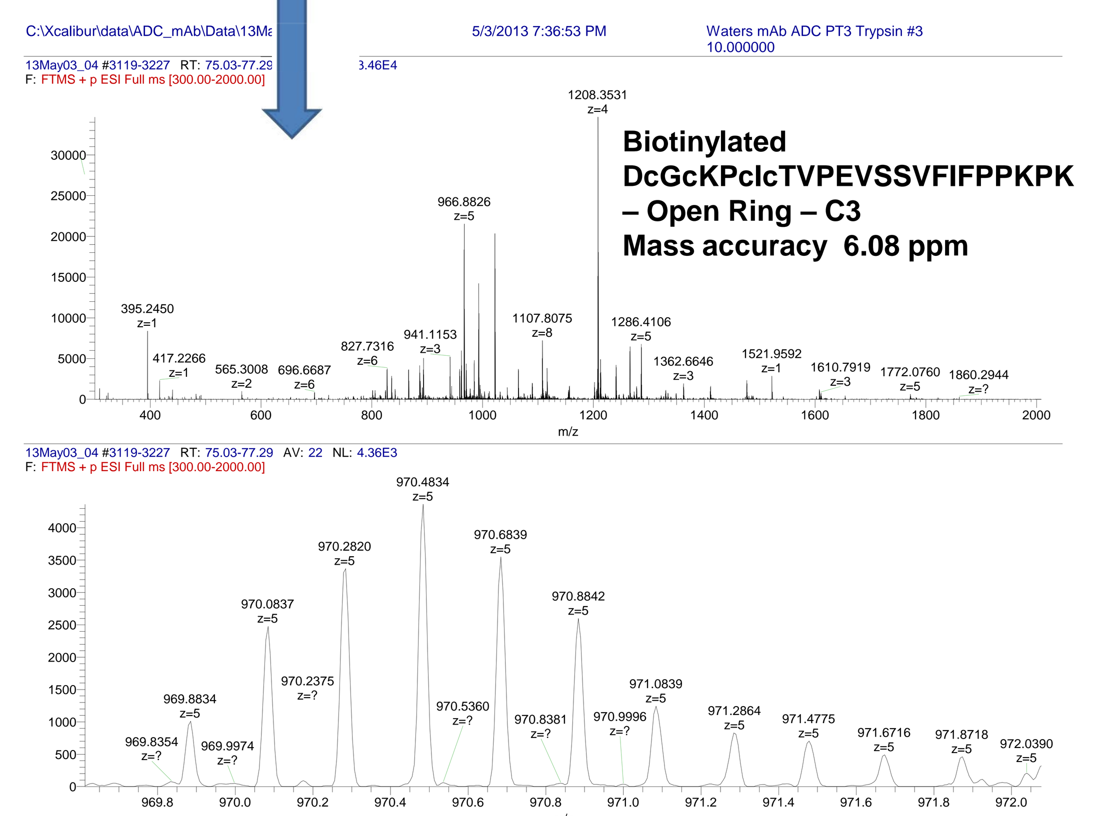


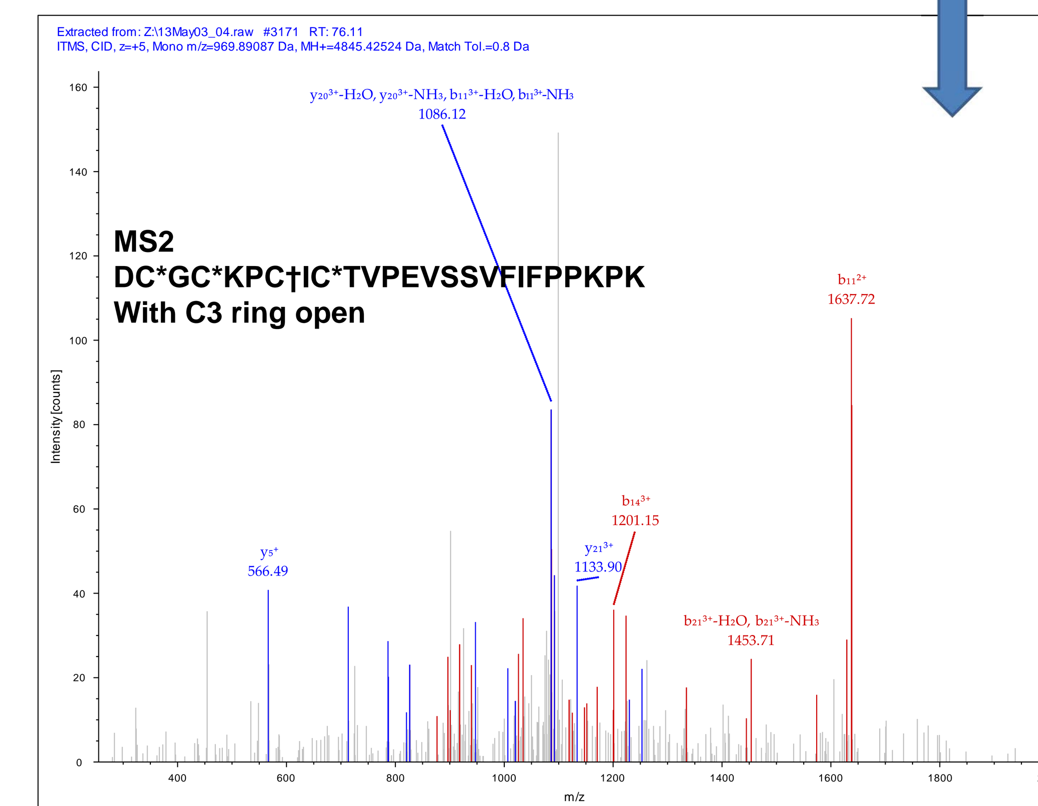




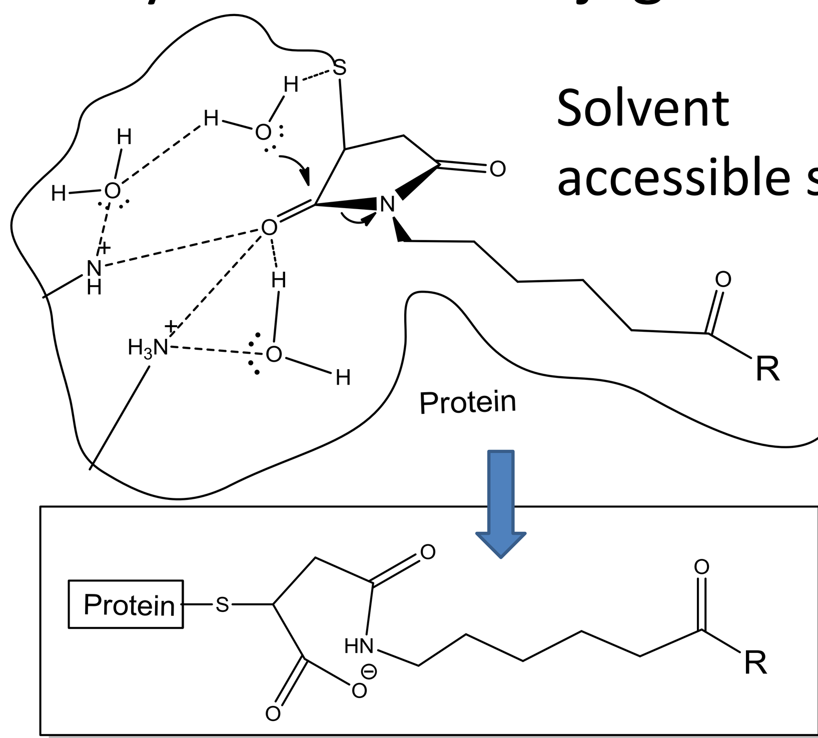








Stability Linked to Conjugation Site



Conclusions

Micro-environment of attachment site can cause chemical changes to linker. This was demonstrated with the multi-cysteine containing peptide where C3 attachment site was hydrolyzed and others were not. Sequence analysis showed neighboring Lysines and Arginines could participate in supporting solvent bound network.

Mass Spectrometry can detect these sites. Detection can be accomplished with High Resolution High Accuracy Mass spectrometers such as the LTQ-Orbitrap.

Open Maleimide Forms are less susceptible to Maleimide Exchange. Albumin and Glutathione can remove linkages by reverse Michael Reaction *in vitro* and *in vivo*.