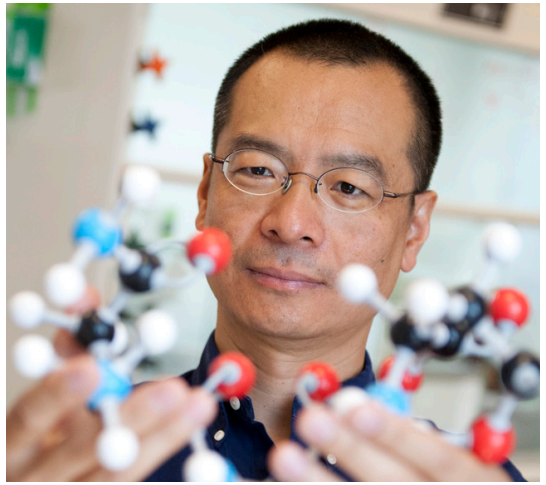
MAM: Multi-Artifacts Monitoring Operators, Processes and Modalities

Zhaohui Sunny Zhou

z.zhou@northeastern.edu

CASSS Mass Spec, 29 Sept 2022, Long Beach, CA



BARNETT INSTITUTE
of Chemical and Biological Analysis

Sunny Zhou is a co-founder, advisor and equity holder of NIRa Biosciences.

analytical chemistry

Article

pubs.acs.org/ac

Min Liu
Amgen

Da Ren

Protein Isoaspartate Methyltransferase-Mediated ^{18}O-Labeling of Isoaspartic Acid for Mass Spectrometry Analysis

Min Liu,[†,§,∥] Janet Cheetham,[†] Nina Cauchon,[†] Judy Ostovic,[†] Wenqin Ni,[§,∥] Da Ren,[*,‡] and Zhaohui Sunny Zhou[*,§,∥]

[†]Analytical Research and Development, Amgen, One Amgen Center Drive, Thousand Oaks, California 91320, United States

[‡]Process and Product Development, Amgen, One Amgen Center Drive, Thousand Oaks, California 91320, United States

[§]Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115, United States

[∥]Barnett Institute of Chemical and 02115, United States

analytical chemistry

Technical Note

pubs.acs.org/ac

Open Access on 08/19/2015

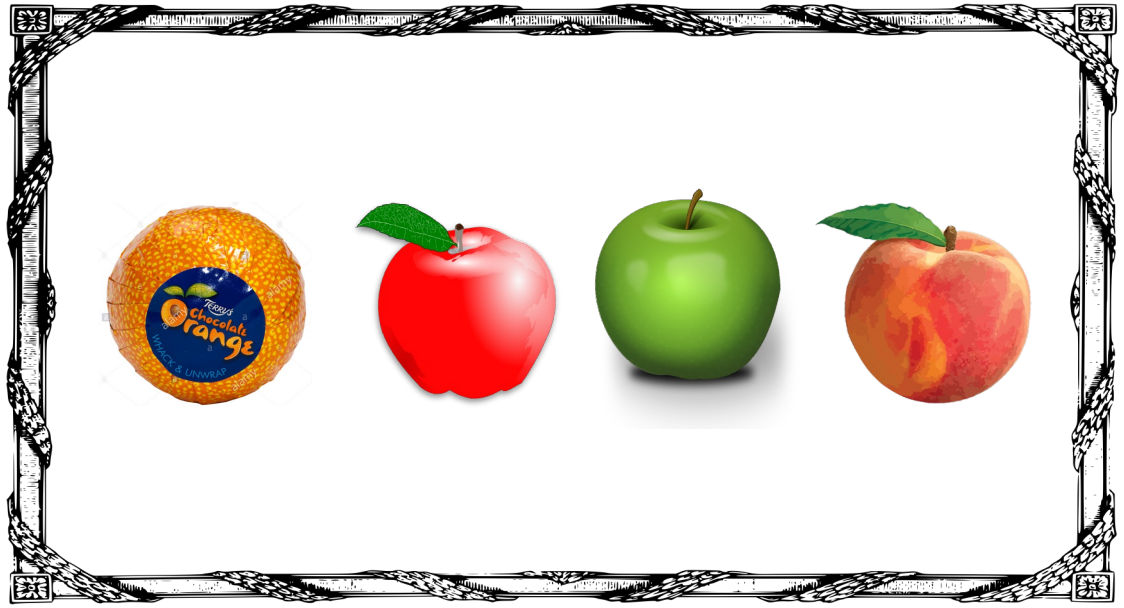
Chris Chumsae
AbbVie, BMS

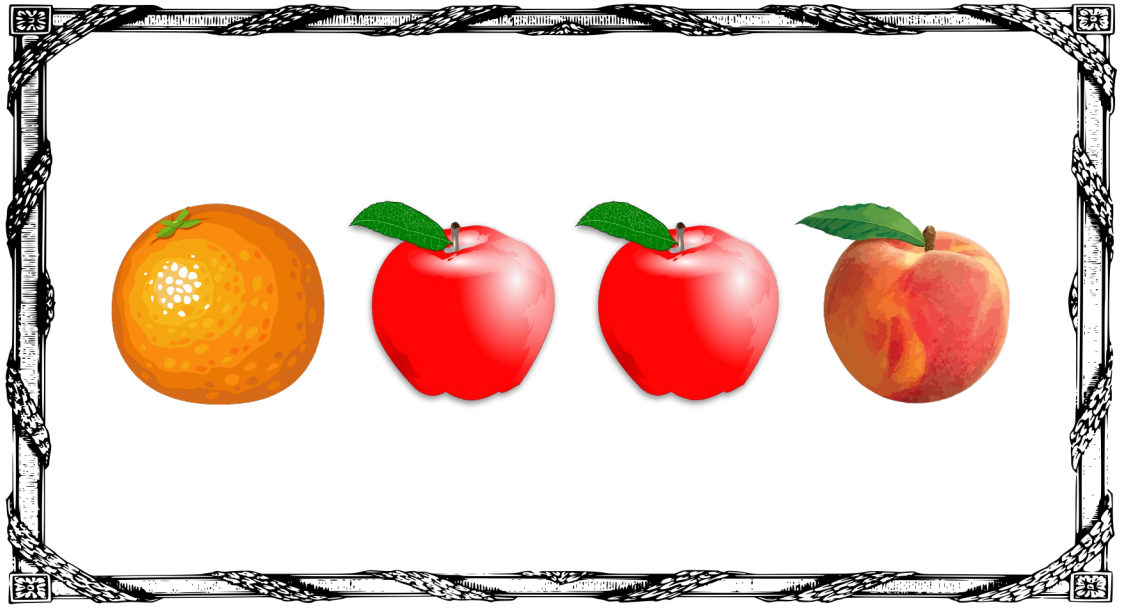
Discovery of a Chemical Modification by Citric Acid in a Recombinant Monoclonal Antibody

Chris Chumsae,[*,†,∥,⊥] Liqiang Lisa Zhou,[†] Yang Shen,[†] Jessica Wohlgemuth,[‡] Emma Fung,[§] Randall Burton,[†] Czeslaw Radziejewski,[*,†] and Zhaohui Sunny Zhou[*,∥]

[†]Protein Analytics, Process Sciences, AbbVie Bioresearch Center, Worcester, Massachusetts 01605, United States

artifact is error in the perception or representation of information





Sources of artifact

- Sample changes during analysis
- Detection
- Data analysis
- Perception

Types of artifact

- False positive
- False negative
- Known unknowns
- Unknown unknowns

Attribute Analytics Performance Metrics from the MAM Consortium Interlaboratory Study

Trina Mouchahoir,* John E. Schiel, Rich Rogers, Alan Heckert, Benjamin J. Place, Aaron Ammerman, Xiaoxiao Li, Tom Robinson, Brian Schmidt, Chris M. Chumsae, Xinbi Li, Anton V. Manuilov, Bo Yan, Gregory O. Staples, Da Ren, Alexander J. Veach, Dongdong Wang, Wael Yared, Zoran Sosic, Yan Wang, Li Zang, Anthony M. Leone, Peiran Liu, Richard Ludwig, Li Tao, Wei Wu, Ahmet Cansizoglu, Andrew Hanneman, Greg W. Adams, Irina Perdivara, Hunter Walker, Margo Wilson, Arnd Brandenburg, Nick DeGraan-Weber, Stefano Gotta, Joe Shambaugh, Melissa Alvarez, X. Christopher Yu, Li Cao, Chun Shao, Andrew Mahan, Hirsh Nanda, Kristen Nields, Nancy Nightlinger, Ben Niu, Jihong Wang, Wei Xu, Gabriella Leo, Nunzio Sepe, Yan-Hui Liu, Bhumit A. Patel, Douglas Richardson, Yi Wang, Daniela Tizabi, Oleg V. Borisov, Yali Lu, Ernest L. Maynard, Albrecht Gruhler, Kim F. Haselmann, Thomas N. Krogh, Carsten P. Sönksen, Simon Letarte, Sean Shen, Kristin Boggio, Keith Johnson, Wenqin Ni, Himakshi Patel, David Ripley, Jason C. Rouse, Ying Zhang, Carly Daniels, Andrew Dawdy, Olga Friese, Thomas W. Powers, Justin B. Sperry, Josh Woods, Eric Carlson, K. Ilker Sen, St John Skilton, Michelle Busch, Anders Lund, Martha Stapels, Xu Guo, Sibylle Heidelberger, Harini Kaluarachchi, Sean McCarthy, John Kim, Jing Zhen, Ying Zhou, Sarah Rogstad, Xiaoshi Wang, Jing Fang, Weibin Chen, Ying Qing Yu, John G. Hoogerheide, Rebecca Scott, and Hua Yuan

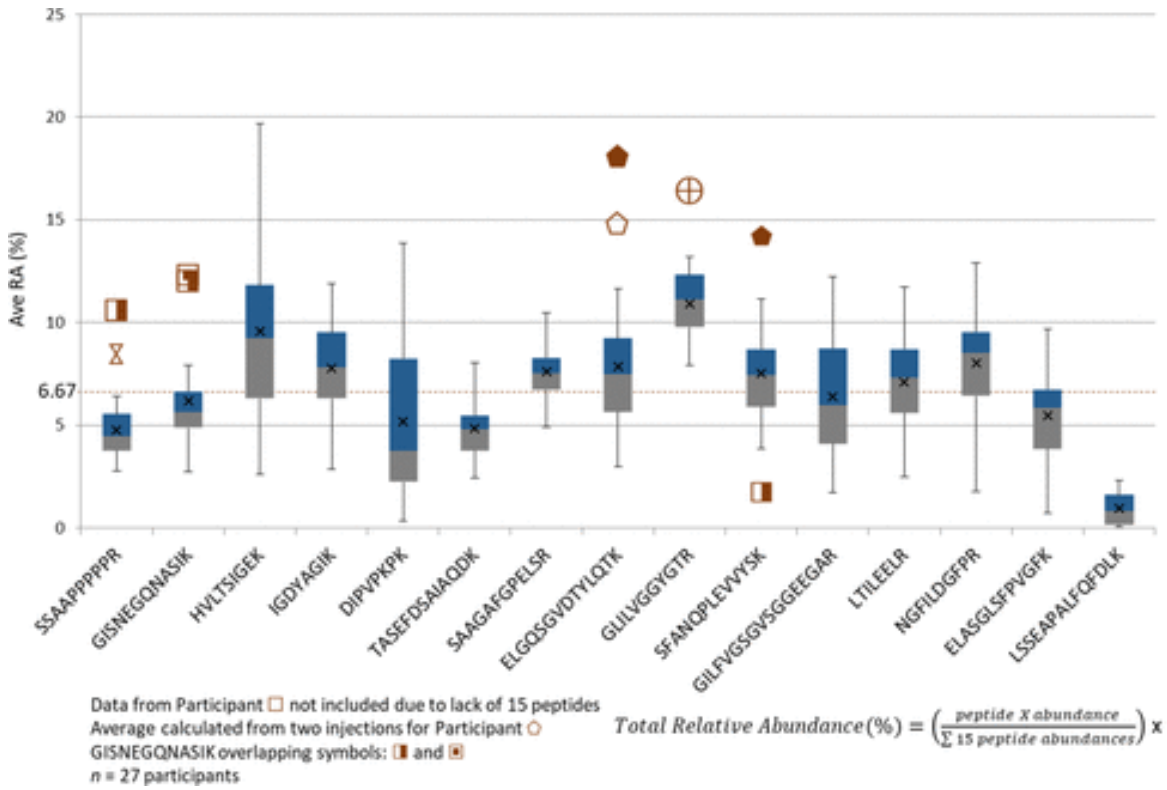
Cite This: *J. Am. Soc. Mass Spectrom.* 2022, 33, 1659–1677

Attribute Analytics Performance Metrics from the MAM Consortium Interlaboratory Study

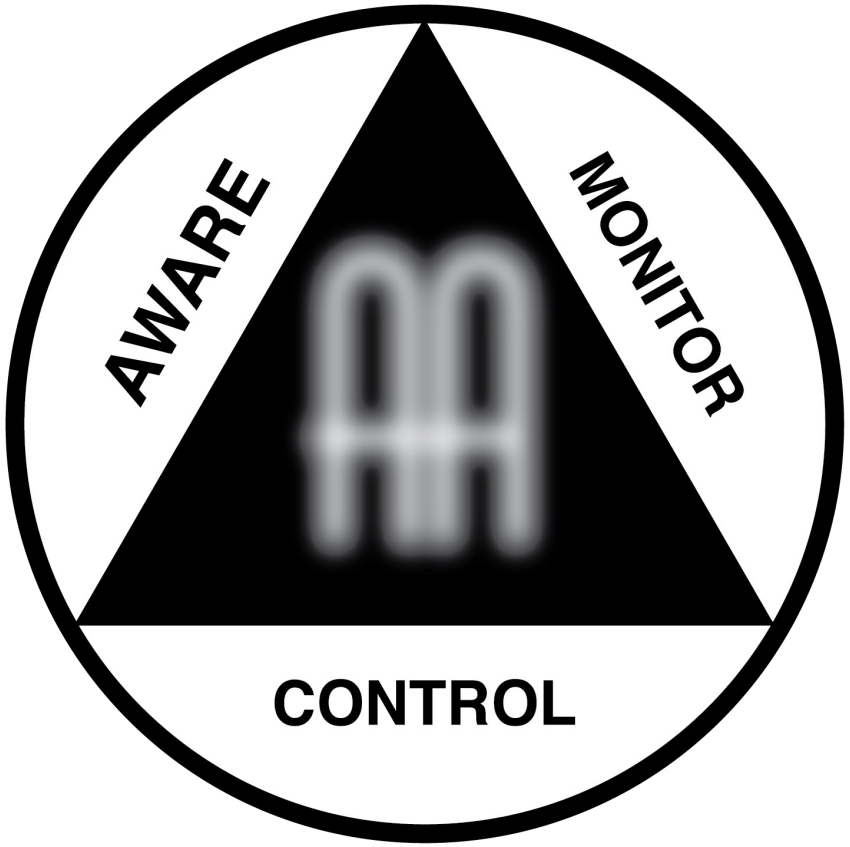
quantitative vs qualitative (detected vs not detected)

The multi-attribute method (MAM) was conceived as a single assay to potentially replace multiple single-attribute assays that have long been used in process development and quality control (QC) for protein therapeutics. MAM is rooted in traditional peptide mapping methods; it leverages mass spectrometry (MS) detection for confident identification and quantitation of many types of protein attributes that may be targeted for monitoring.

While MAM has been widely explored across the industry, it has yet to gain a strong foothold within QC laboratories as a replacement method for established orthogonal platforms. Members of the MAM consortium recently undertook an interlaboratory study to evaluate the industry-wide status of MAM. Here we present the results of this study as they pertain to the targeted attribute analytics component of MAM, including investigation into the sources of variability between laboratories and comparison of MAM data to orthogonal methods. These results are made available with an eye toward aiding the community in further optimizing the method to enable its more frequent use in the QC environment.



Artifact Anonymous (AA)



Communication:

reviews, white pages and guidelines

Collaboration:

academia

industry: biopharma, vendors

regulatory agencies: FDA, NIST

Control:

standardization and optimization

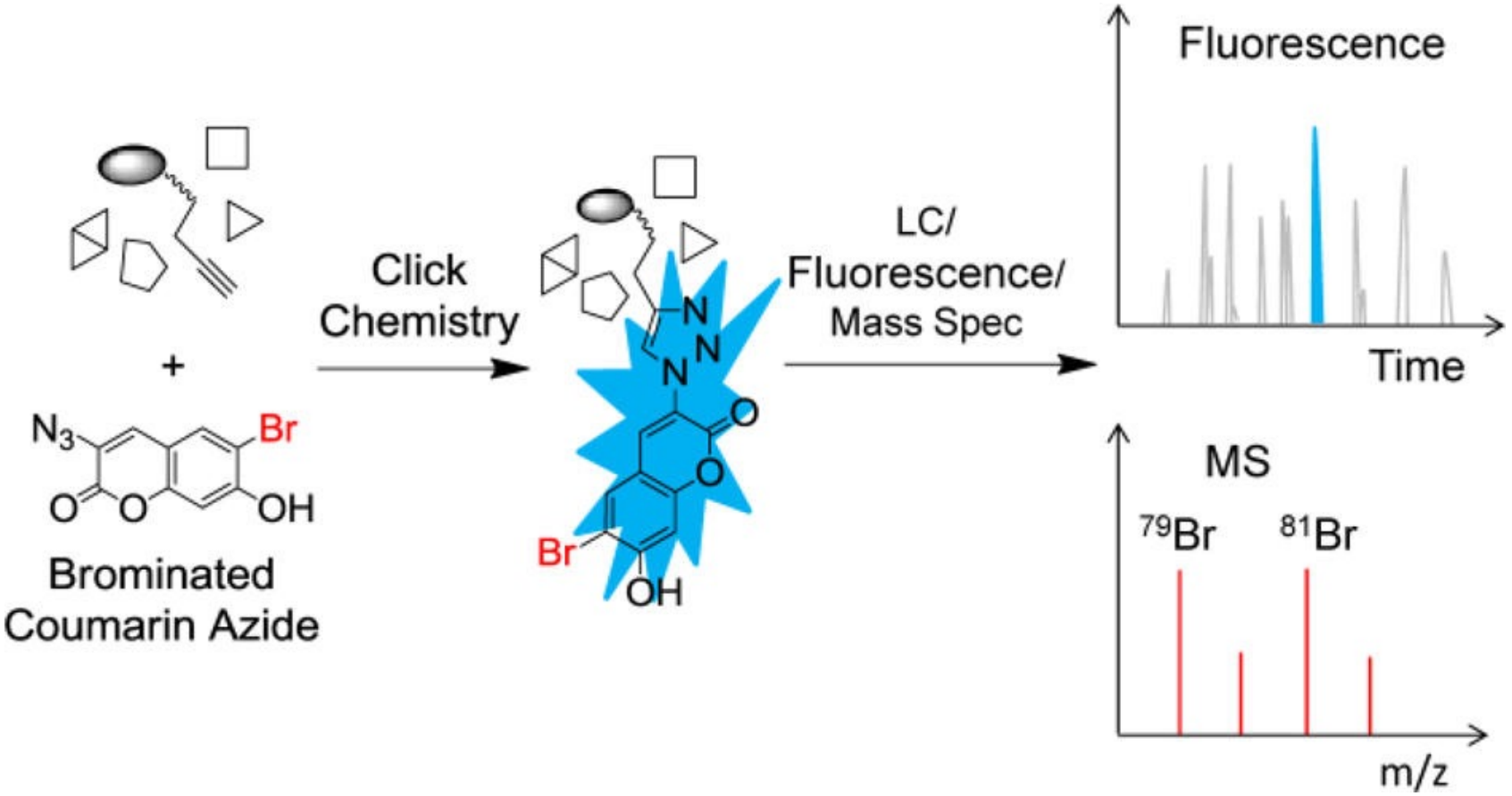
You are invited to participate and contribute, please contact Sunny Zhou at z.zhou@northeastern.edu. Thank you!

mass, structures, properties-reactivities

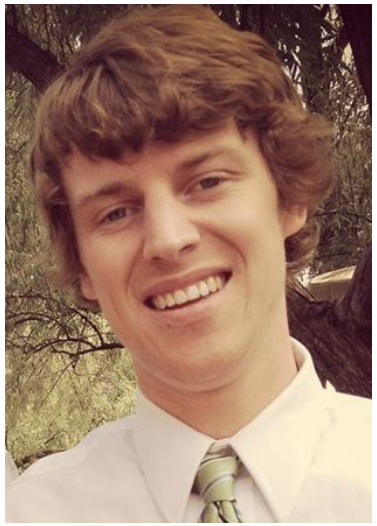
- Mass spectrometry
  - ionization
  - identification: isotopic labeling
- New labeling tool: glutamine (Gln) via transglutaminase (TGase)
- Knowledge: elucidation of structures: new, unknown species (PTM's)
  - reactive species: metabolites, methylglyoxal (MGO)
  - crosslinking
- Sample preparation:
  - deamidation, isoaspartic acid (isoAsp), succinimide
  - meta-stable species: chemical trapping, native mass spec
- New modalities and new opportunities: higher-order structure (HOS)
  - virus-like particles (VLPs): adeno-associated virus (AAV)

Bioconjugate Chemistry

Detection of Alkynes via Click Chemistry with a Brominated Coumarin Azide by Simultaneous Fluorescence and Isotopic Signatures in Mass Spectrometry



Chris Chumsae
AbbVie, BMS



Kevin Moulton
Northeastern
Vertex

Yang L, Chumsae C, Kaplan JB, Moulton KR, Wang D, Lee DH, Zhou ZS. Bioconjug Chem. 2017, 28, 2302. DOI: 10.1021/acs.bioconjchem.7b00354

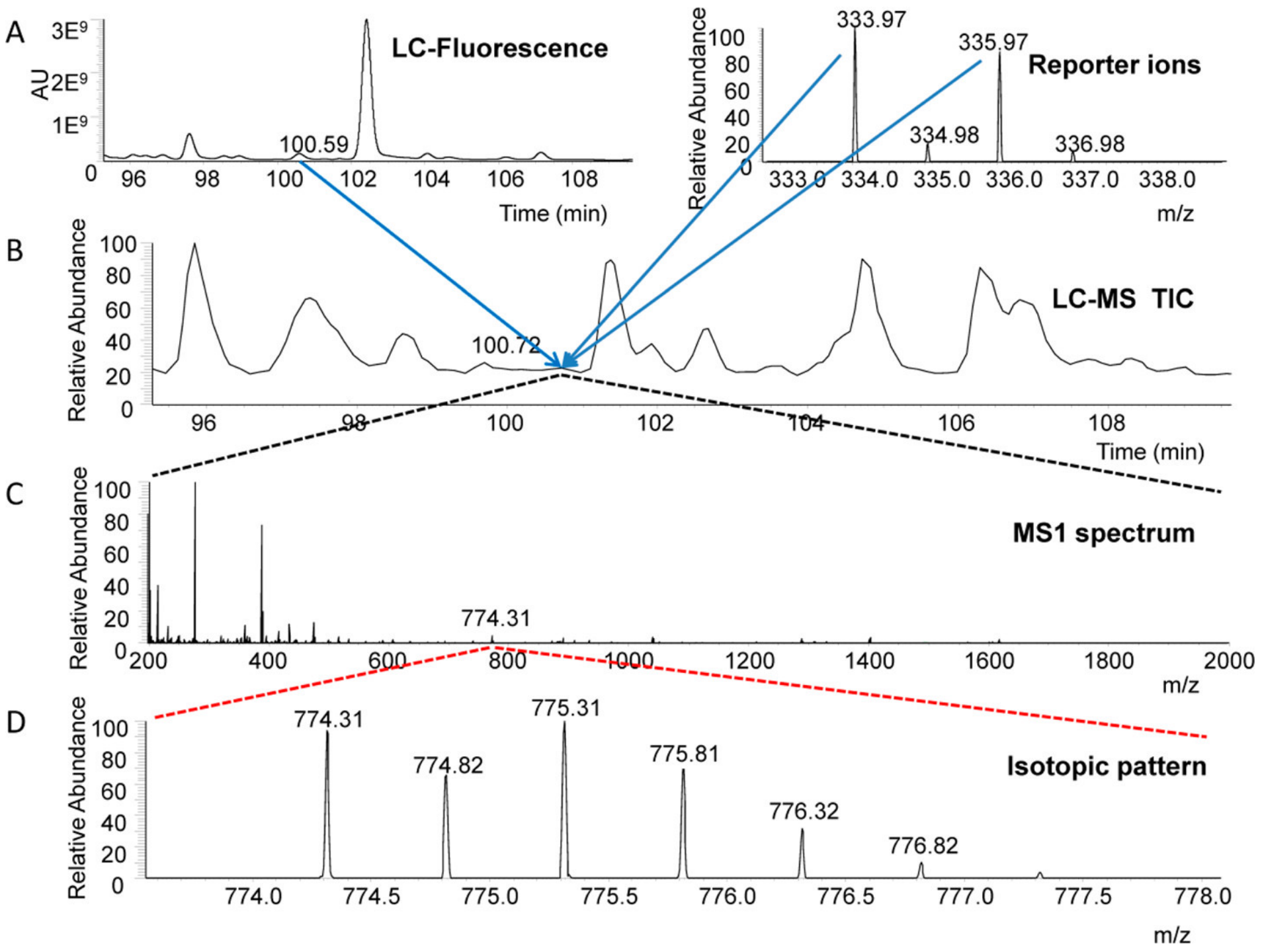


Figure 4. LC–fluorescence–MS analysis of tryptic peptides of a mAb triazole product.

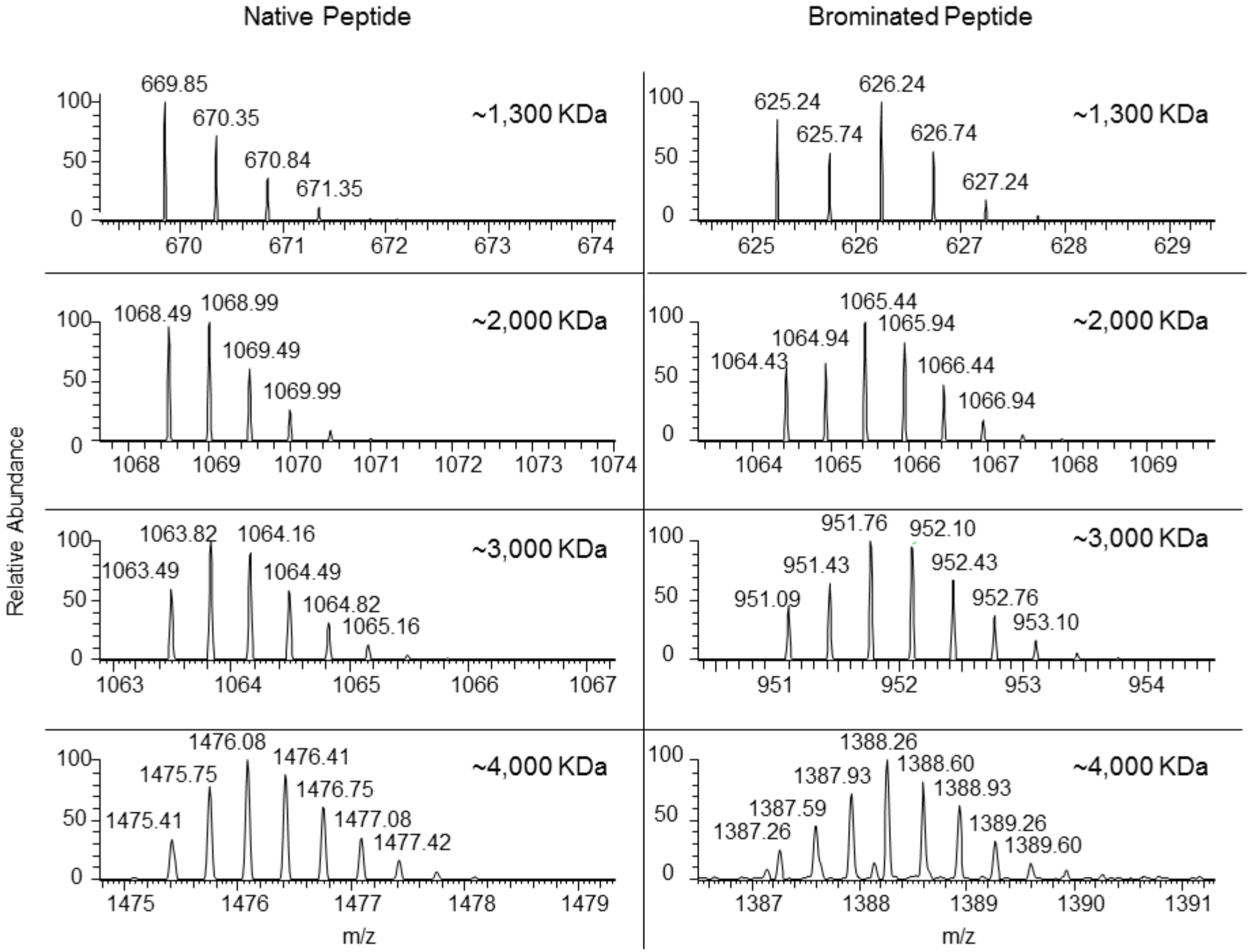
(A) Fluorescence chromatogram and reporter ion pair leading to tagged peptides in MS;

(B) MS total ion current containing numerous peaks;

(C) MS1 spectra at one scan containing multiple *m/z*;

(D) unique isotopic pattern identifying the tagged peptide.

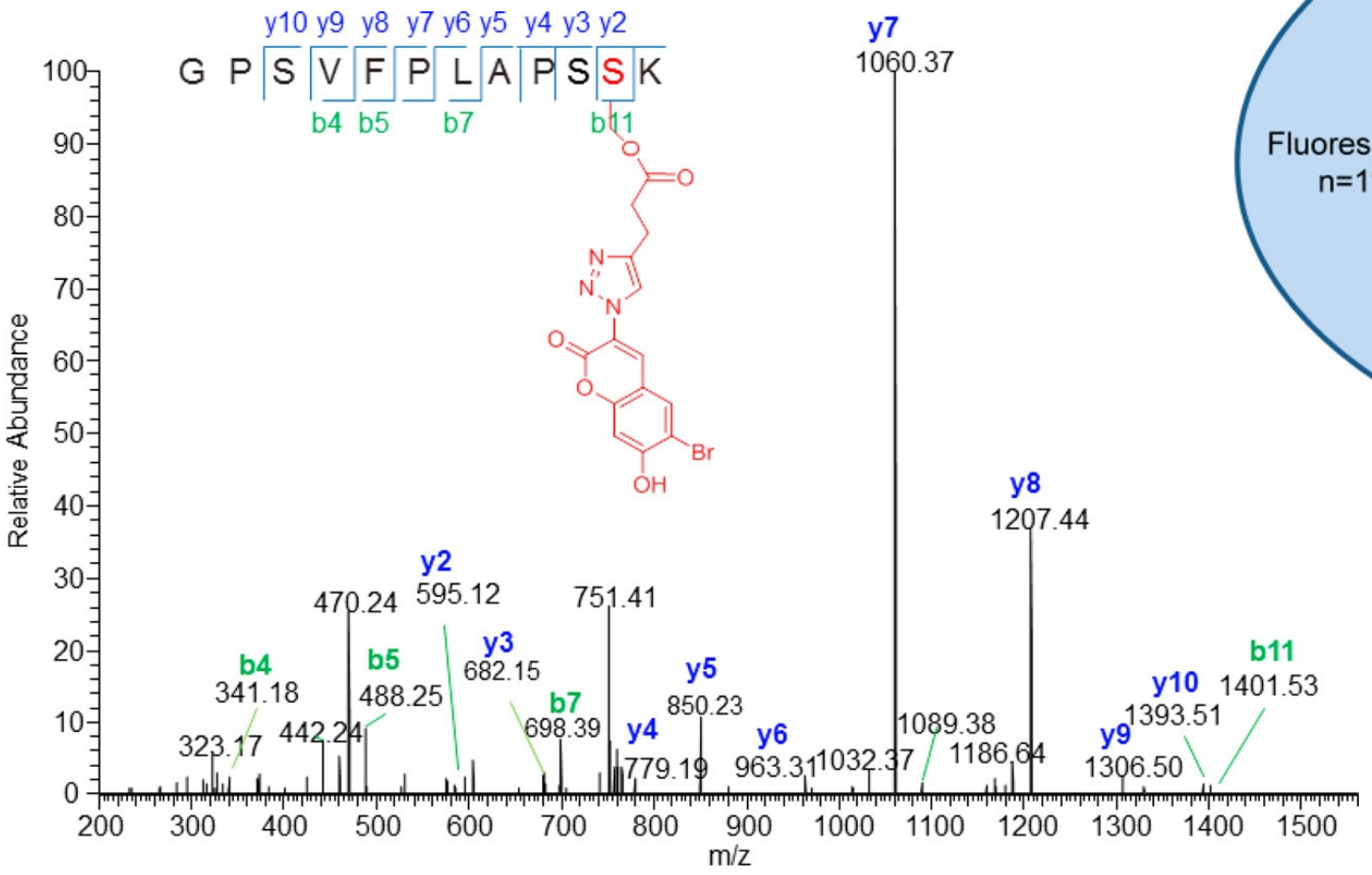
Bromine: Unique Isotope Patterns

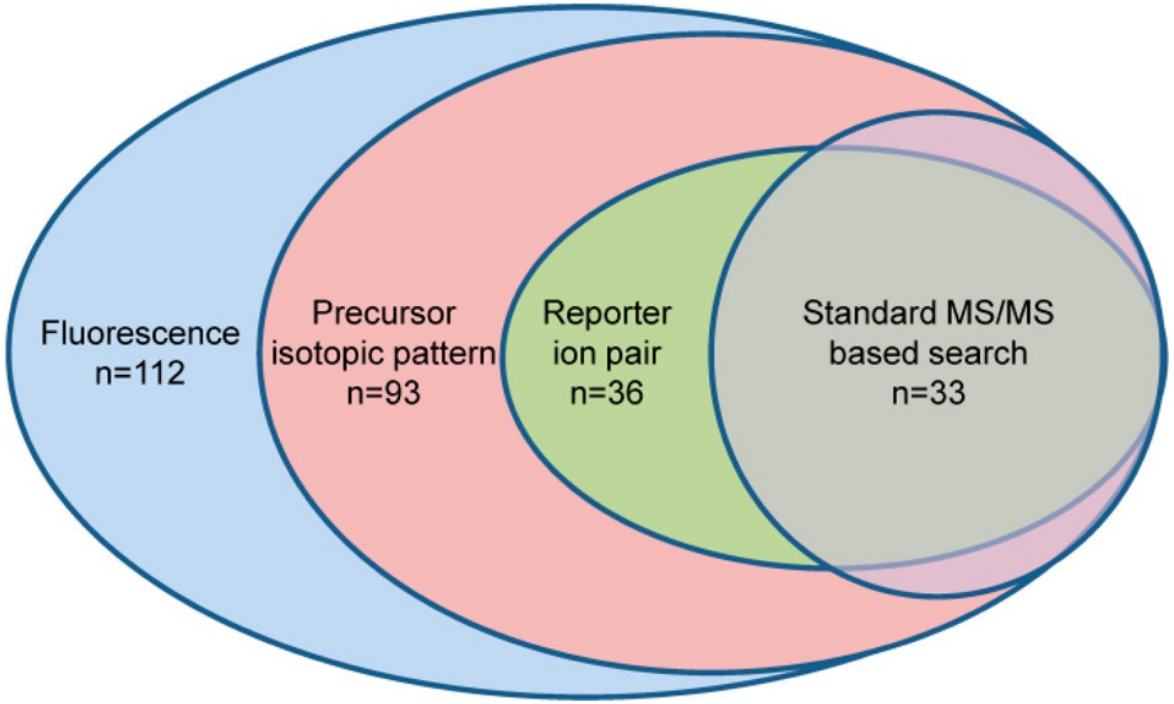


DongDong Wang
AbbVie
Takeda

Systematically Evaluate Various Workflows

Limitations





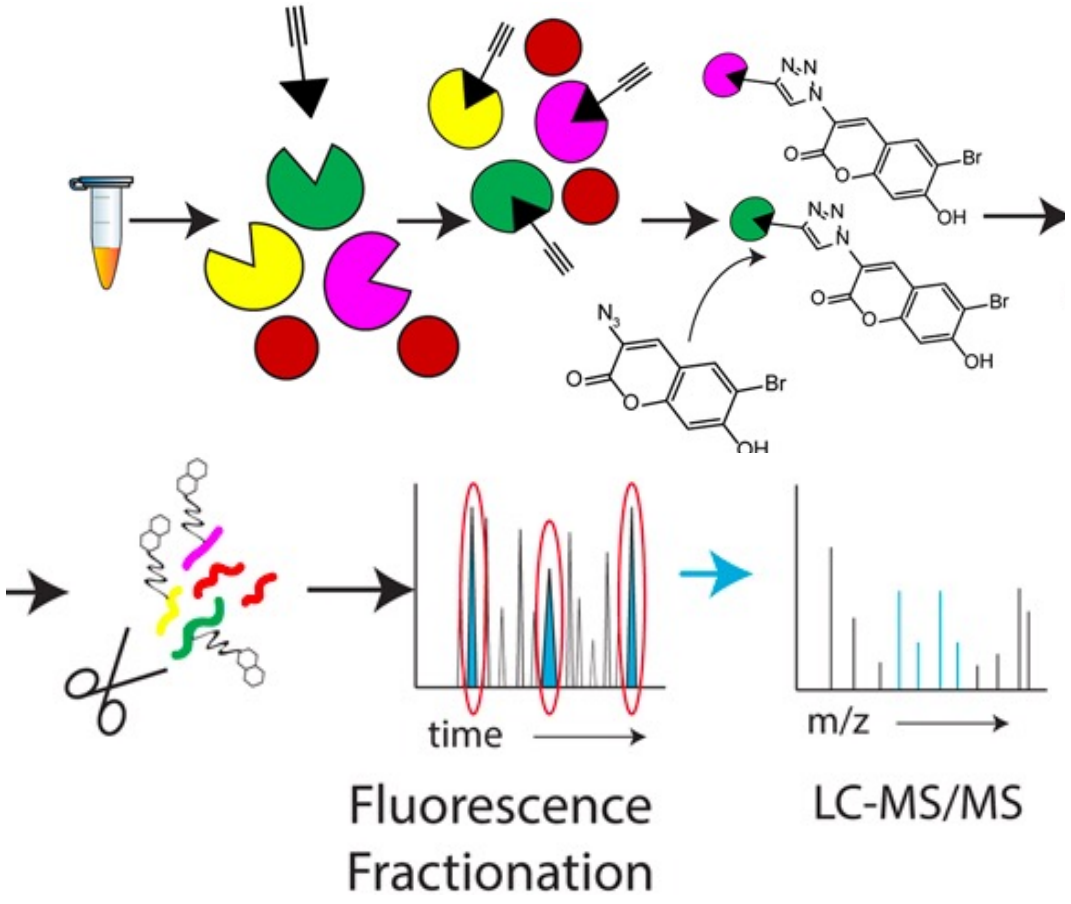
David Lee

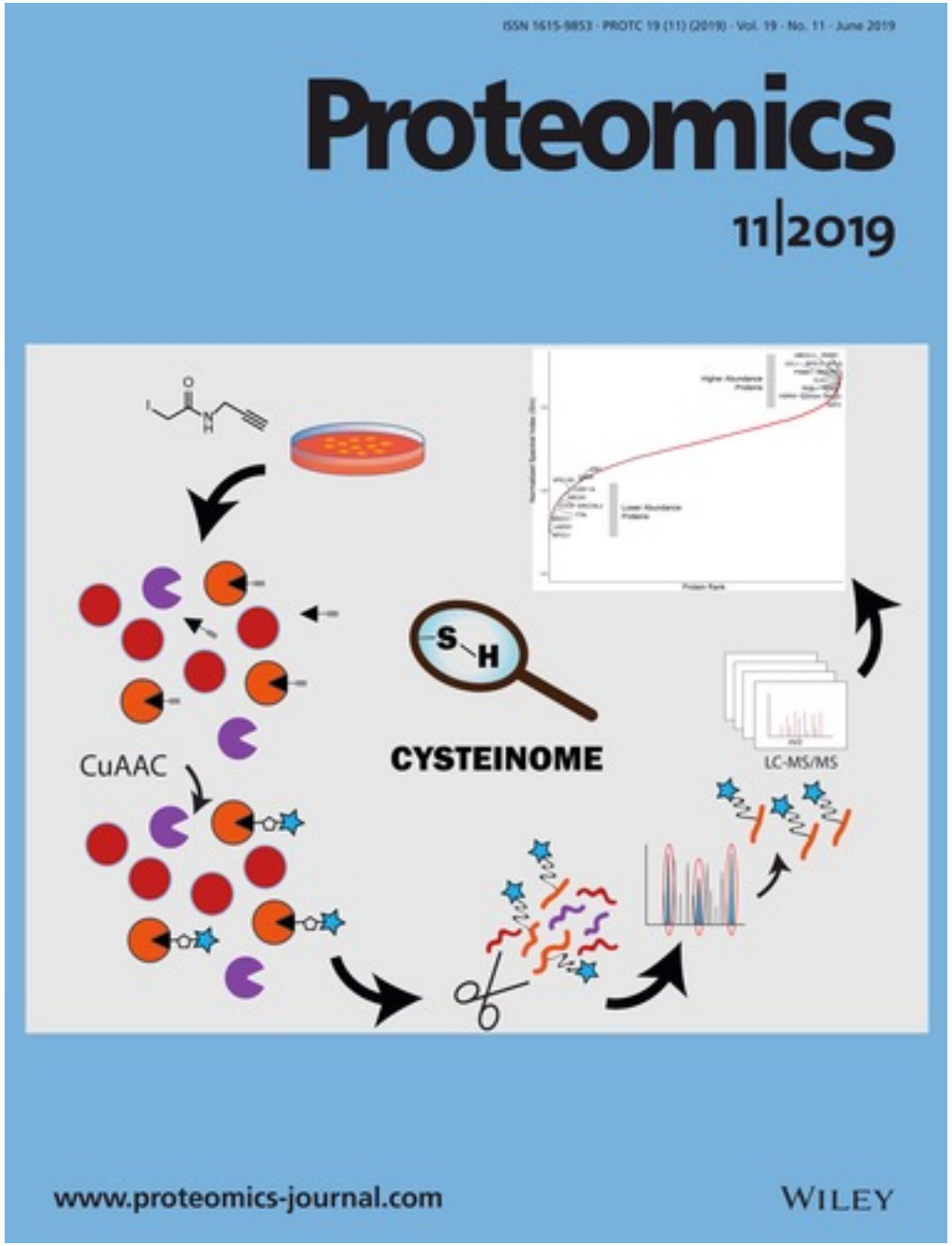
Lihua Yang

AbbVie

Yang L, Chumsae C, Kaplan JB, Moulton KR, Wang D, Lee DH, Zhou ZS. Bioconjug Chem. 2017, 28, 2302. DOI: 10.1021/acs.bioconjchem.7b00354

A Dual-Purpose Bromocoumarin Tag Enables Deep Profiling of the Cellular Cysteinome (from AbbVie)



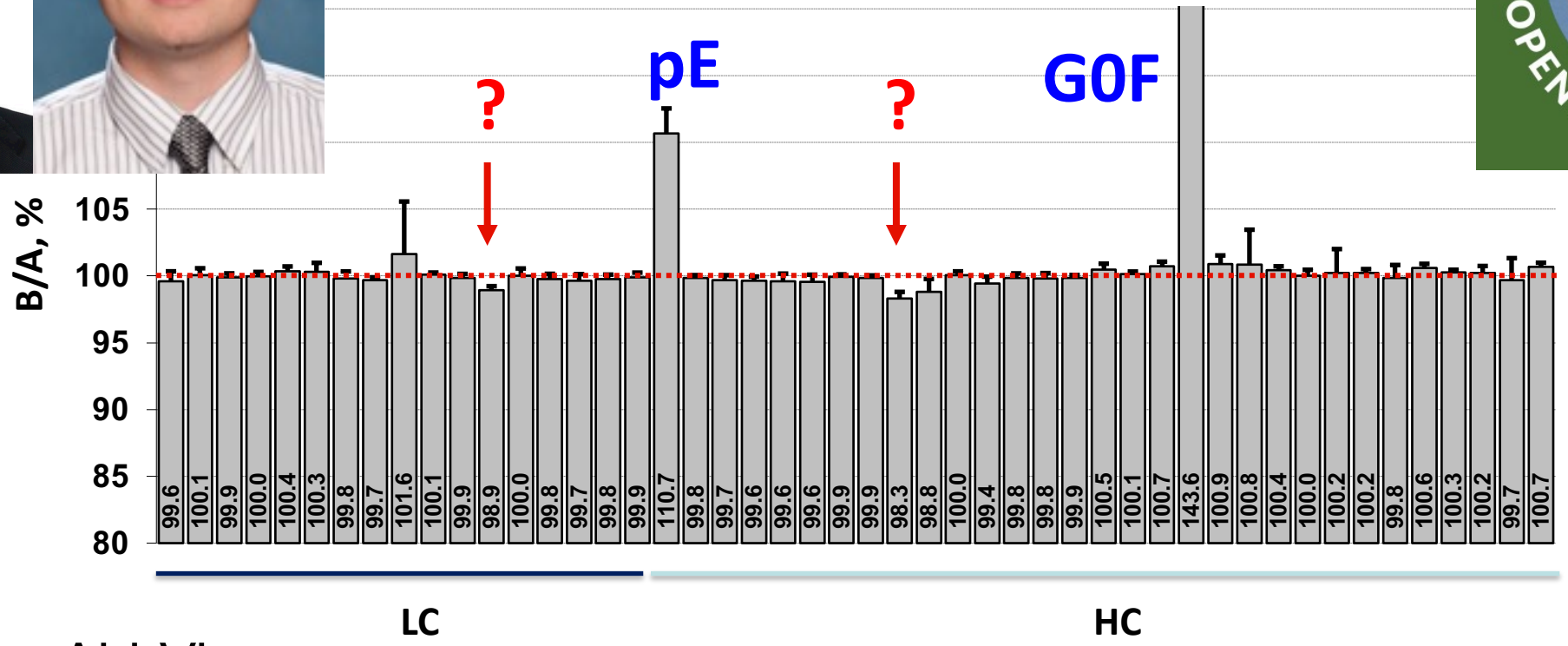


Rabalski AJ, Williams JD, McClure RA, Vasudevan A, Baranczak A. Proteomics. 2019, 19. e1800433. doi: 10.1002/pmic.201800433.

Identify Sequence of Unknown Peptide:

stable isotope-tagged reference standard (SITRS): fermentation

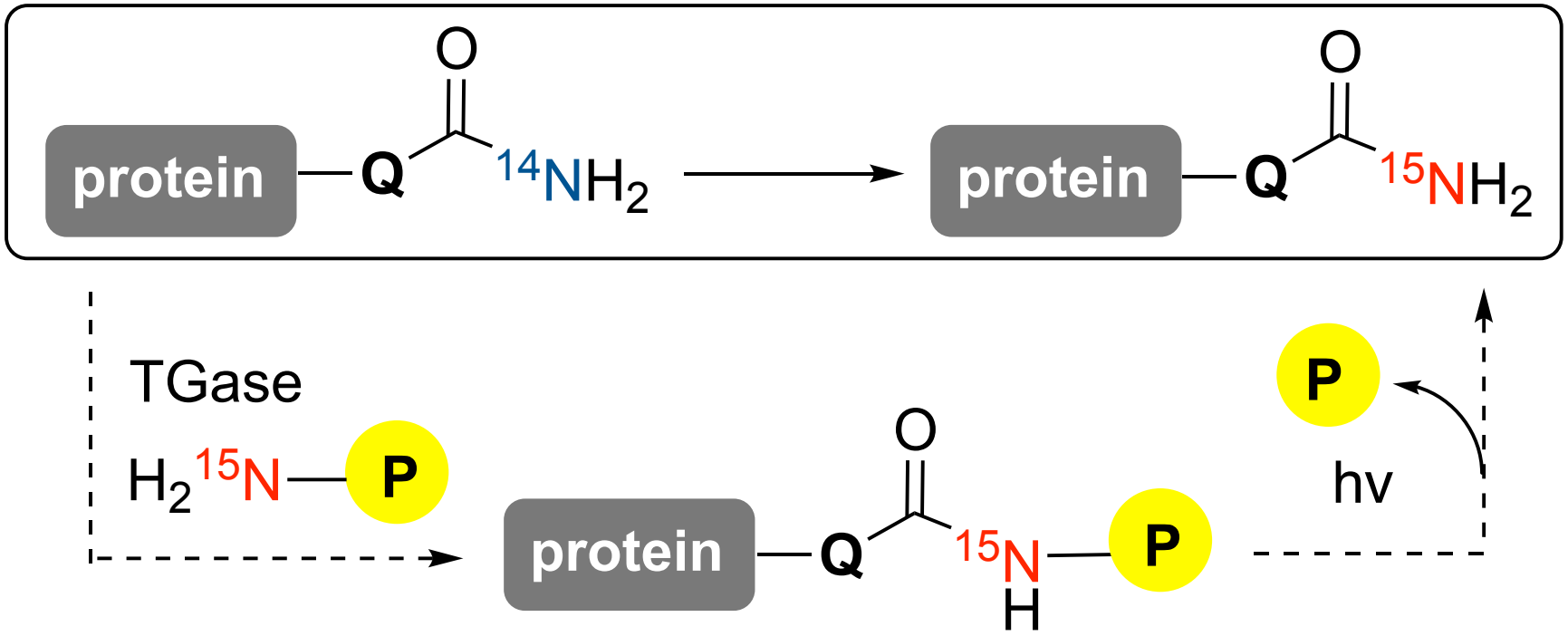
putative "new" related peptides



AbbVie

Anton Manuilov, Czes Radziejewski, David Lee; mAbs, 2011, 3, 387

Install 15N to Glutamine in Proteins/Peptides: Isotope Tracer

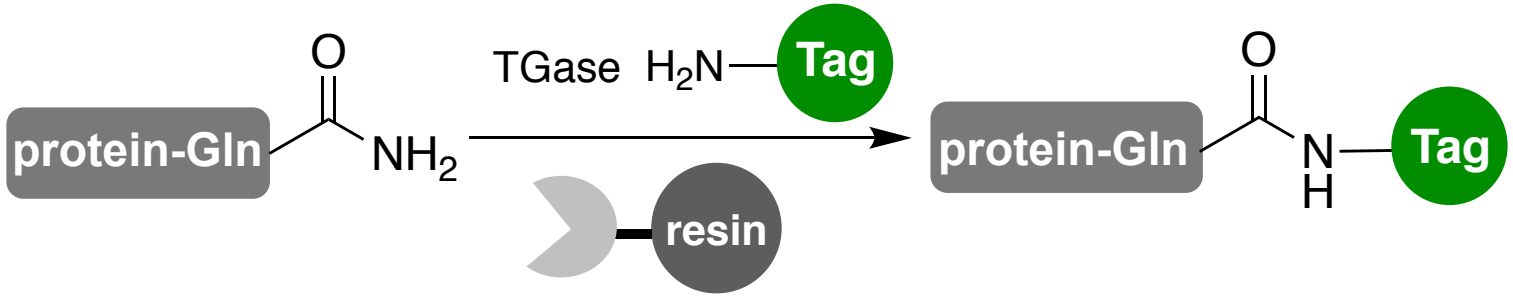


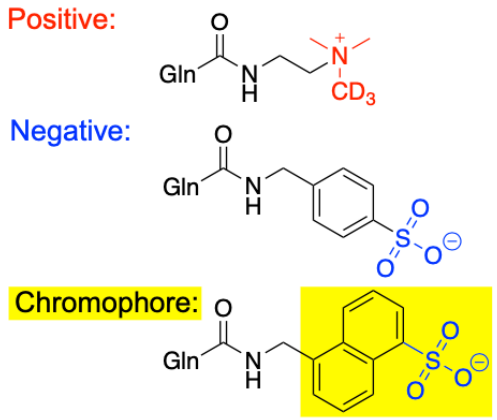
Molly Blevins
Northeastern
Texas-Austin
Genentech

- Post production
- Native and intact proteins
- Mixture of proteins: host cell proteins
- Site-specific: specificity can be tuned
- Peptides as well

Please talk to us: applications and collaborations.

Site-specific incorporation of tags to enhance analysis of peptides and proteins





Tags: positive, negative, UV chromophore

Mass spectrometry*:* enhanced ionization efficiency (improve sequence coverage), altered charge states, alternative fragmentation pathways, quantification

Common methods are chemical and target nucleophilic residues

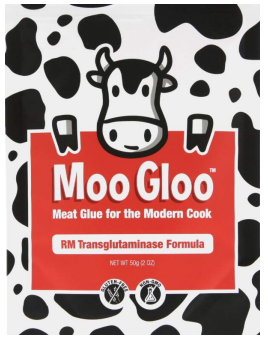
- Lysine: variable charge, frequency 6%
- Cysteine: frequency 2%

Our approach target neutral amide on glutamine (4%)

- Multiple isoform of TGase: broad and narrow
- Selectivity: conformational and site
- Tunable: both positive and negative tags can be incorporated

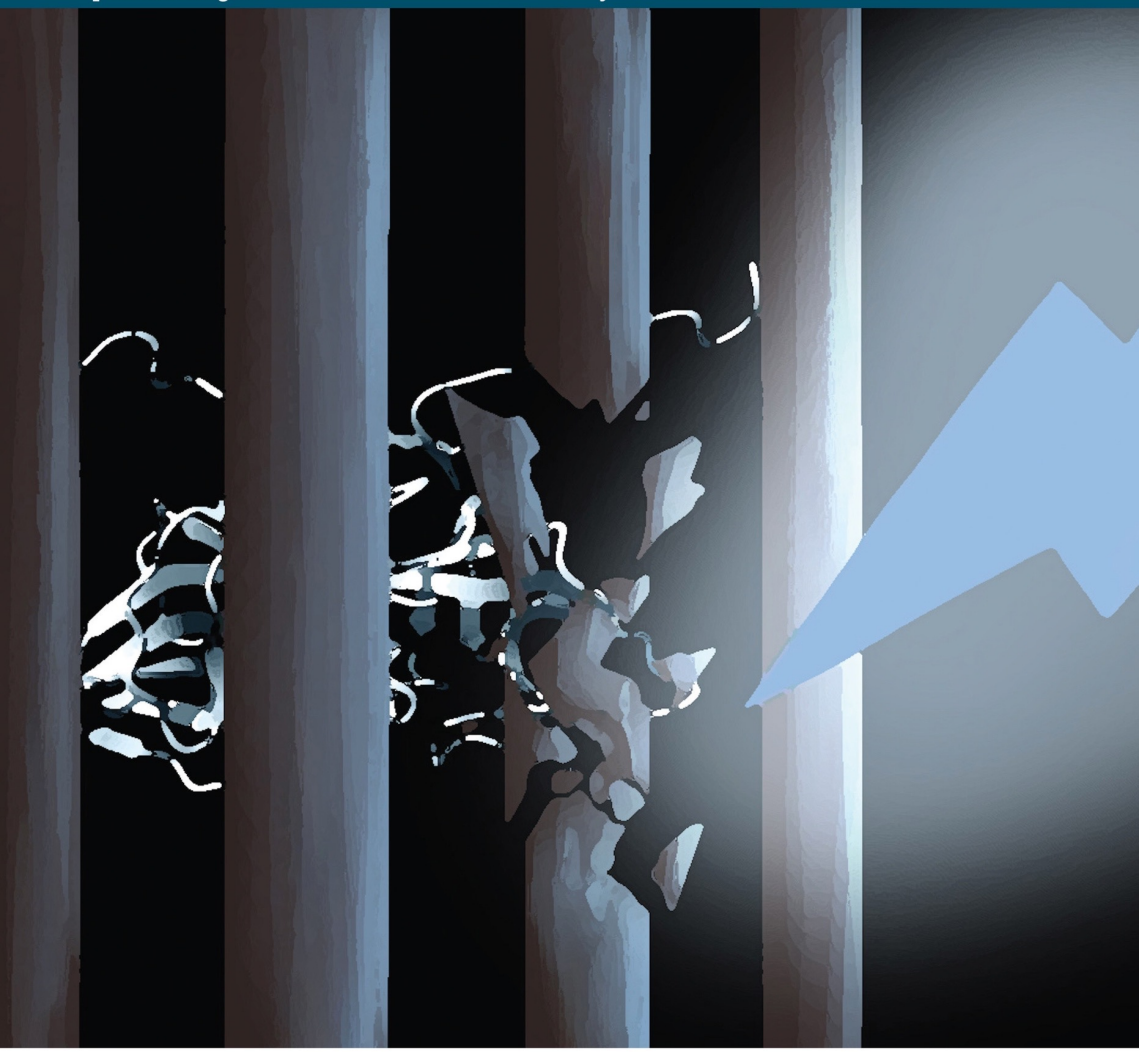
Please talk to us: applications and collaborations.

meat glue
Amazon



Bioconjugate Chemistry

pubs.acs.org/bc June 2019 Volume 30, Number 6

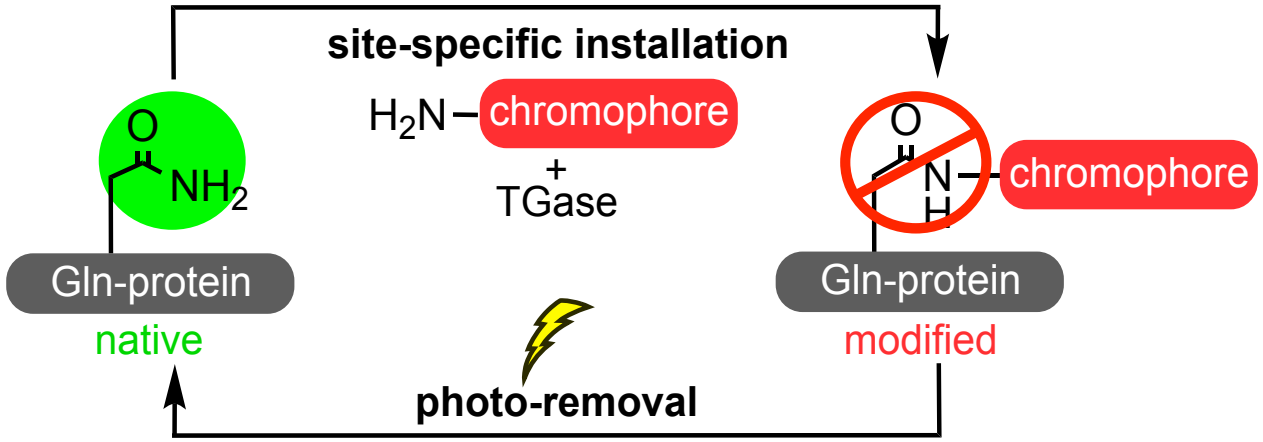


ACS Publications
Most Trusted. Most Cited. Most Read.

www.acs.org

Site-Specific Reversible Protein and Peptide Modification

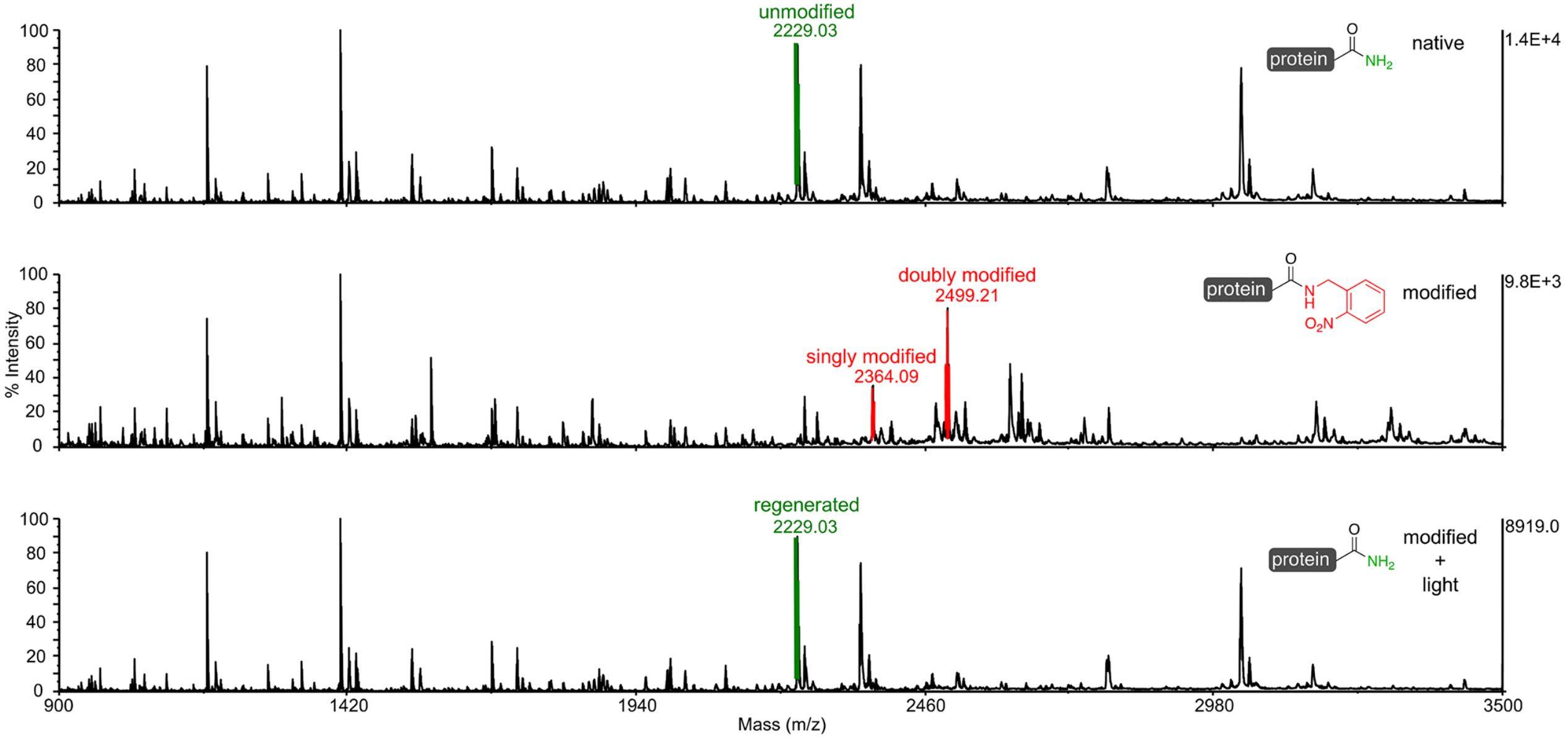
Transglutaminase-Catalyzed Glutamine Conjugation and Bioorthogonal Light-Mediated Removal



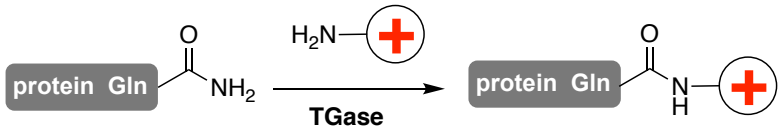
Kevin Moulton, Amissi Sadiki, Bilyana Koleva, Lincoln Ombelets, Tina Tran, Shanshan Liu, Bryan Wang, Hongyan Chen, Emily Micheloni, Penny Beuning, George O'Doherty and Zhaohui Sunny Zhou.

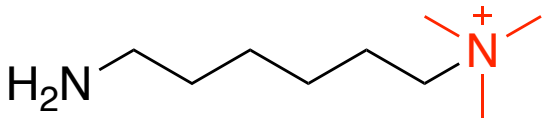
Bioconjugate Chemistry, **2019**, 1617, DOI: 10.1021/acs.bioconjchem.9b00145.

Photo-Caging: Reversible and No Damage to the Protein

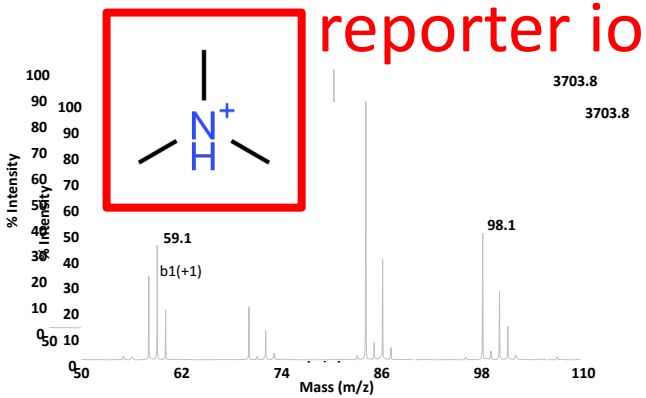


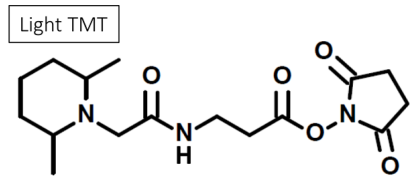
MALDI mass spectra of tryptic peptides of native UmuD (top), nitrobenzylamine-modified UmuD by TGase (middle), and modified UmuD after photolysis (bottom).

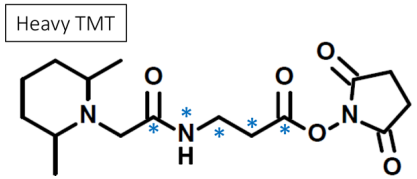




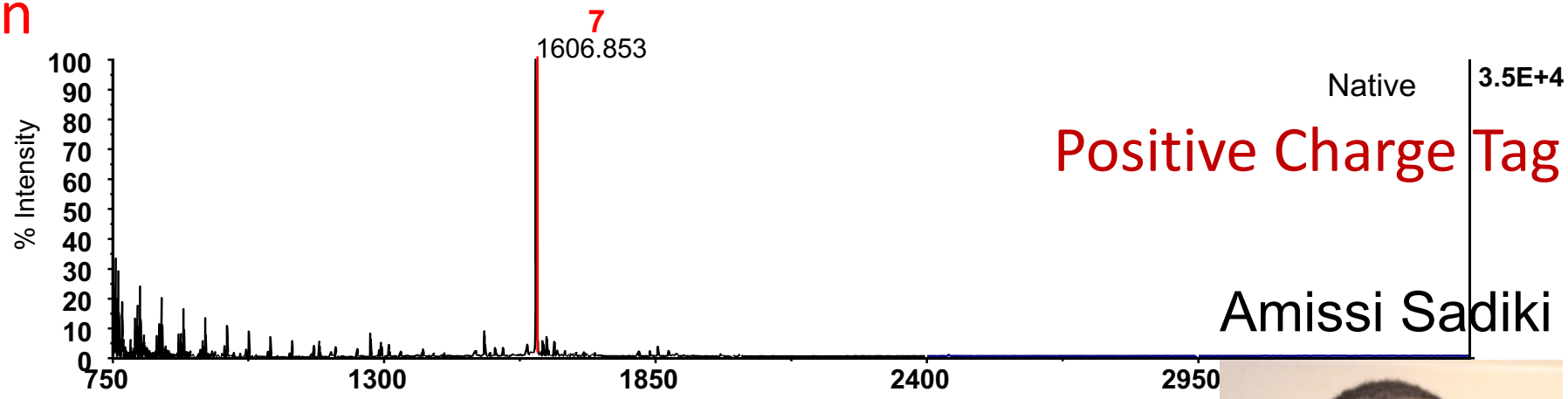
Sequence	Modified	Theoretical pI	Theoretical (m/z)
1. (-)GLSDGEWQ*QVLNVWGK(V)	Yes	4.4	1957.05
2. (-)GLSDGEWQ*Q*VLNVWGK(V)	Yes	4.4	2098.18
3. (-)GLSDGEWQQ*VL(N)	Yes	3.7	1372.76
4. (-)GLSDGEWQQ*VLNVWG(K)	Yes	3.7	1828.97
5. (-)GLSDGEWQ**Q*VLNVWGK(V)	Yes	4.4	2039.11
6. (S)DGEWQQ*VLNVWGK(V)	Yes	4.4	1699.92
7. (K)VEADIAGHGQEVLIR(L)	No	4.7	1606.86
8. (K)GHHEAELKPLAQ*SHATK(H)	Yes	7.0	1995.12
9. (K)HPGDFGADAQ*GAMTK(A)	Yes	5.2	1643.82
10. (K)ELGFQ*G(-)	Yes	4.0	791.465
11. (K)YKELGFQ*G(-)	Yes	6.0	1082.63





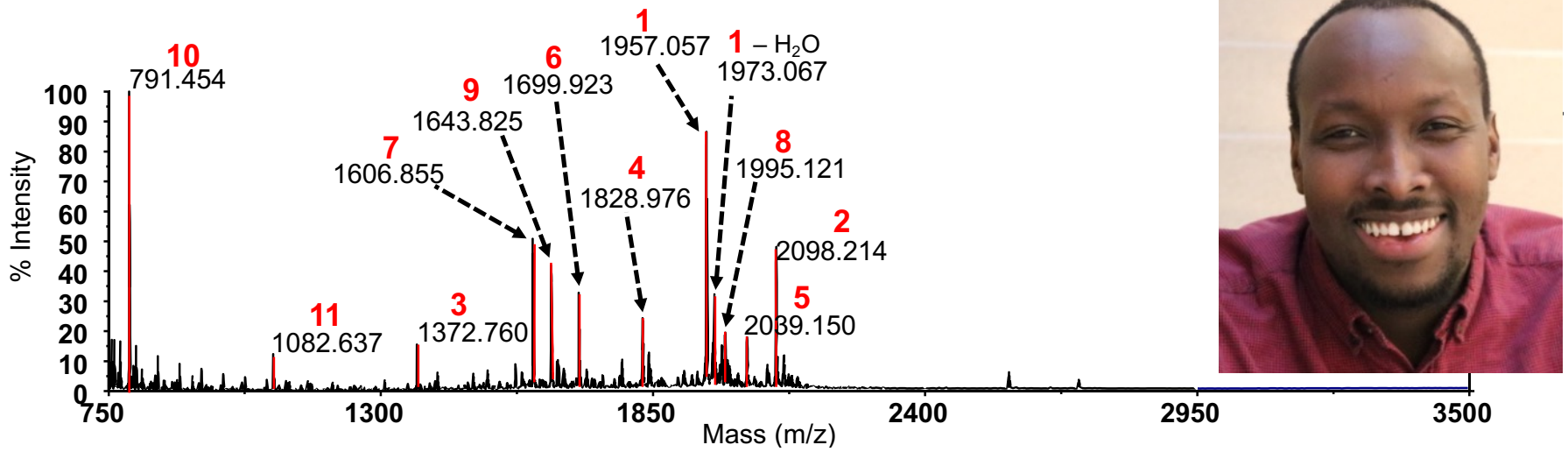


tandem mass tag (TMT)
iTRAQ

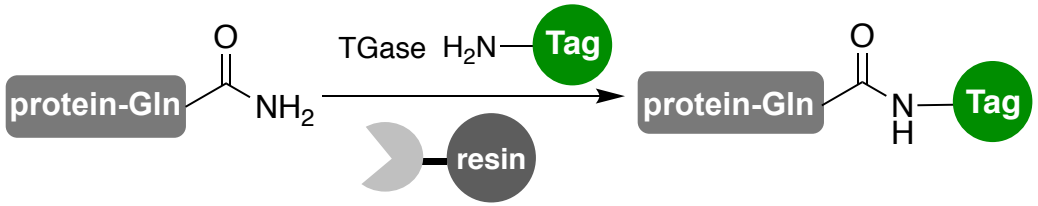


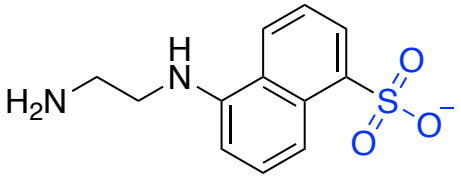
Positive Charge Tag

Amissi Sadiki

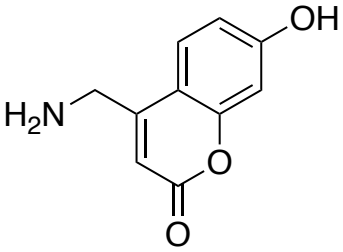


site-specific modification of glutamines in proteins for selective fragmentation





EDANS, λ_{max} = 336 nm



Coumarin, λ_{max} = 350 nm

Current methods are nonselective activation (e.g., CID, ETD)

large data sets, redundancy

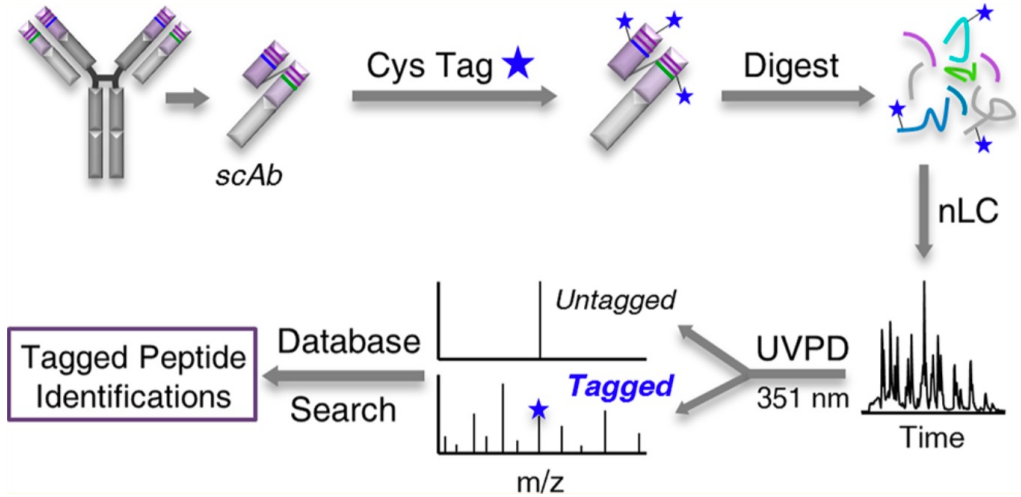
long search times

high false discovery rate (FDR)

UVPD: alternative dissociation

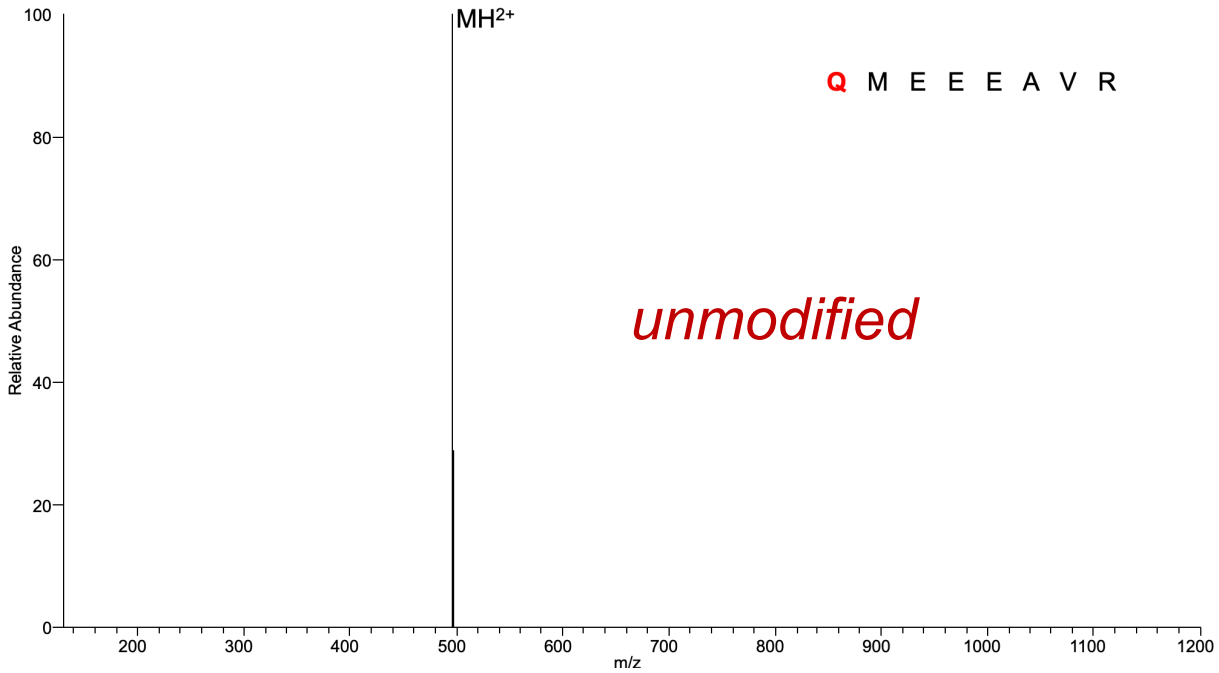
Peptides: minimal gas-phase absorption at ~350 nm

Enhance selectivity of MS/MS workflow



Enjalbert, *Rapid Commun. Mass Spectrom.*, **2011**, 25, 3375; Cotham, Brodbelt, *Anal Chem*, **2013**, 85, 5577

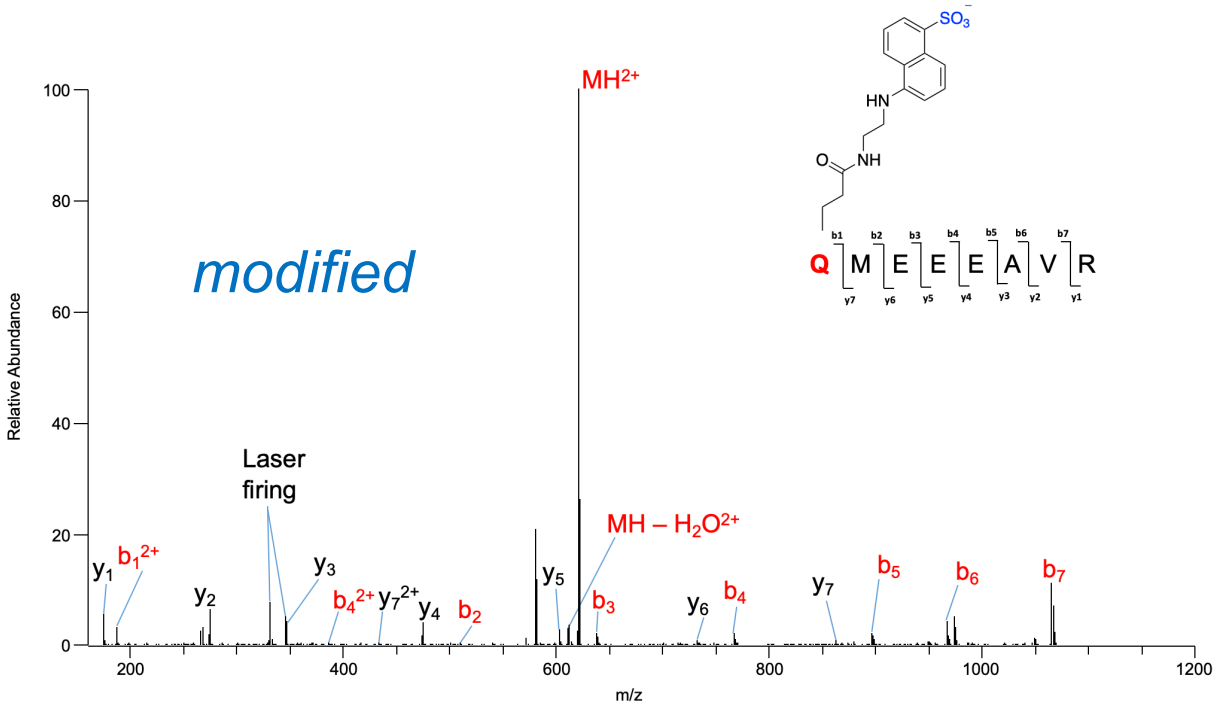
Selective photodissociation (355 nm) of glutaminyl residues on Exenatide



- modification of whole proteins and native confirmation: enable top down MS
- de-novo sequencing
- photo-selected reaction monitoring (photo-SRM)
- enable analysis of complex mixtures

Jenny Brodbelt
UT-Austin

Amanda Helms



mass, structures, properties-reactivities

- Mass spectrometry
  - ionization
  - identification: isotopic labeling
- New labeling tool: glutamine (Gln) via transglutaminase (TGase)
- Knowledge: elucidation of structures: new, unknown species (PTM's)
  - reactive species: metabolites, methylglyoxal (MGO)
  - crosslinking
- Sample preparation:
  - deamidation, isoaspartic acid (isoAsp), succinimide
  - meta-stable species: chemical trapping, native mass spec
- New modalities and new opportunities: higher-order structure (HOS)
  - virus-like particles (VLPs): adeno-associated virus (AAV)

Knowns vs Unknowns

Analytical chemists are very good at finding what we look for.

Rob Garnick

Reports that say that something hasn't happened are always interesting to me, because as we know, there are known knowns; there are things we know we know.

We also know there are known unknowns; that is to say we know there are some things we do not know.

But there are also unknown unknowns – the ones we don't know we don't know. And if one looks throughout the history of our country and other free countries, it is the latter category that tend to be the difficult ones.

-Donald Rumsfeld, 12 Feb 2002

Crosslinking in Proteins: Unknown Chemistry and Site

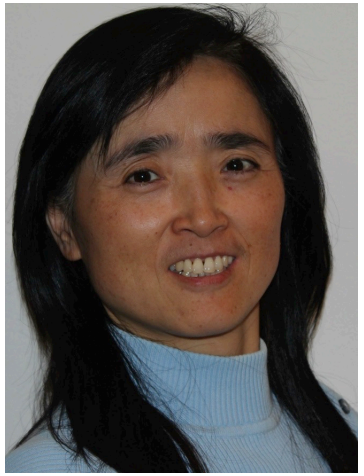
Stressed:

Basic (pH 8) or

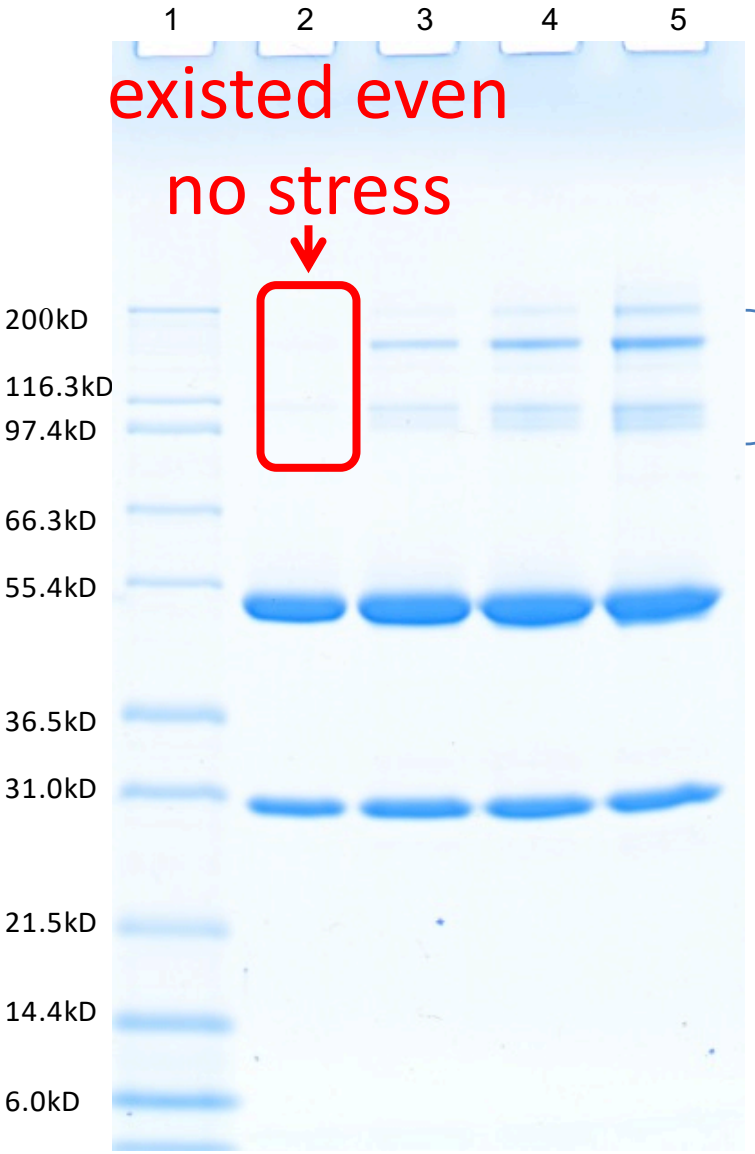
Light

Non-reducible

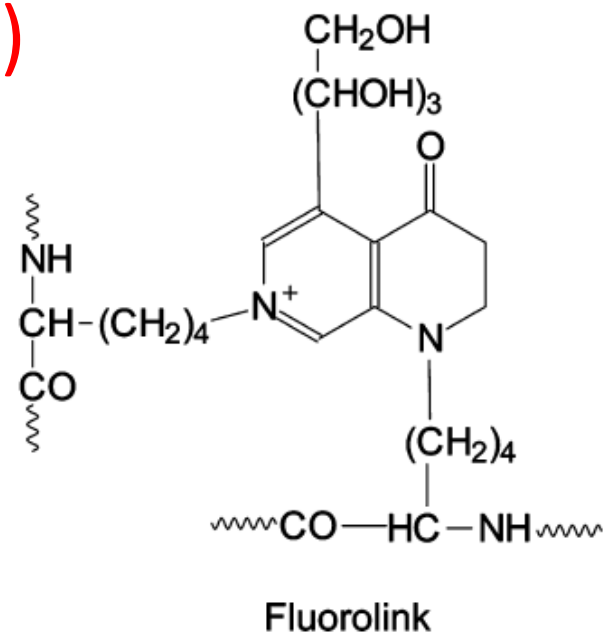
(not disulfide)



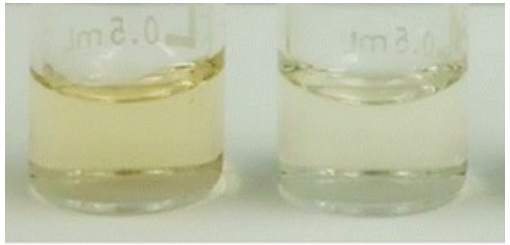
Min Liu, Amgen



no idea on mass change (too big)



coloration

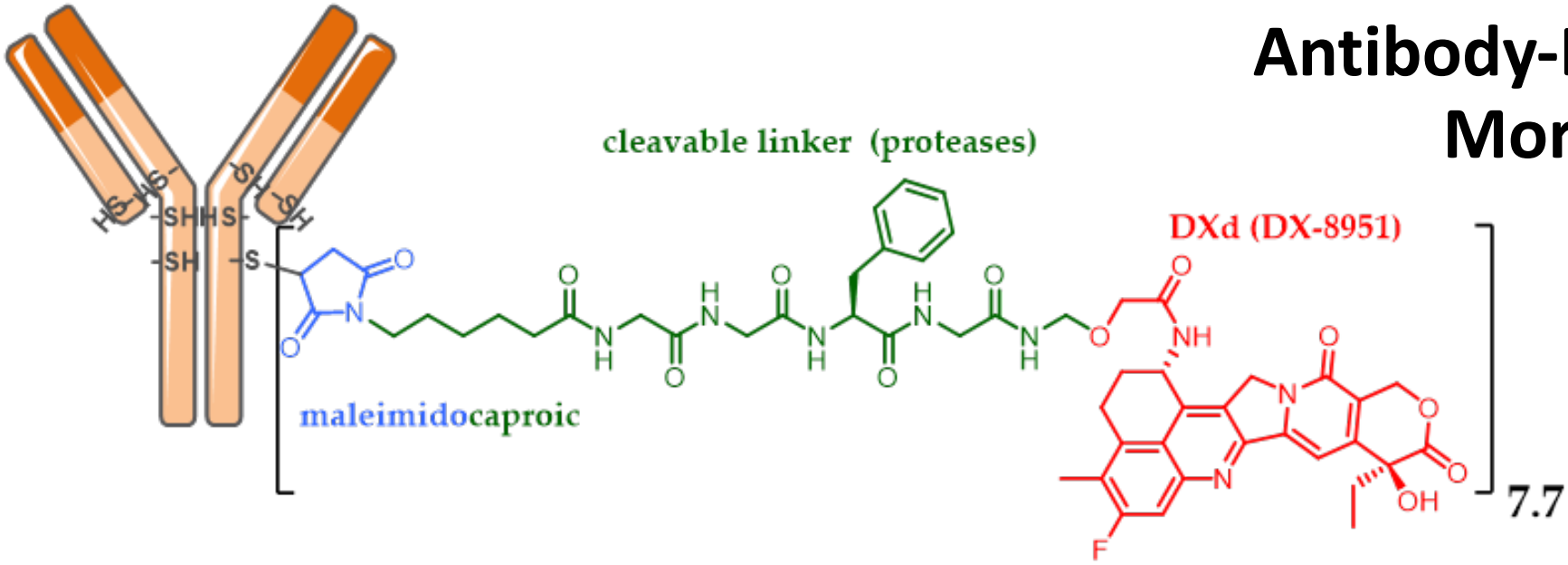


300-800 nm

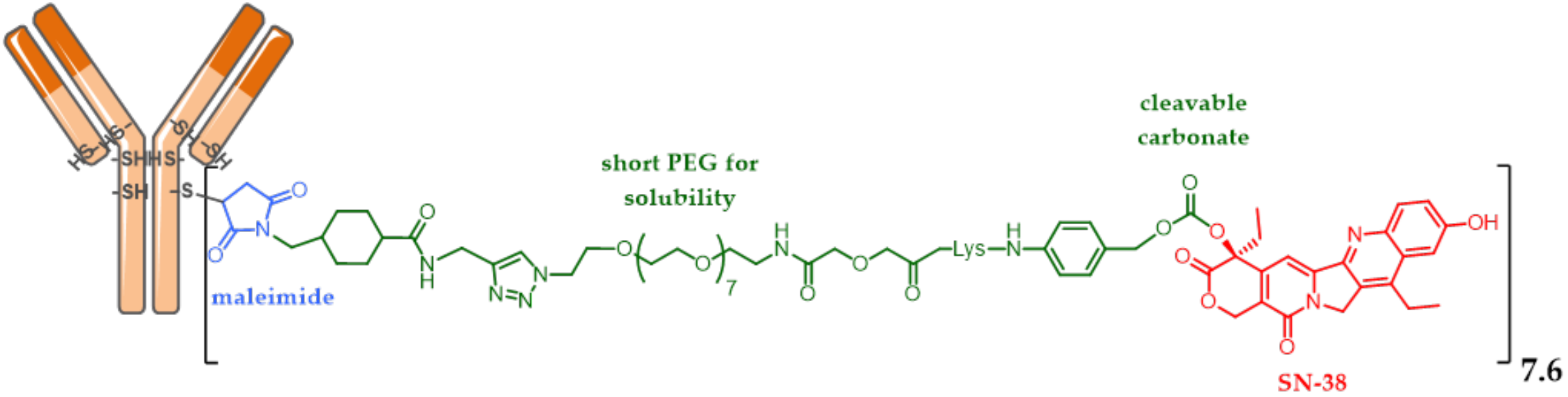
1, 2, 3x ICH

Antibody-Drug Conjugates (ADC): More Prone to Crosslinking

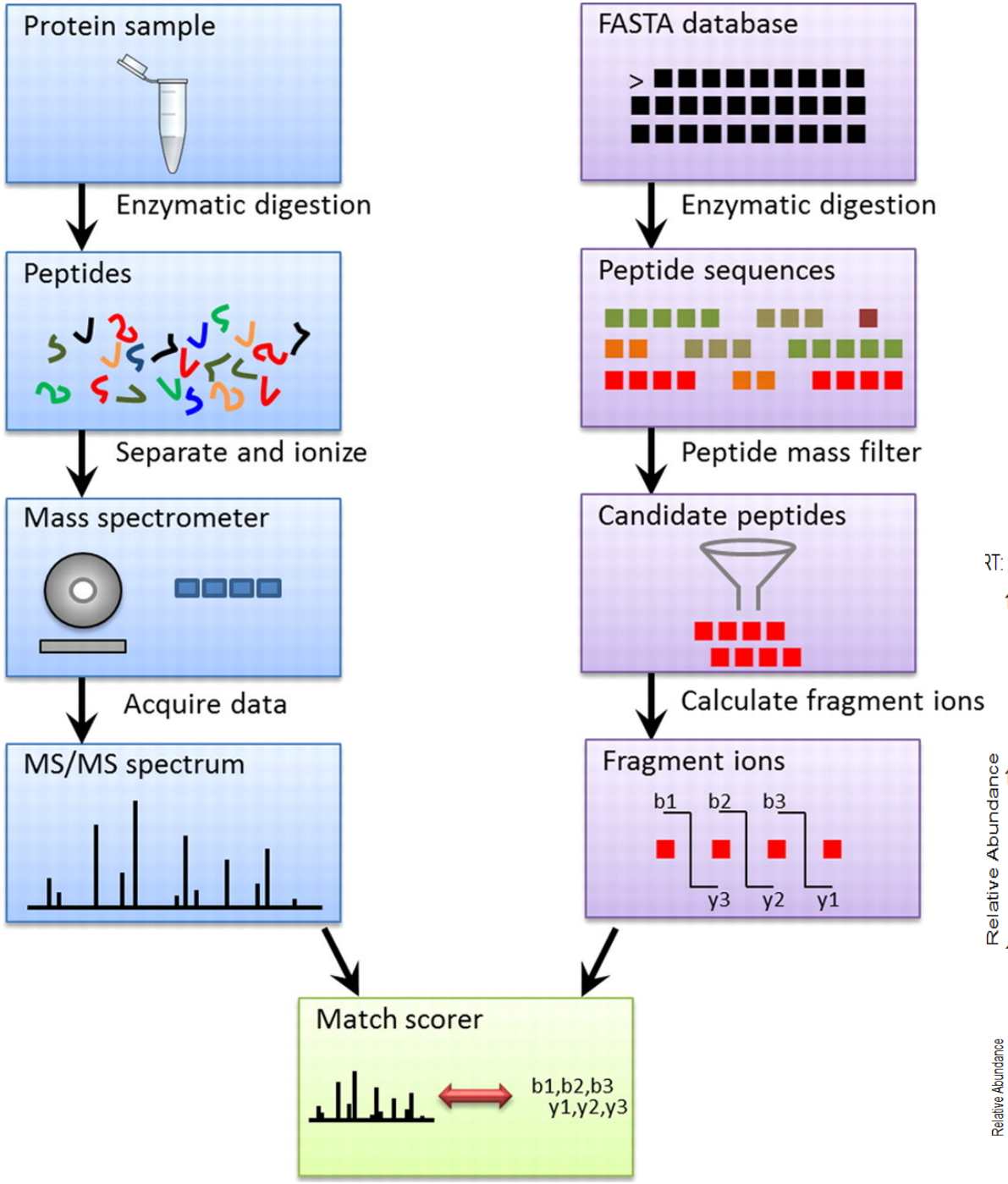
payload chemically more reactive



anti-HER2 Enhertu® (fam-trastuzumab deruxtecan-nxki or DS-8201a)



anti-TROP2 Trodelvy® (sacituzumab govitecan or IMMU-132) encyclopedia.pub/entry/2300



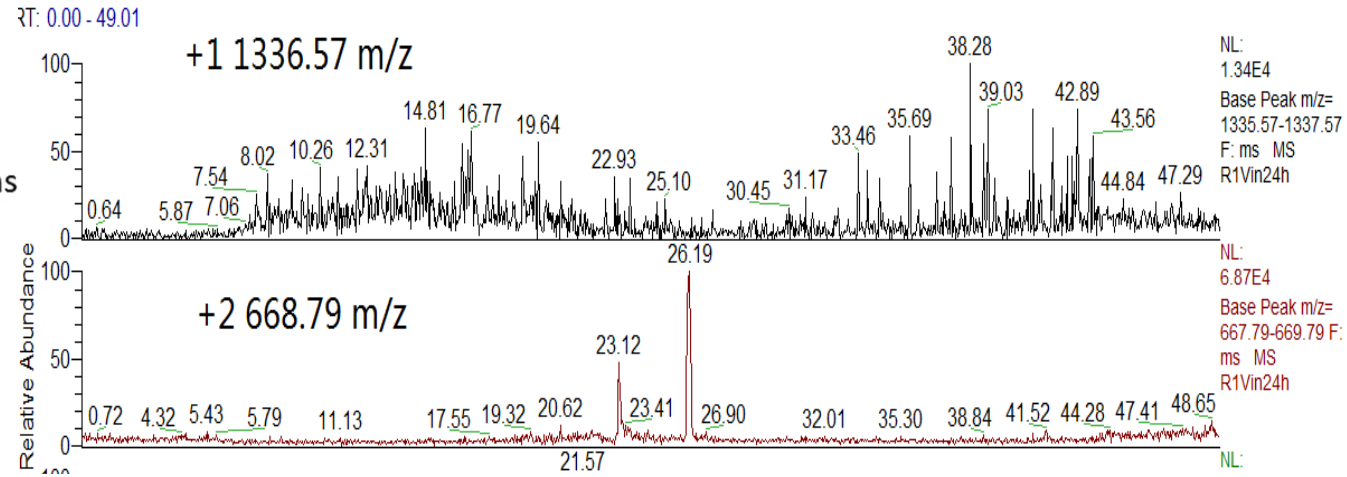
Limitations of Mass Spec

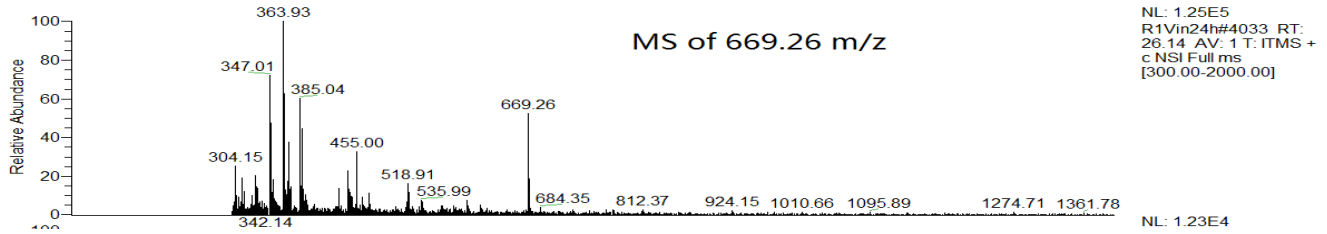
many peaks, which one?

identification: database search

pre-defined chemistry (mass, site)

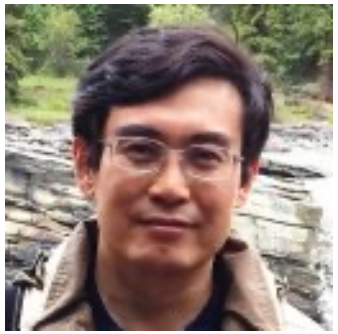
theoretical: unknowns





Pan-Specific for the Target Chemistry:
Telltale Sign of X-Linked Peptide: ^{18}O-Labeling

Ren Da



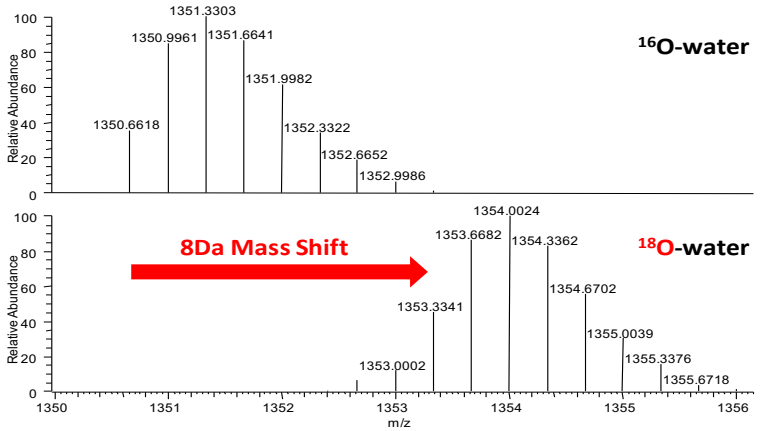
Zhongqi Zhang "ZZ"

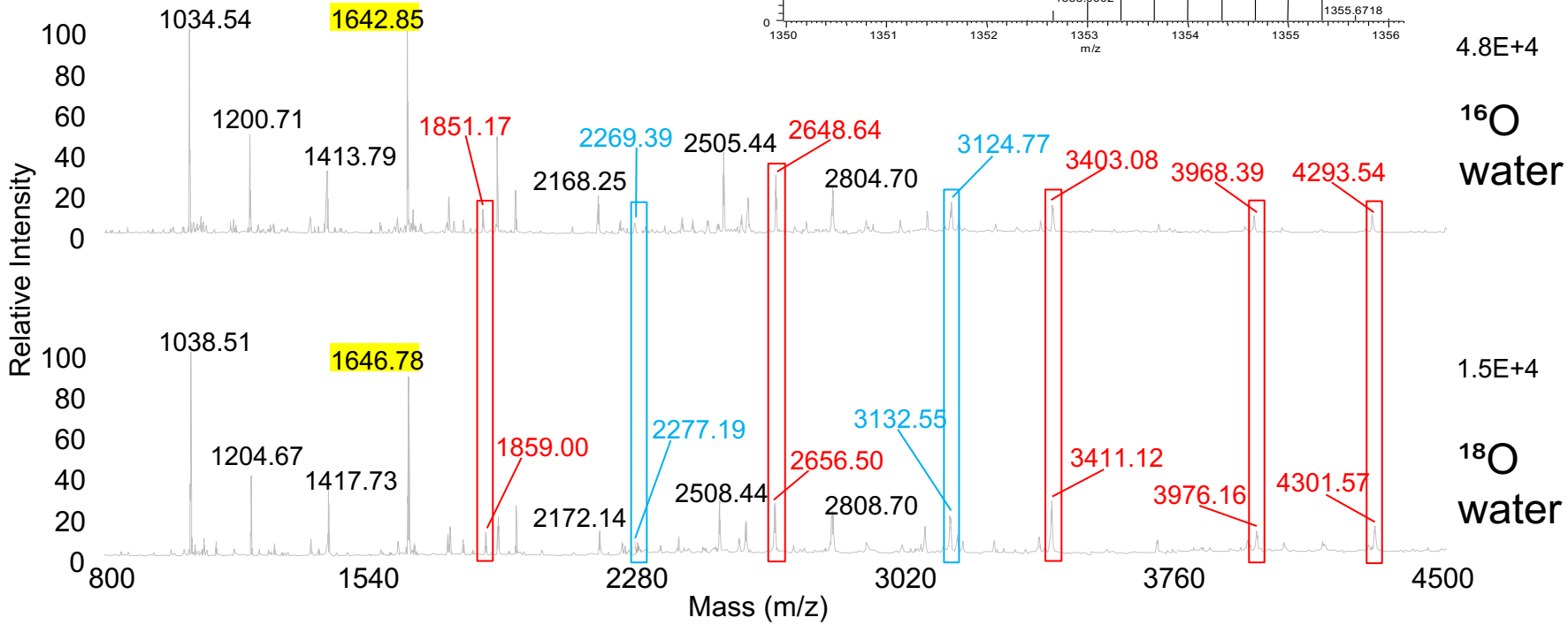
NH_2-abce---m---wxyz-COOH

X

NH_2-ABCD---N---WXYZ-COOH

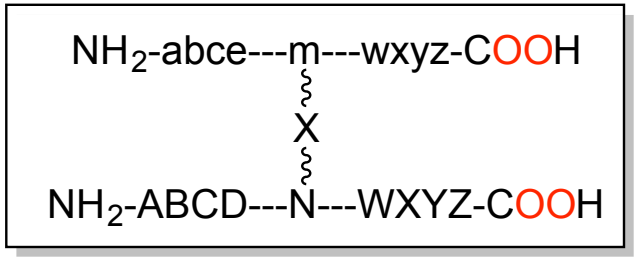
2 termini
4 18O
+8 Da





Crosslinked peptide pairs (indicate with boxes): characteristic +8 Da mass shift.
Specific (blue, predictable) and non-specific (red, unexpected) trypsin cleavage.

Fragmentation of Crosslinked Peptides

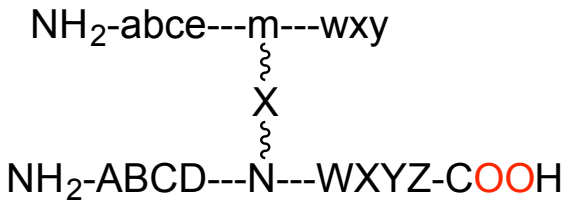


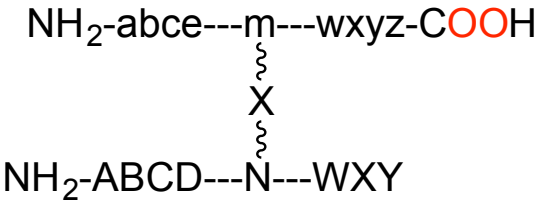
NH_2-abcd
NH_2-abc
NH_2-ab

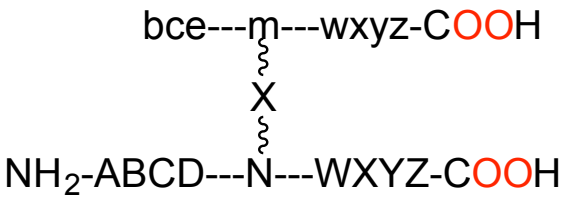
NH_2-ABCD
NH_2-ABC
NH_2-AB

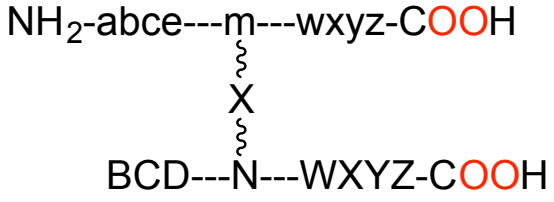
wxyz-COOH
xyz-COOH
yz-COOH

WXYZ-COOH
XYZ-COOH
YZ-COOH





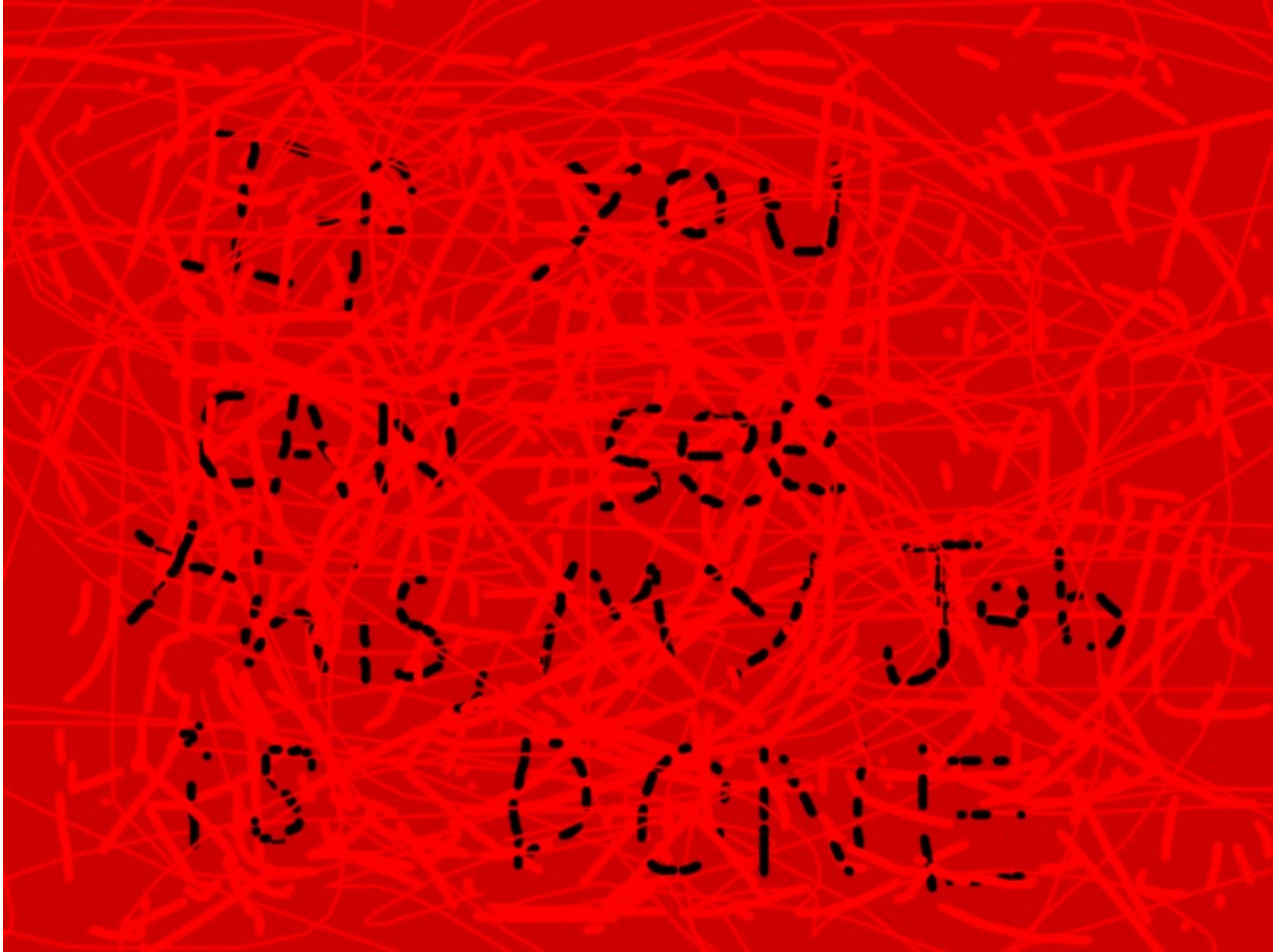




☹ information overload: multiple sets of ions ☹

☺ sufficient data to deduce sequences ☺

http://www.district196.org/schools/avhsold/dept/science/physics/physicsweb04/AVHSPhysics/color-notes.html


If YOU
CAN SEE
THIS MY JOB
IS DONE

Elucidation of the Unknown Chemistry Workflow: Chemistry (Property) Guided

Detection of cross-linked peptides	Determination of sequences		Deduction of cross-link site & chemistry
Tryptic digestion in ^{16}O- or ^{18}O-water	Match mass of linear fragment ions	de novo sequencing of cross-linked fragment ions	Mass difference (combined native chains vs cross-linked peptide) for formula & chemistry
Mass shift of 8 Da	Partial peptide sequences	Sequence tag	Confirmed structure
NH_2-abcd---m---wxyz-COOH ? NH_2-ABCD---N---WXYZ-COOH	NH_2-abcd wxyz-COOH NH_2-abc xyz-COOH NH_2-ab yz-COOH	bcd---m---wxyz-COOH ? NH_2-ABCD---N---WXYZ-COOH cd---m---wxyz-COOH ? NH_2-ABCD---N---WXYZ-COOH d---m---wxyz-COOH ? NH_2-ABCD---N---WXYZ-COOH	NH_2-abcd---m---wxyz-COOH S NH_2-ABCD---N---WXYZ-COOH

Table S2. Deduction of elemental formula for the crosslinked S215-K244/S215-K244 peptide.

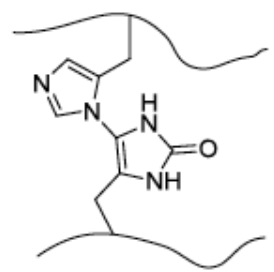
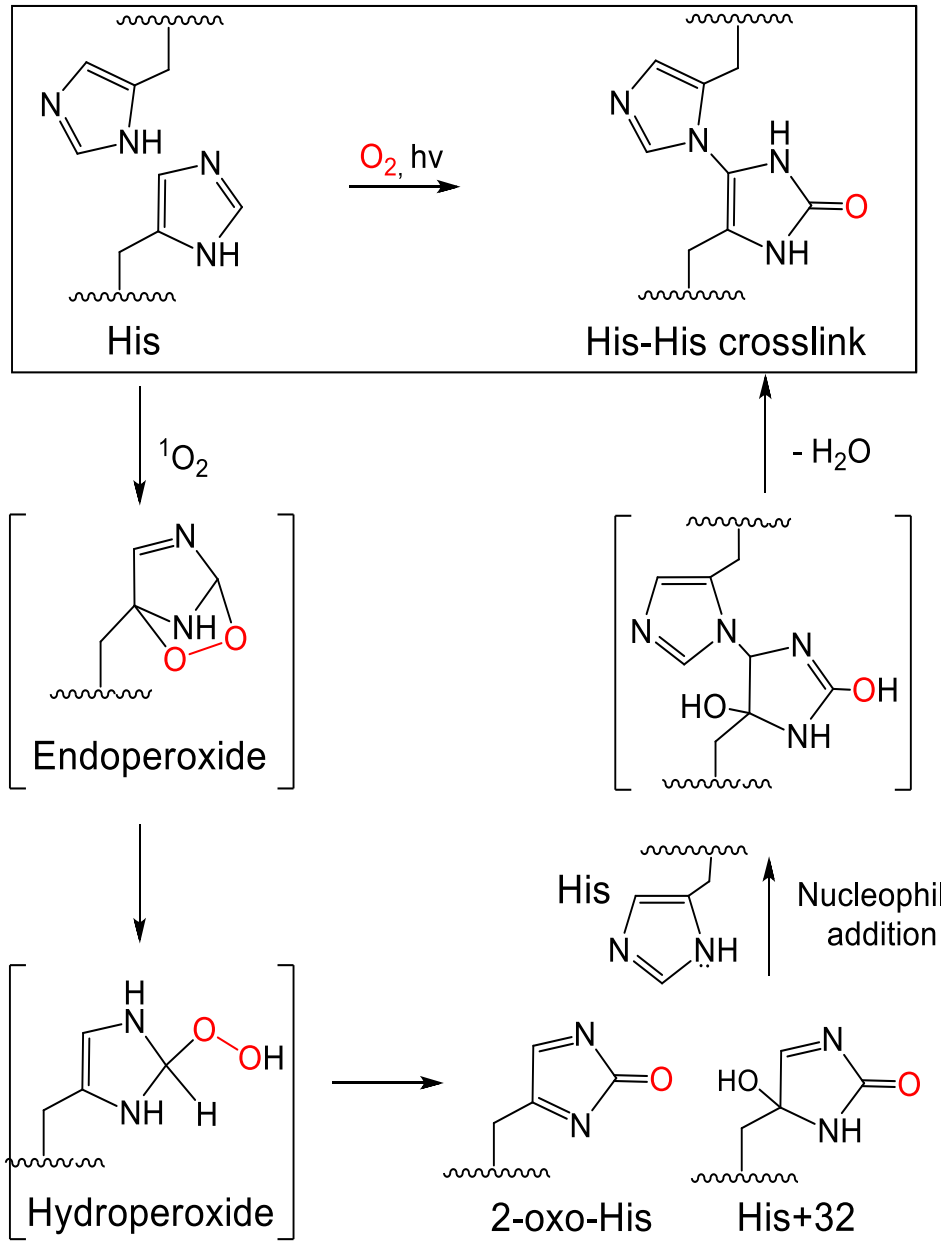
Name	Mass (Da)		
Calculated mass of S215-K244 (single chain)	3336.587		
Sum of the mass of two unmodified chains	6673.174		
Observed mass of the crosslinked peptide	6687.149		
Mass difference	+13.975		
Proposed formula and calculated mass (mass error in Da)	+O-2H: 13.979 (0.004)	+N: 14.003 (0.028)	+CH2: 14.016 (0.041)
Comments	Most likely	Unlikely	Unlikely
Proposed structure		-	-

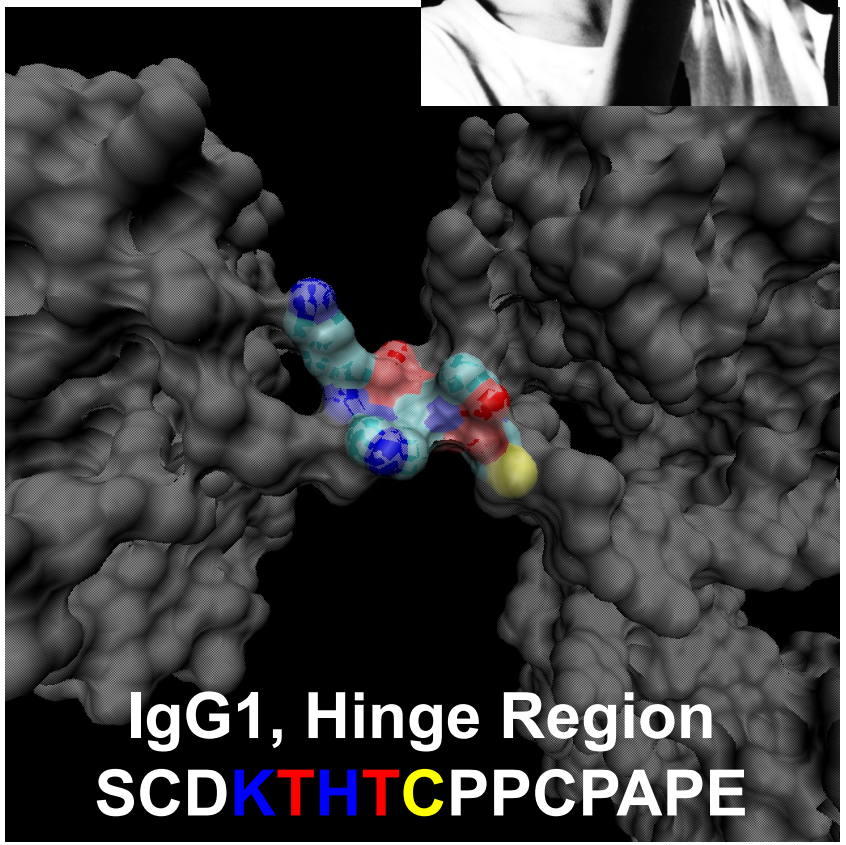
Photo-oxidative Crosslinking of Histidine



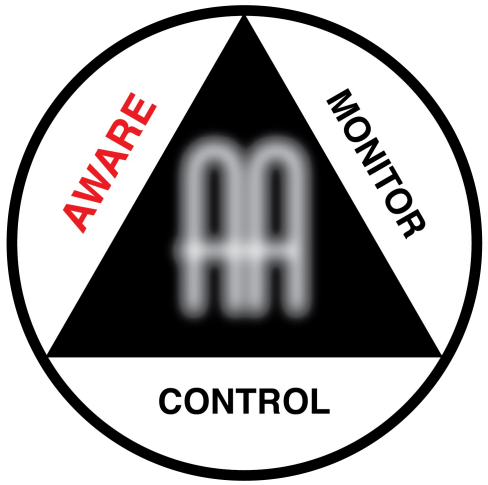
1st in protein

unknown to known

Eddie Zhou



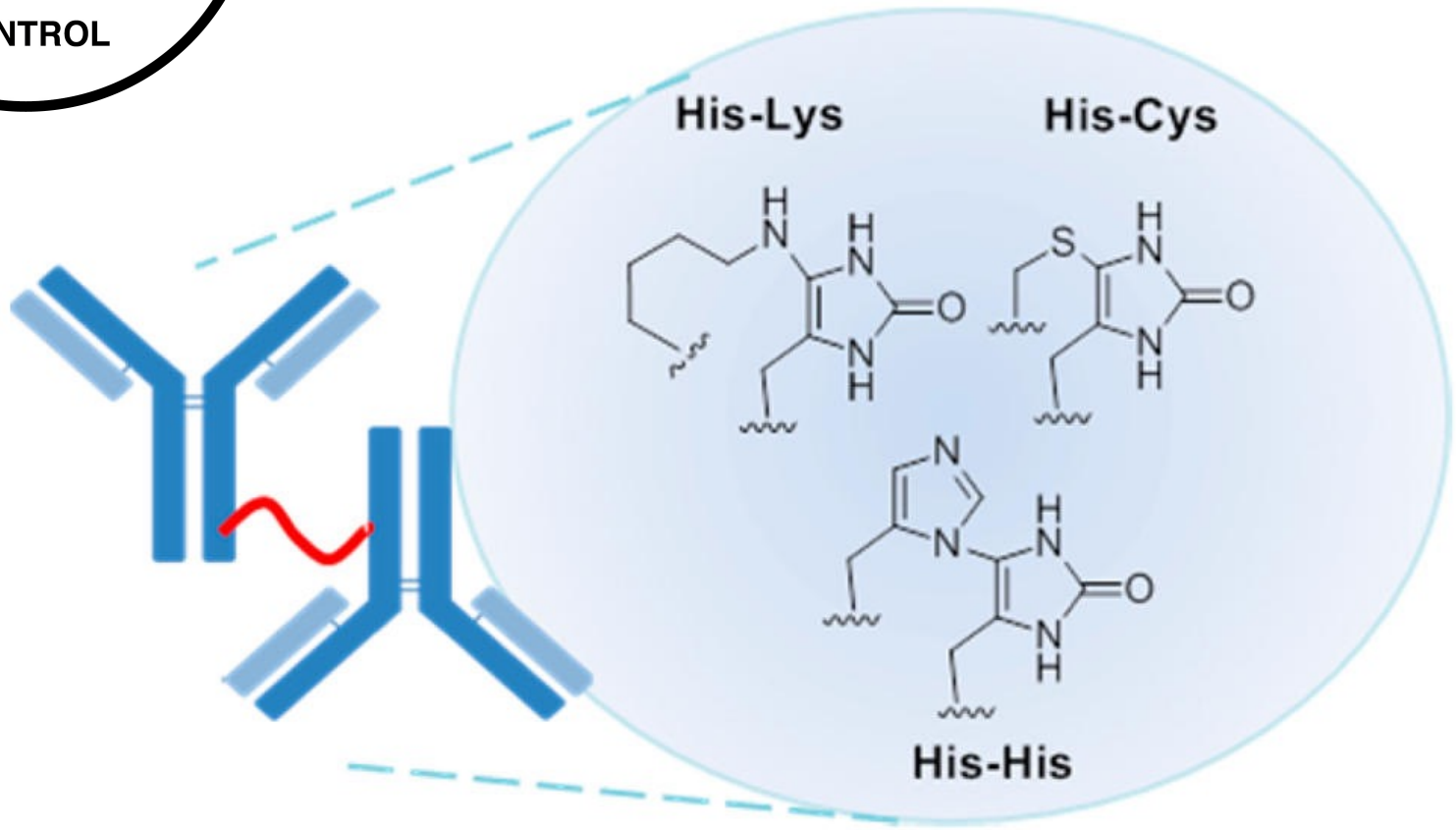
Min Liu, Zhongqi Zhang, Janet Cheetham, Da Ren, and Zhaohui Sunny Zhou, Northeastern and Amgen, *Analytical Chemistry, 2014, 86, 4940.*



Histidine Oxidation and X-linking is Common

Safe Light: Manufacture and Storage

observed without stress



reactors:
glass vs opaque
light variation
visible light
may be bad too

histidine as buffer

safe light

Anal Chem, 2017, *89*, 7915. Xu, et a. Biogen
J Proteome Res, 2018. doi: 10.1021/acs.jproteome.7b00881. Mariotti et al
Eur J Pharm Biopharm, 2018, 127, 37. Cheng Du, et al. BMS

NH_2-abce---m---wxyz-COOH
X
NH_2-ABCD---N---WXYZ-COOH

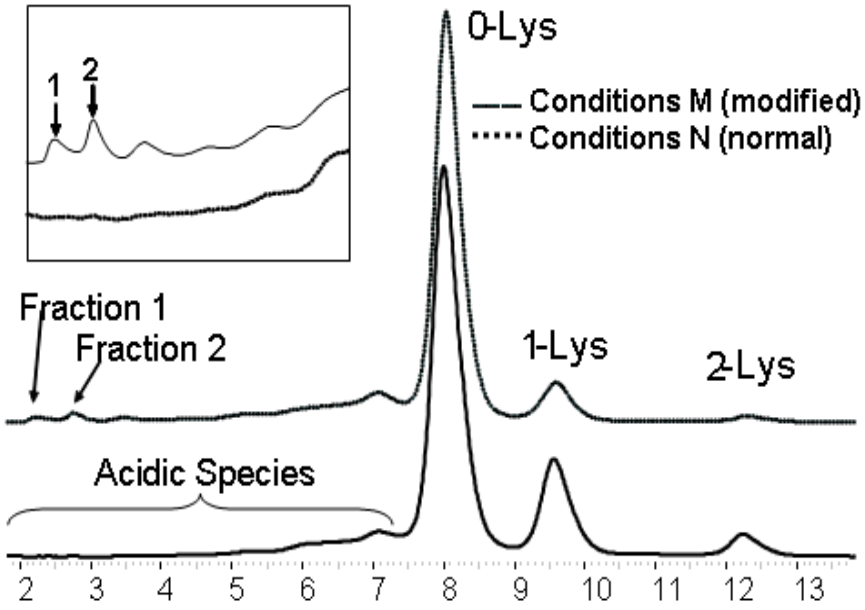
2 termini

Crosslinked Proteins: Pan-Specific Method for Quantification and Enrichment

Kevin Moulton
Vertex

Cell Culture Affects Product Quality

- Different cell culture conditions
- Acidic species (weak cation exchange)
- Mass increases of 54 and 72 Da (LC/HC)
- Not in the PTM databases



Localization of Sites
Manual search +54/72 peptides
None for tryptic peptides

Several mis-cleaved peptides

Potential site: arginine

Czeslaw Radziejewski

AbbVie
now retired

Chumsae C, Gifford K, Lian W, Liu H, Radziejewski CH, Zhou ZS.
Anal Chem, 2013, 85, 11401

Mass to Structure: Elucidation of Unknown Chemistry

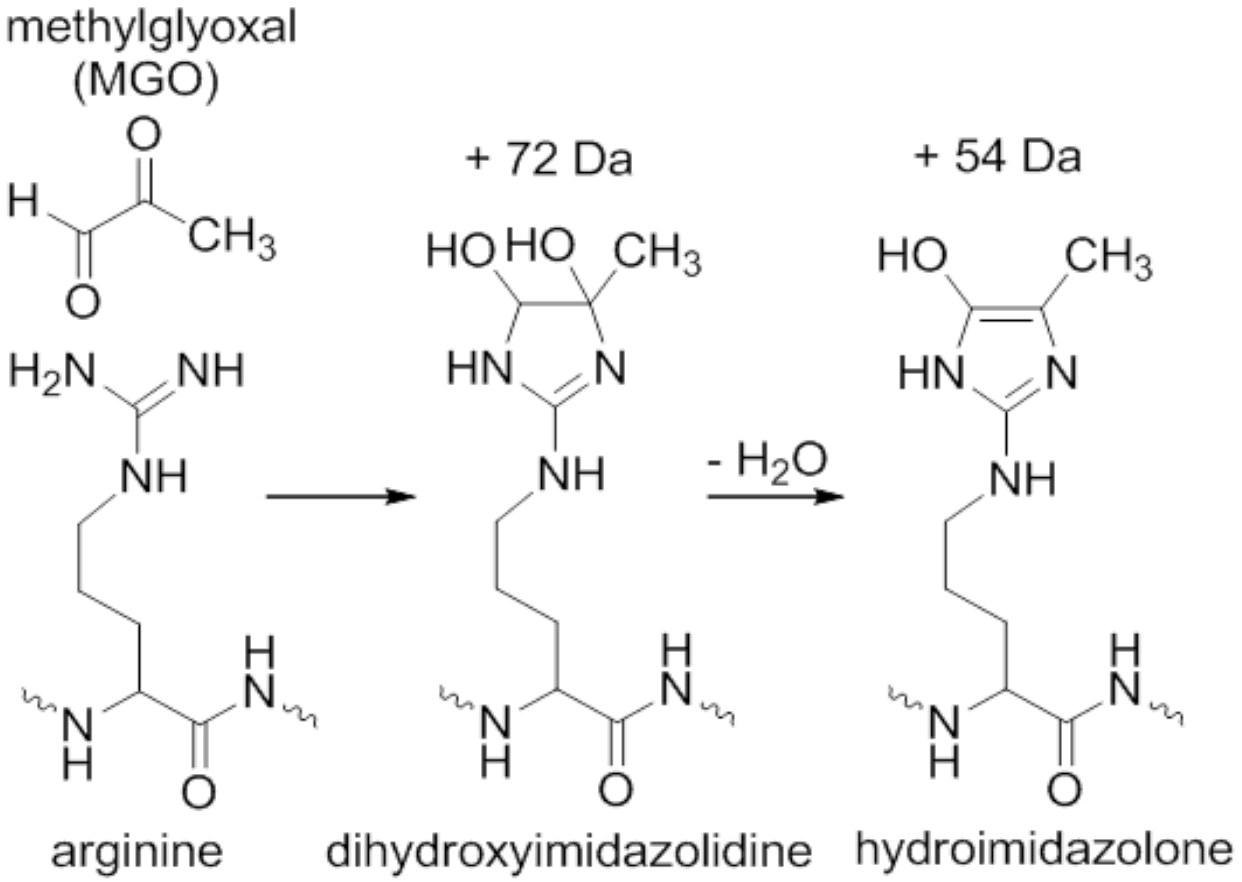
- Mass increases: 54 and 72 Da;
  2 reactive species?
- Not in the PTM databases
- Potential site: arginine

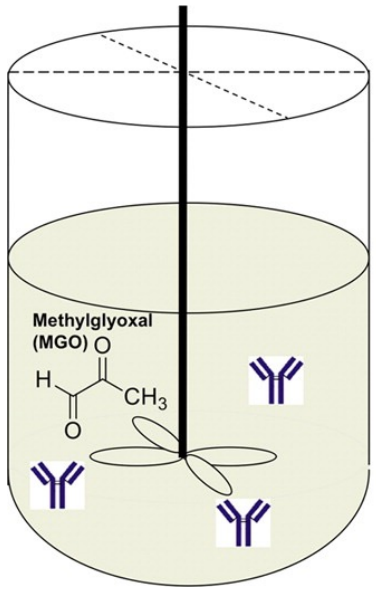
- 72–54 =18, water
- eliminate a hydroxyl (OH) group
- single molecule may be responsible
- mechanism: substitution or
- addition to carbonyl: 72 or 90 Da
- secondary metabolite

Chumsae C, Gifford K, Lian W, Liu H, Radziejewski CH, Zhou ZS.
Anal Chem, 2013, 85, 11401

Methylglyoxal (MGO): Reactive Metabolite

- Mass increases of 54 and 72 Da
- First for mAbs: cell stress, engineering

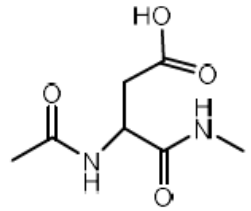




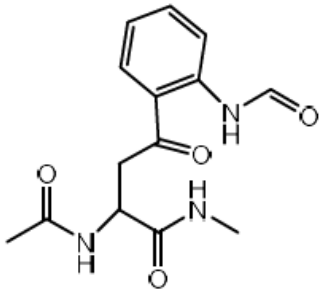
Chris Chumsae

AbbVie
now BMS

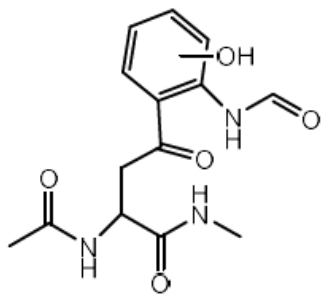
Chumsae C, Gifford K, Lian W, Liu H, Radziejewski CH, Zhou ZS.
Anal Chem, 2013, 85, 11401



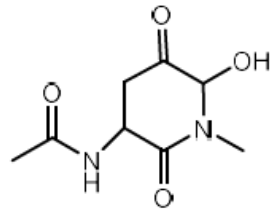
Asp (-71)



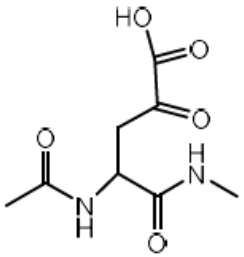
N-formalkynurenine (+32)



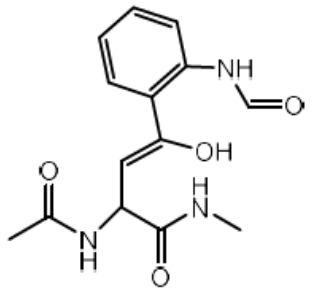
Hydroxy-N-formalkynurenine (+48)



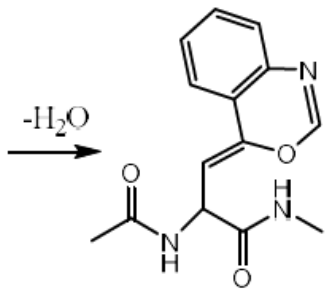
(-59)



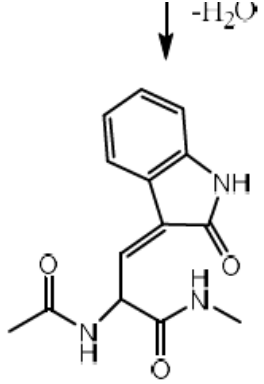
Oxoglutamic acid (-43)



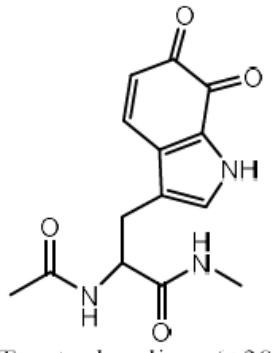
N-formalkynurenine (+32)



N-formalkynurenine-H2O (+

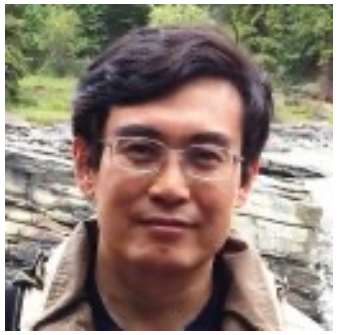


Dehydrooxindolealanine (+14)



Tryptophandione (+30)

Zhongqi Zhang "ZZ"



Journal of Pharmaceutical Sciences 111 (2022) 1556–1564

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Tell me the mass change,
I will tell you the chemistry.

Consortium Project

Pharmaceutical Biotechnology

A Mass Spectrometric Characterization of Light-Induced Modifications in Therapeutic Proteins

Zhongqi Zhang[a,*], Sih-Yao Chow[a], Ronandro De Guzman[a], Nathan H. Joh[a], Marisa K. Joubert[a], Jason Richardson[a], Bhavana Shah[a], Mats Wikström[a], Zhaohui Sunny Zhou[b], Jette Wypych[a]

[a] *Process Development, Amgen Inc. One Amgen Center Drive, Thousand Oaks, CA 91320, USA*
[b] *Department of Chemistry and Chemical Biology, Barnett Institute for Chemical and Biological Analysis, Northeastern University, Bo 02115, USA*

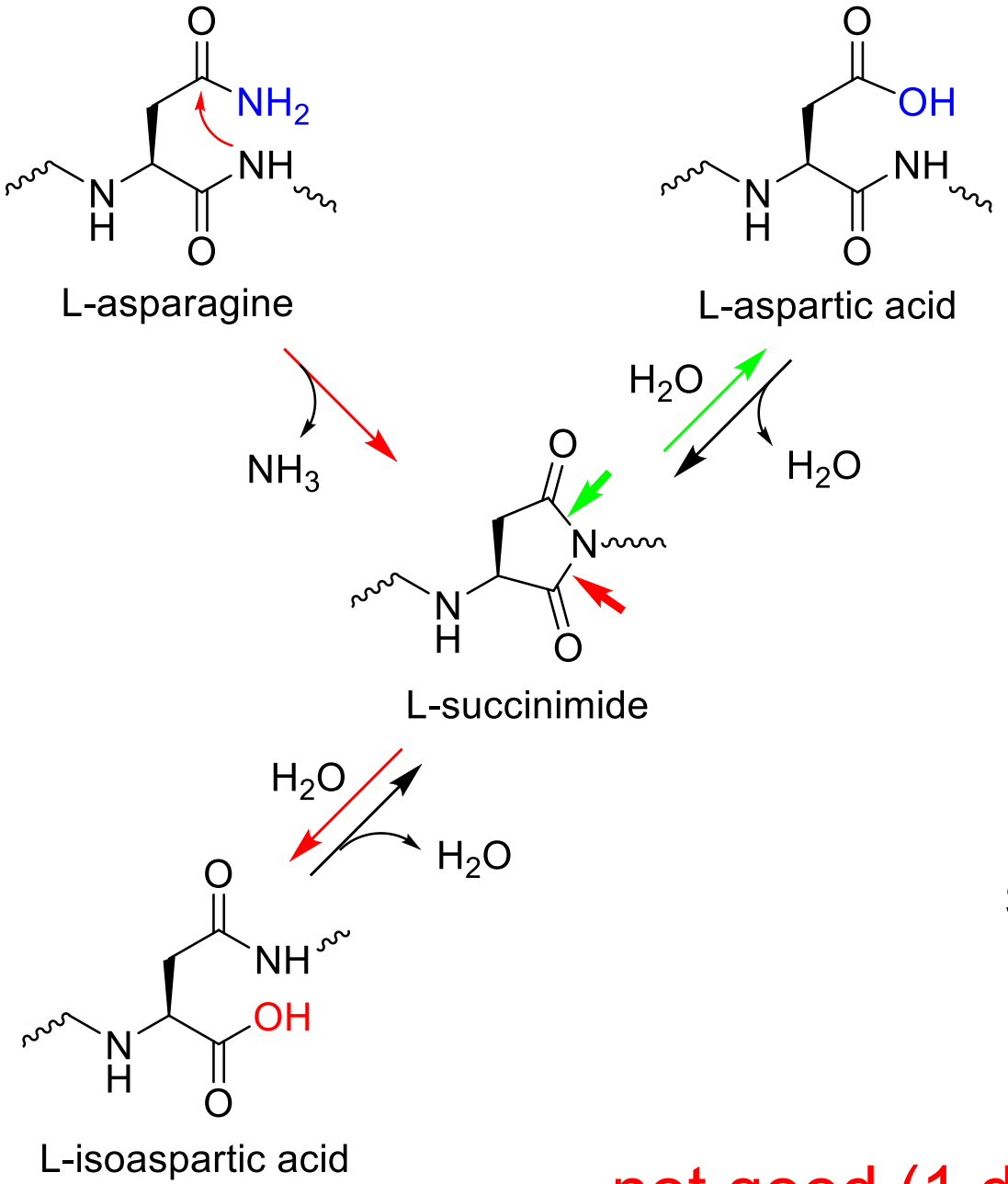
Table I. Chemical Instabilities Reported for Proteins of Therapeutic Interest

Deamidation
Asp–isoAsp interconversion/isomerization
Racemization
Proteolysis
Beta-elimination
Oxidation
Metal-Catalyzed Oxidation (MCO)
Photooxidation
Free radical cascade oxidation
Disulfide exchange
DKP formation
Condensation reactions
pGlu formation
Hinge region hydrolysis
Trp hydrolysis

many more:
cross-linking
coloration
formulation
new modalities
Unknowns ???

mass, structures, properties-reactivities

- Mass spectrometry
  - ionization
  - identification: isotopic labeling
- New labeling tool: glutamine (Gln) via transglutaminase (TGase)
- Knowledge: elucidation of structures: new, unknown species (PTM's)
  - reactive species: metabolites, methylglyoxal (MGO)
  - crosslinking
- Sample preparation:
  - deamidation, isoaspartic acid (isoAsp), succinimide
  - meta-stable species: chemical trapping, native mass spec
- New modalities and new opportunities: higher-order structure (HOS)
  - virus-like particles (VLPs): adeno-associated virus (AAV)



Deamidation and Isomerization

isoaspartic acid (isoAsp, isoD)

Asparagine (Asn) deamidation

Aspartic acid (Asp) isomerization

Succinimide (Asu) intermediate

spontaneous and ubiquitous

deamidation rate

$t_{1/2}$ = 1 to 500 days

sequence, structure and pH dependence

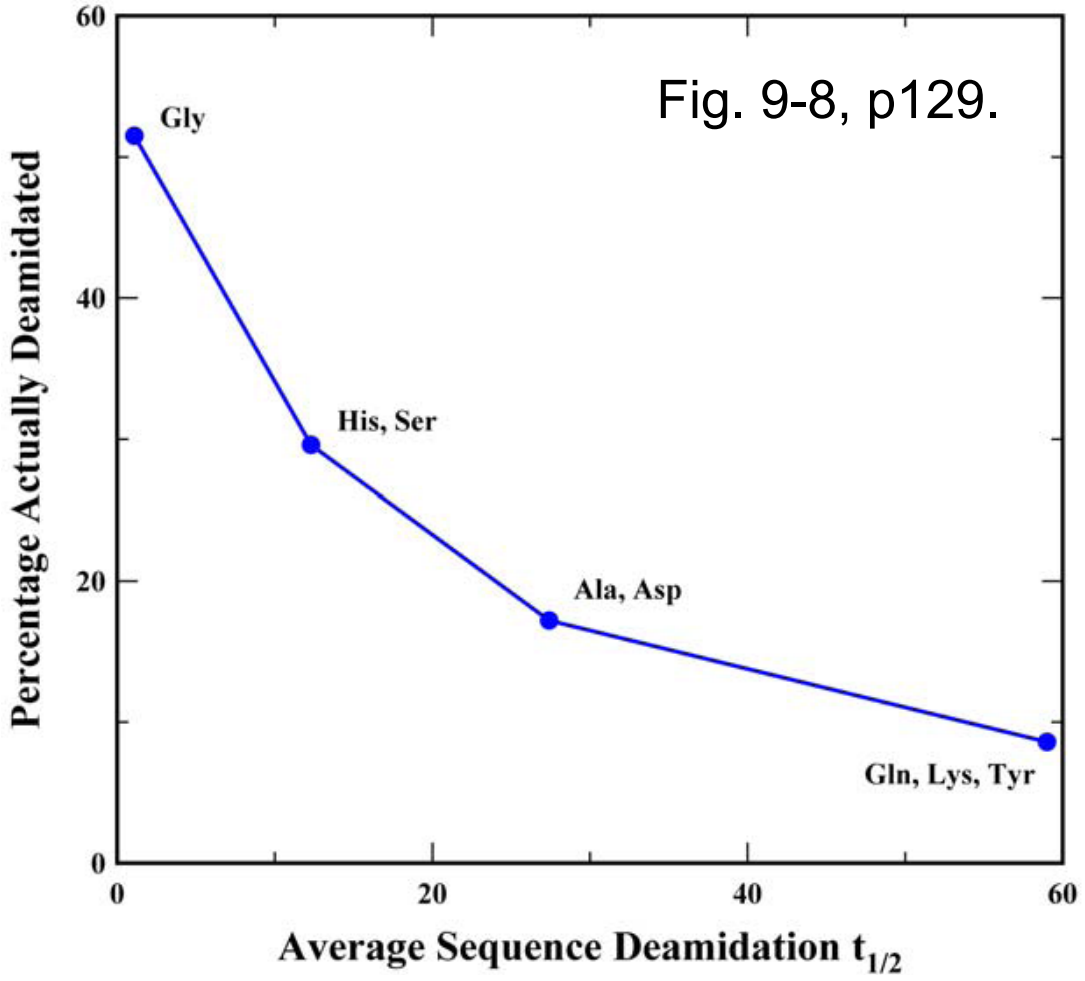
Asn-Gly > Asn-Ser, Asn-His >> Asn-Pro

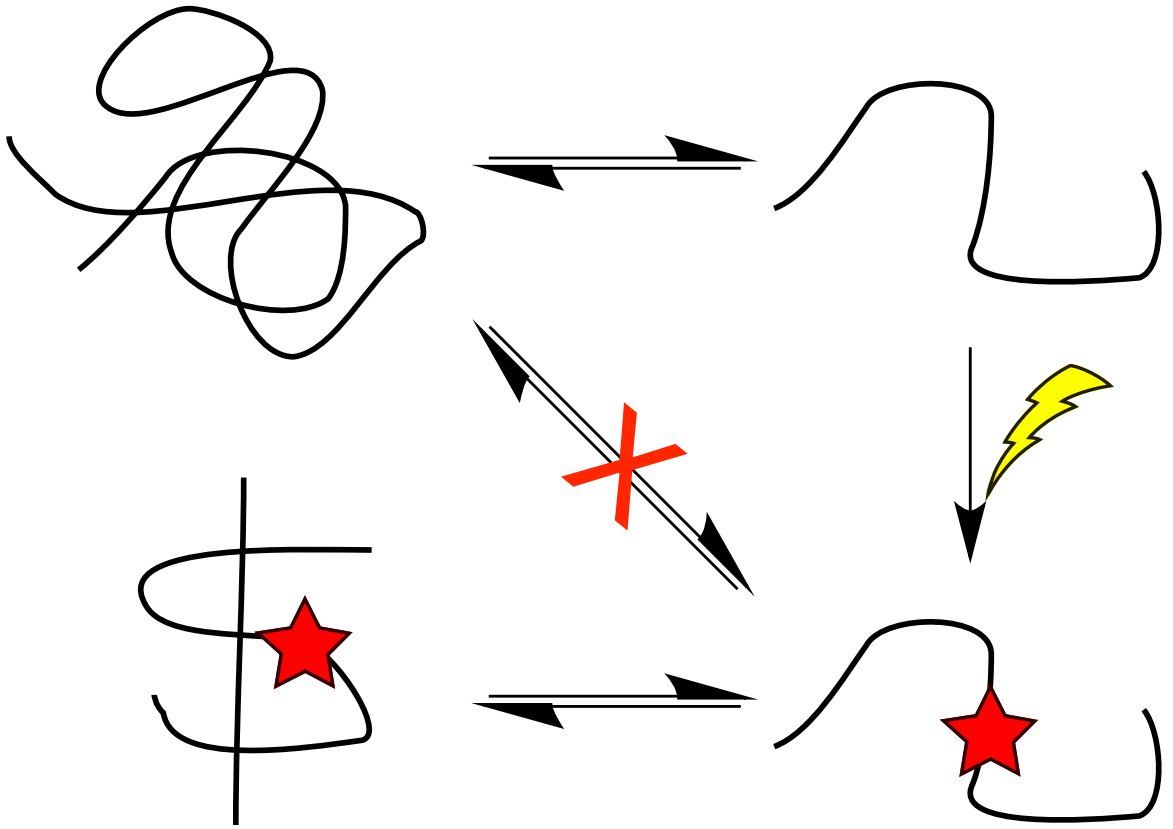
(NG > NS, NH > NP)

not good (1 d), not sure, not happy (10 d), no problem

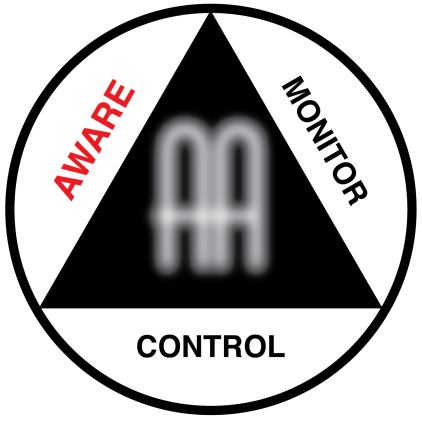
Dependence on Sequences and Structures

sample preparation: changes in structures





Robinson, N. E.; Robinson, A. B. *Molecular Clocks: Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins*; Althouse Press: Cave Junction, Oregon, USA, 2004.



Deamidation

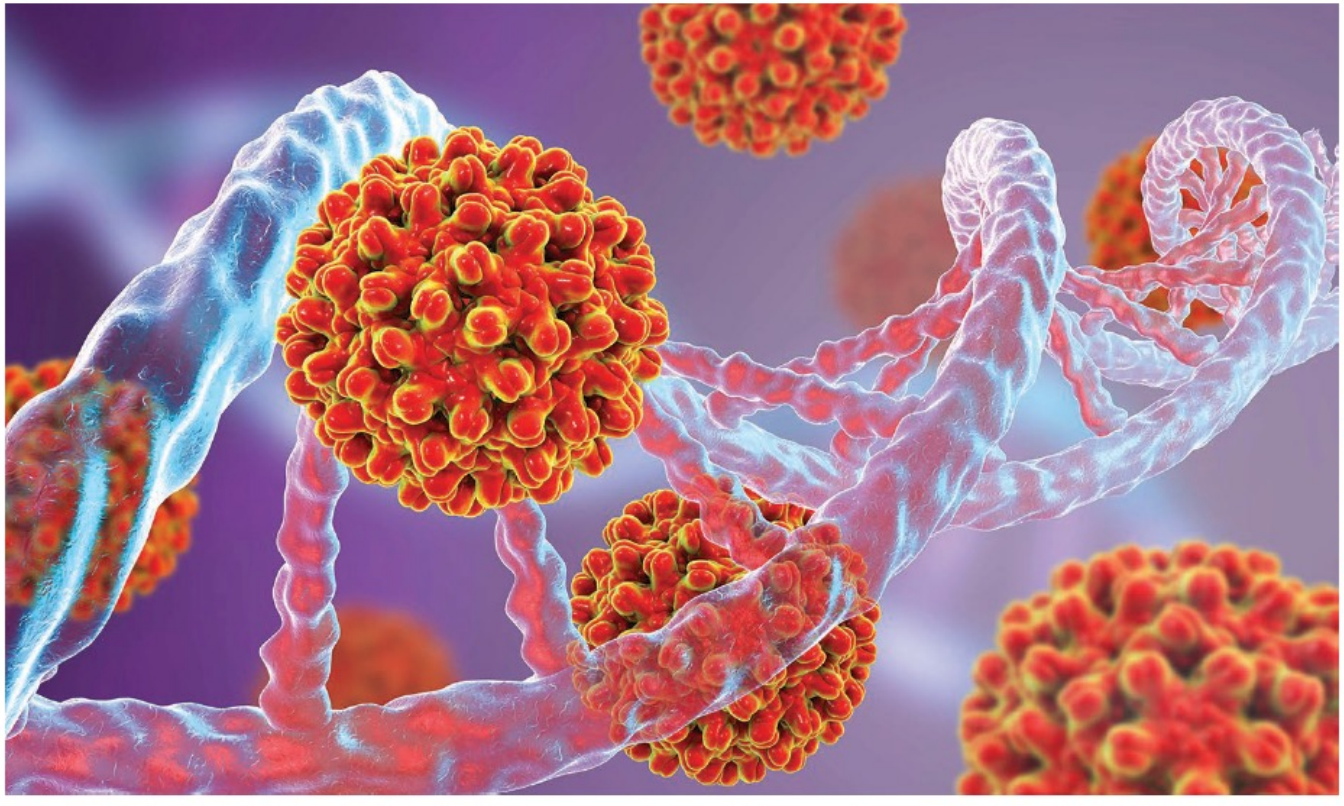
Aditya Ansodaria

To win at gene therapy, companies pick viruses with production credentials

Nature Biotechnology, 2019, 37, 5. Cormac Sheridan

Is a spontaneous chemical change on the proteins that coat an adeno-associated virus (AAV) a problem for gene therapy developers? According to a recent controversial paper from the lab of gene therapy pioneer Jim Wilson, professor of medicine and pediatrics at the University of Pennsylvania, the hitherto overlooked phenomenon of protein deamidation can affect the capsid of AAV, the vector most widely adopted in gene therapy, reducing the efficiency with which vectors enter their target cells. Because the reaction is unpredictable it may impair lot-to-lot consistency in manufacturing. "My first response to the paper was here we go again," says Michael Linden, newly appointed CSO of Hampton, UK-based Touchlight Genetics. "Let's see if this one explodes."

The findings (*Mol. Ther.* https://doi.org/10.1016/j.ymthe.2018.09.013, 2018) have not been universally accepted. But at the same time, Linden adds, the paper serves to highlight the lack of standardization in gene therapy vector analytics and manufacturing. Every lab has its own way of measuring vector titer and purity. "These things have probably a much bigger effect on the potency of the vector than deamidation," he says.



Kateryna Kon / Science Photo Library / Getty images

Gene therapies require viral particles to deliver genetic material into the various tissues. For companies scaling up to run trials, clinical-grade viral vector production can be a challenge.

aggregation and oxidation. "We are able to substantially stabilize viruses regarding their functionality," says CEO Michael Scholl. Purification methods influence vector potency as well. The final product may also contain DNA from the bacterial plasmids used to transfect the producer

measure of the actual level of viral activity. "We dose based on genome copy [number], which is about the only assay we can reliably perform," says Wilson. The cell-based assays in use are suboptimal, Linden notes. "They're not reflective of the bioactivity of the virus. What you

Table 1. Characteristics of Deamidated Residues of Interest

	N+1 Residue	Structural Topology	Structural Motif	Average % Deamidation
N35	Q	N/A	N/A	1
N57	G	N/A	N/A	80
N94	H	N/A	N/A	7
N254*	N	surface exposed	not assigned	9
N255*	H	surface exposed	not assigned	N/A
N263	G	surface exposed	HVR I	99
N305	N	buried	alpha helix	8
N385	G	surface exposed	HVR III	88
N410	N	buried	not assigned	3
N459	T	surface exposed	HVR IV	7
N499	N	surface exposed	HVR V	17
N514*	G	surface exposed	HVR V	84
N517*	S	surface exposed	HVR V	4
N540*	G	buried	HVR VII	79
N630*	F	buried	not assigned	1
N653	T	surface exposed	HI loop	1

Asterisks represent residues selected for further analysis. NA, not applicable.

Molecular Therapy
Original Article

Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function

April R. Giles,[1,2] Joshua J. Sims,[1,2] Kevin B. Turner,[1] Lakshmanan Govindasamy,[1] Mauricio R. Alvira,[1] Martin Lock,[1] and James M. Wilson[1]

[1]Gene Therapy Program, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Trypsin Digestion

We prepared stock solutions of 1 M DTT and 1.0 M IAM. Capsid proteins were denatured and reduced at 90°C for 10 min in the presence of 10 mM DTT and 2 M GndHCl. We allowed the samples to cool to room temperature and then alkylated them with 30 mM IAM at room temperature for 30 min in the dark. We quenched the alkylation reaction with the addition of 1 mL of DTT. We added 20 mM ammonium bicarbonate (pH 7.5–8) to the denatured protein solution at a volume that diluted the final GndHCl concentration to 200 mM. We added trypsin solution for a 1:20 trypsin-to-protein ratio and incubated at 37°C overnight. After digestion, we added TFA to a final concentration of 0.5% to quench the digestion reaction.

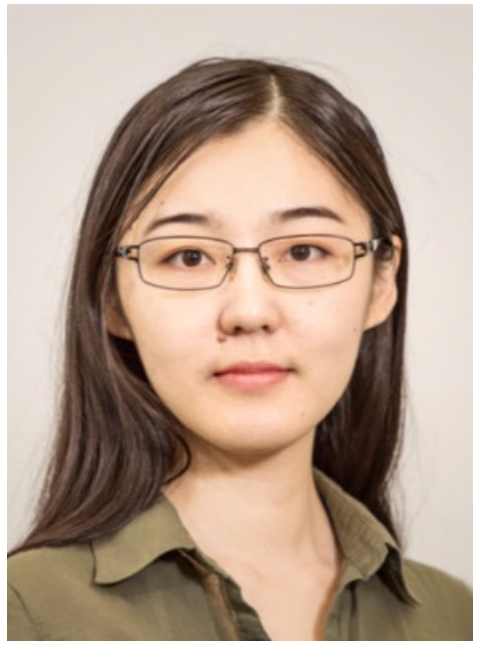
Analytical Artifacts: Ambiguity

Asn Asn
Asn Asn

Asn Asn
Asp Asp

Asn Asn
Asn Asp

Asn Asn
Asp Asp



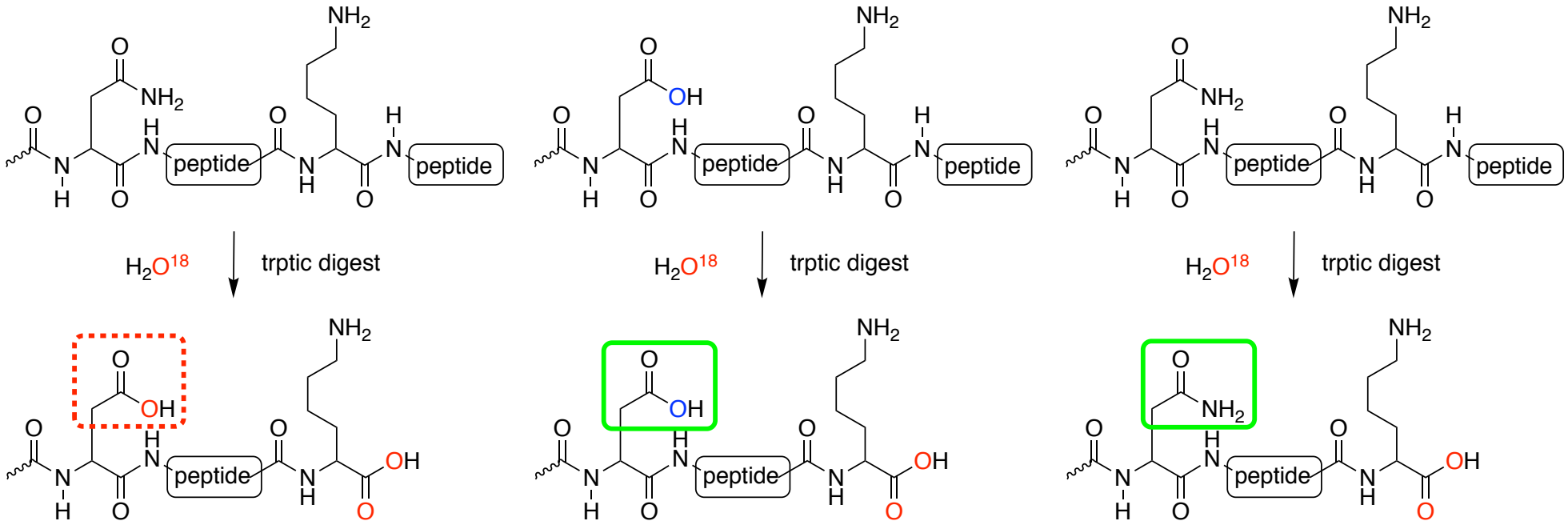
Shanshan Liu
Northeastern,
Takeda, NIRa

Asn-Gly > Asn-Ser, Asn-His >> Asn-Pro

(NG > NS, NH > NP)

not good (1 d), not sure, not happy (10 d), no problem

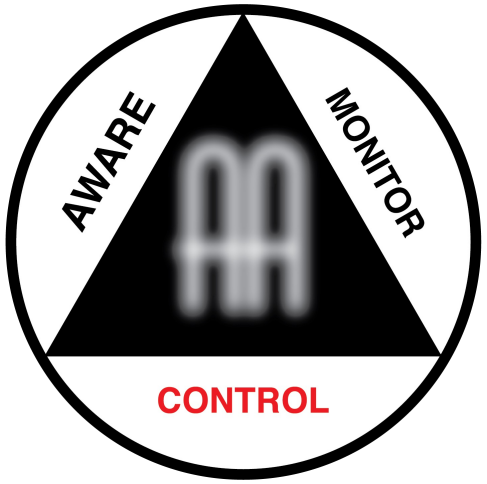
Distinguish Artifact and Real



David Verrill

- Deamidation prior to sample prep w/ $H_2{}^{16}O$: + 1 Da
- Deamidation during sample prep (*artifact*) w/ $H_2{}^{18}O$: + 3 Da
- b ions generated from Asp or isoAsp
- not interfered with ^{18}O atom incorporation into C-termini

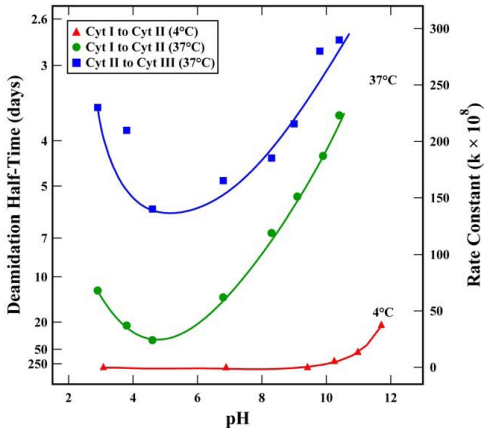
Anal Chem, 2012, 84, 6355, Yi Du, Fengqiang Wang, Kimberly May, Wei Xu, Hongcheng Liu

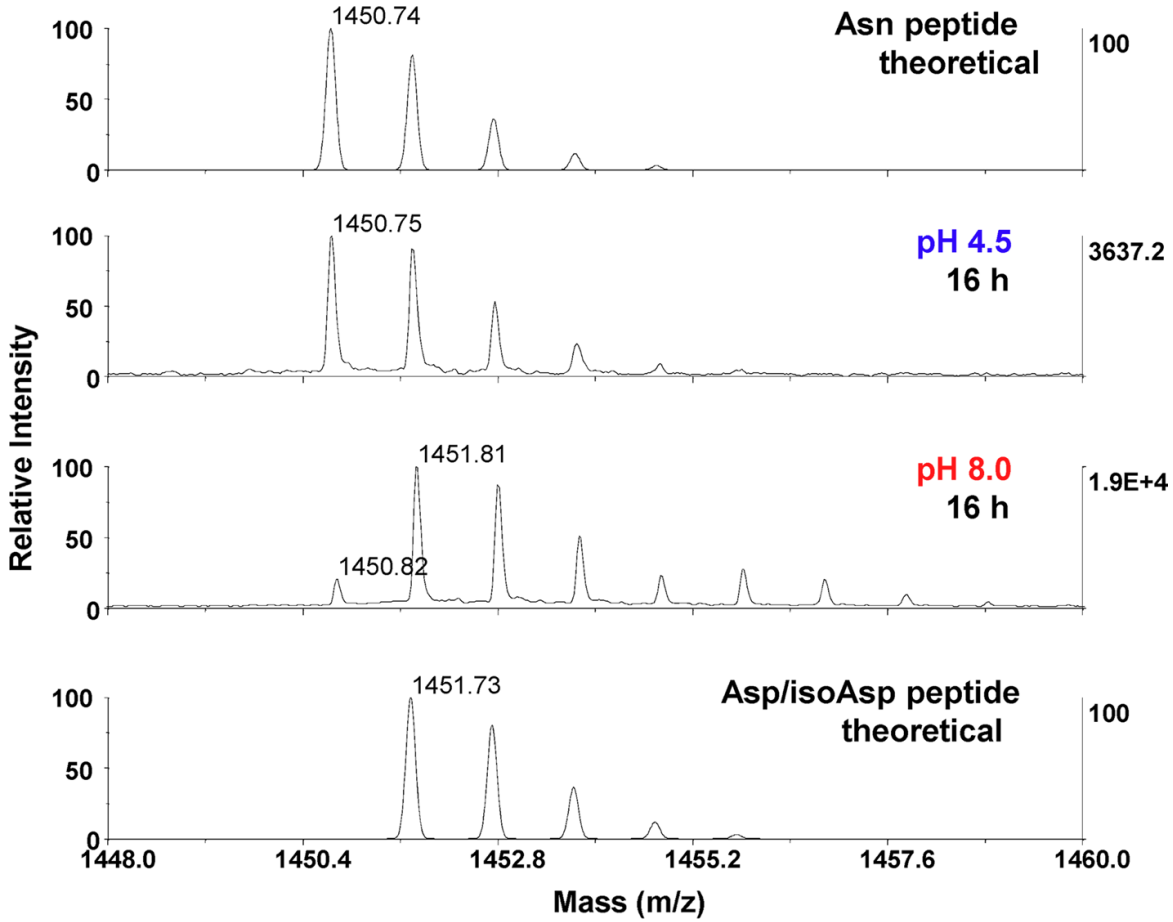


Mildly Acidic Conditions Eliminate Deamidation Artifact during Proteolysis:

Digestion with Glu-C at pH 4.5 vs pH 8

first principle mechanism-based





exenatide Glu-C peptide
WLKNGGPSSGAPPPS-NH_2
(25-39C-terminal amide)

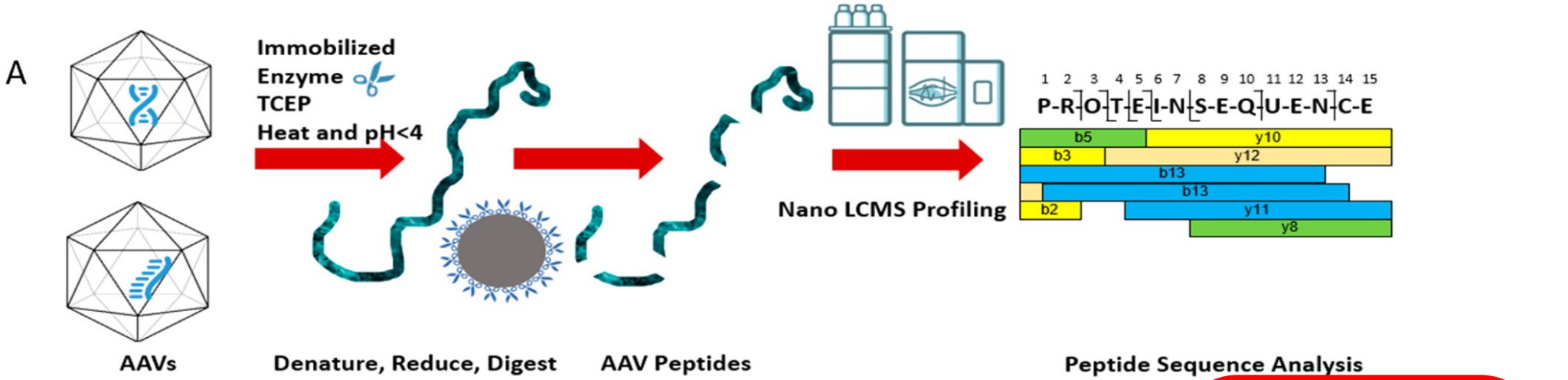
Jared Auclair Shanshan Liu

Amino Acids, 2016, 48, 1059, Liu, Moulton, Auclair, Zhou

Correction to Rapid Highly-Efficient Digestion and Peptide Mapping of Adeno-Associated Viruses

Estee Naggar Toole, Craig Dufresne, Somak Ray, Alexander Boris Schwann, Amissi Sadiki, Zhaohui Sunny Zhou, Ken Cook, and Alexander R. Ivanov*

Anal. Chem. **2021**, *93*, 30, 10403−10410. DOI: 10.1021/acs.analchem.1c02117



B

```
1   MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY  50
51  KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF 100
101 QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP 150
151 VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT 200
201 NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP 250
251 TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI 300
301 NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL 350
351 PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS 400
401 QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT 450
451 PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY 500
501 SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT 550
551 NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV 600
601 LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN 650
651 TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY 700
701 TSNYNKSVNGVYSEPRPIGTRYLTRNL                        735
```

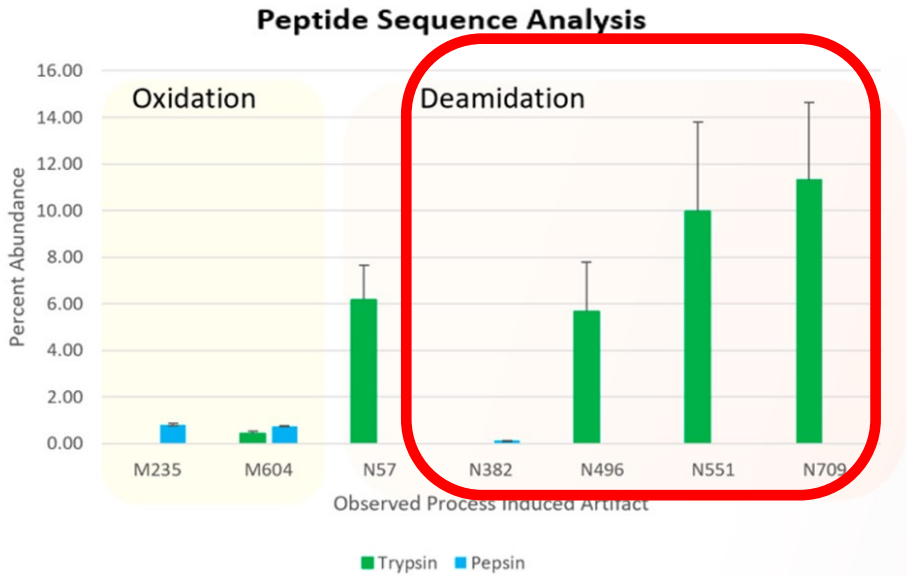
Gap 1 Residues 162 -245

Gap 2 Residues 361 -389

Gap 3 Residues 666 -688

Structural Characterization of AAV Proteome

C



Characterization of Adeno-Associated Virus (AAV)

Deamidation and Isomerization:

Challenges, Artifacts and Opportunities

Zhaohui Sunny Zhou

z.zhou@northeastern.edu

Bioprocessing Summit, 16 August 2021, virtual

BARNETT INSTITUTE

of Chemical and Biological Analysis

linkedin.com/in/sunnyzhou

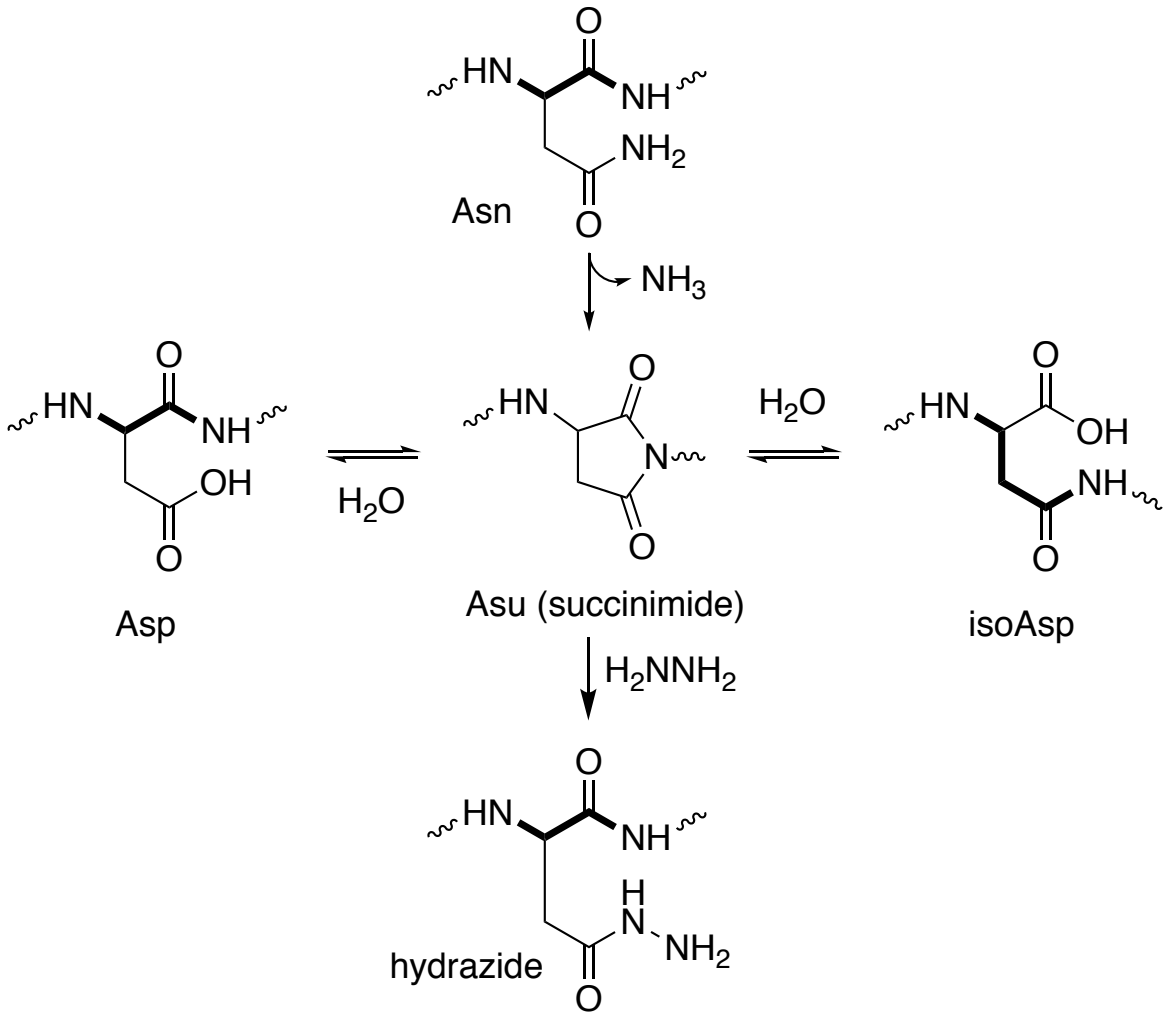
Play (k)

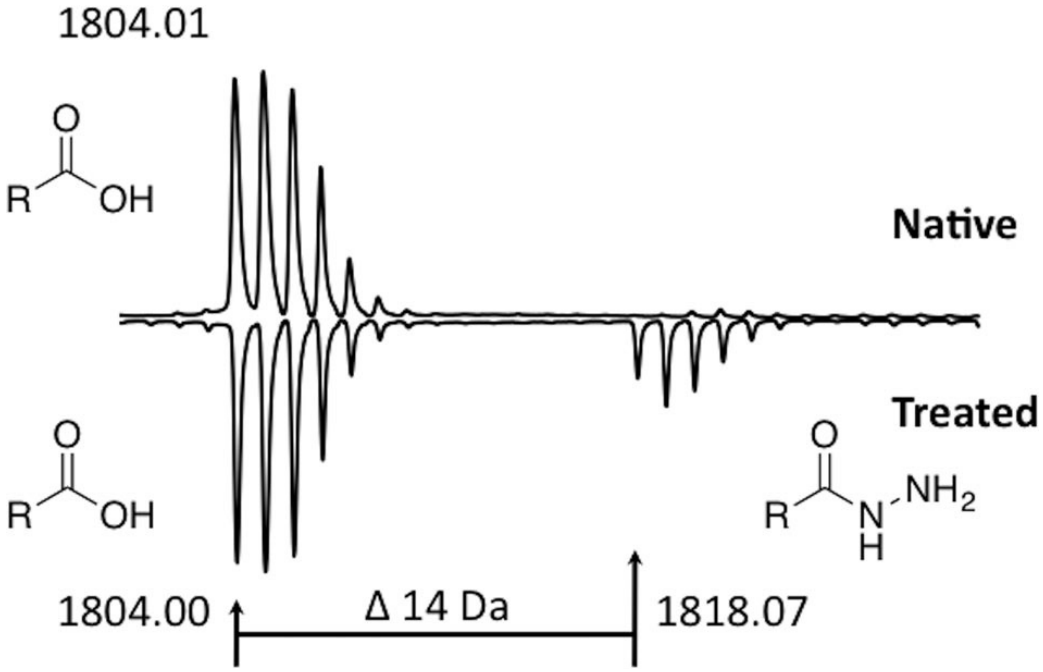
0:02 / 22:46 • Northeastern University

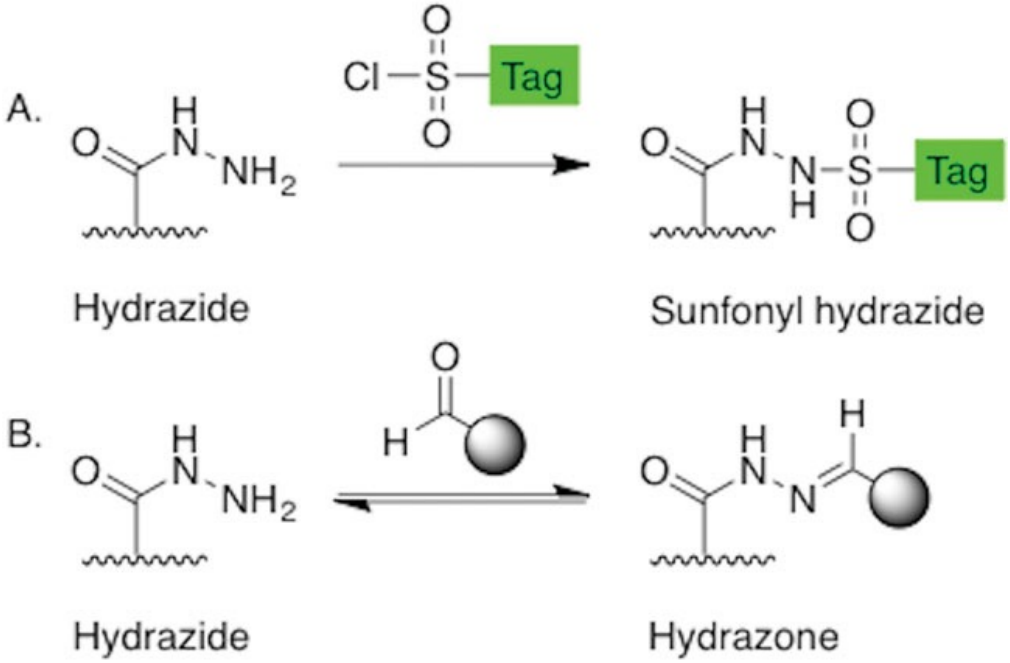
Deamidation of AAV: Sunny Zhou 2021-08-14

youtu.be/7EiRvAVrr4o

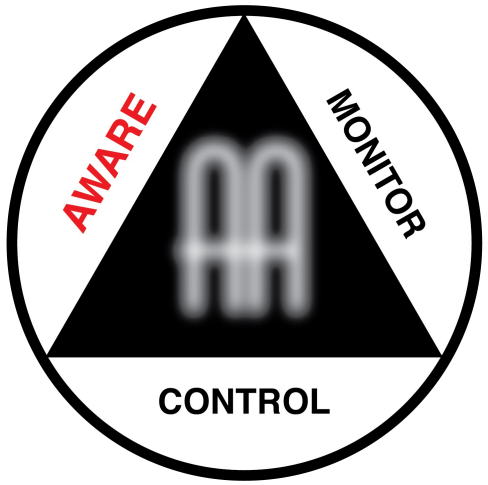
False negative: labile PTMs trap succinimide (Asu)

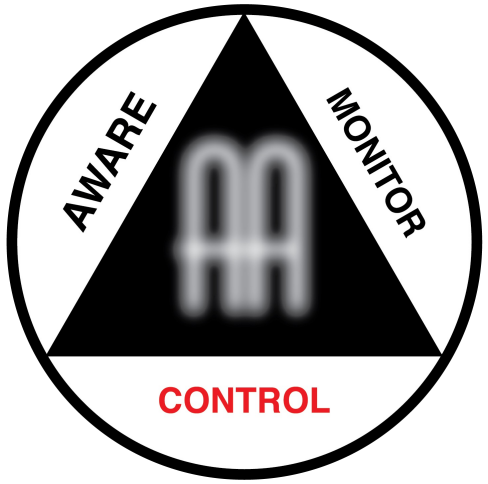






J Pharm Sci, 2014, 103, 3033, Klaene, Ni, Alfaro, Zhou

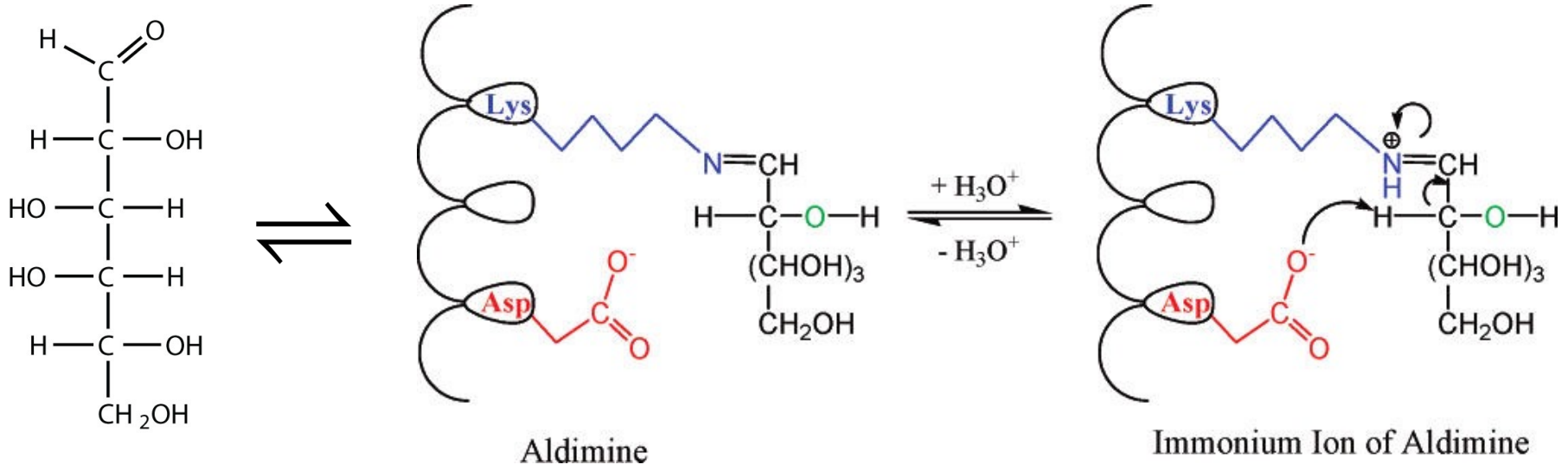




Anal. Chem. 2008, 80, 2379-2390

Unveiling a Glycation Hot Spot in a Recombinant Humanized Monoclonal Antibody

Boyan Zhang,*,† Yi Yang,† Inn Yuk,‡ Roger Pai,§ Patrick McKay,§ Charles Eigenbrot,|| Mark Dennis,⊥ Viswanatham Katta,† and Kathleen Champion Francissen†



Semi-stable modifications: false negative

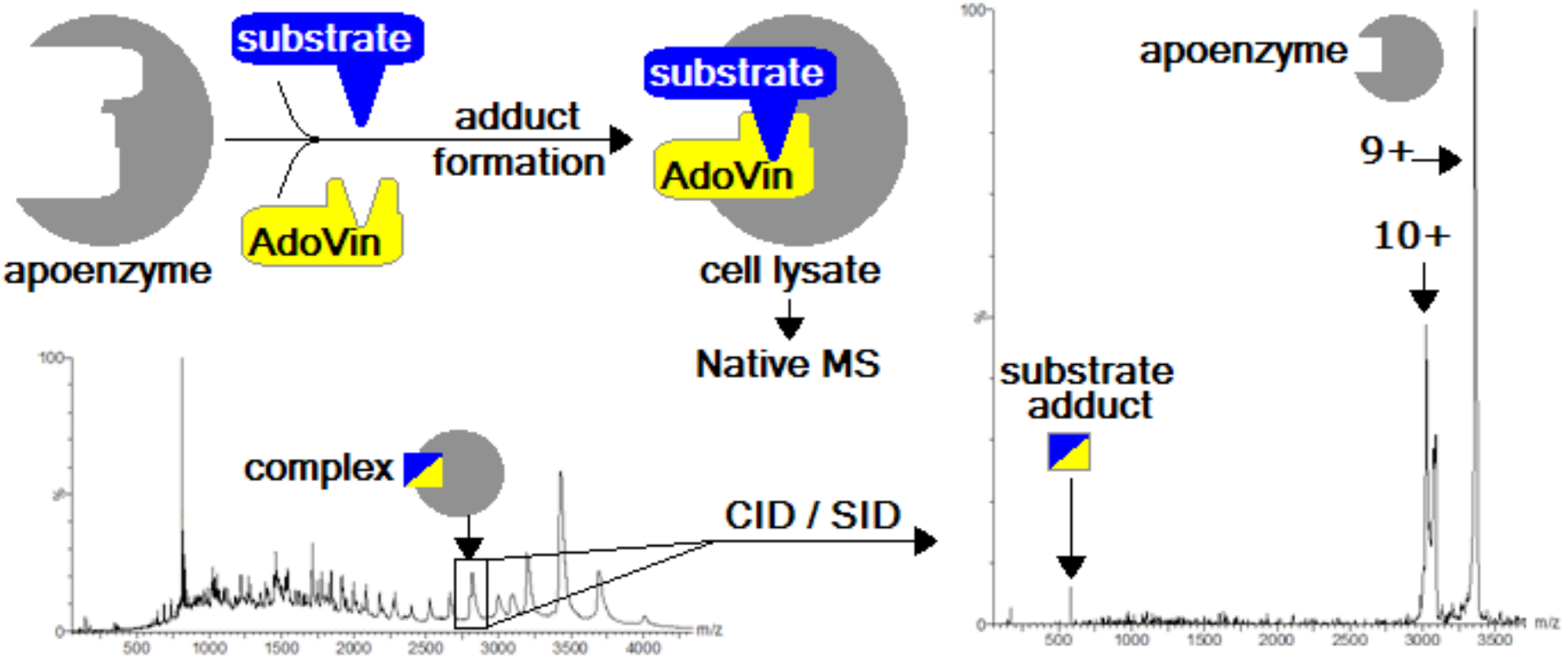
Reversible, stabilized by protein interactions

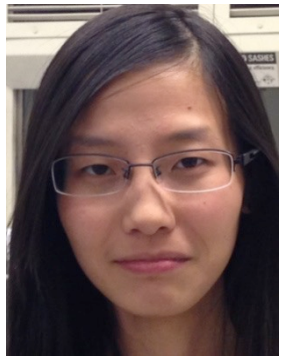
Unstable during sample preparation, e.g., trisulfide

Trapping (reduction), analyze small molecule fraction

Identify Substrate-Enzyme in Non-Covalent Complex: Native Mass Spectrometry

Kalli Catcott
Mersana
Wanlu Qu
Northeastern
Novartis



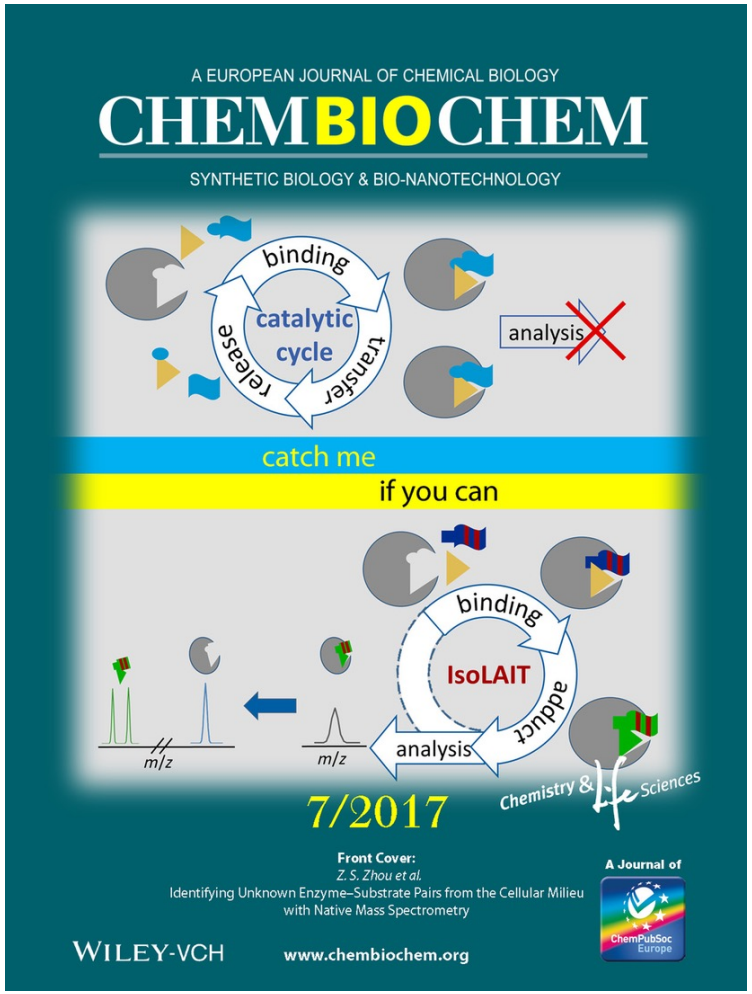


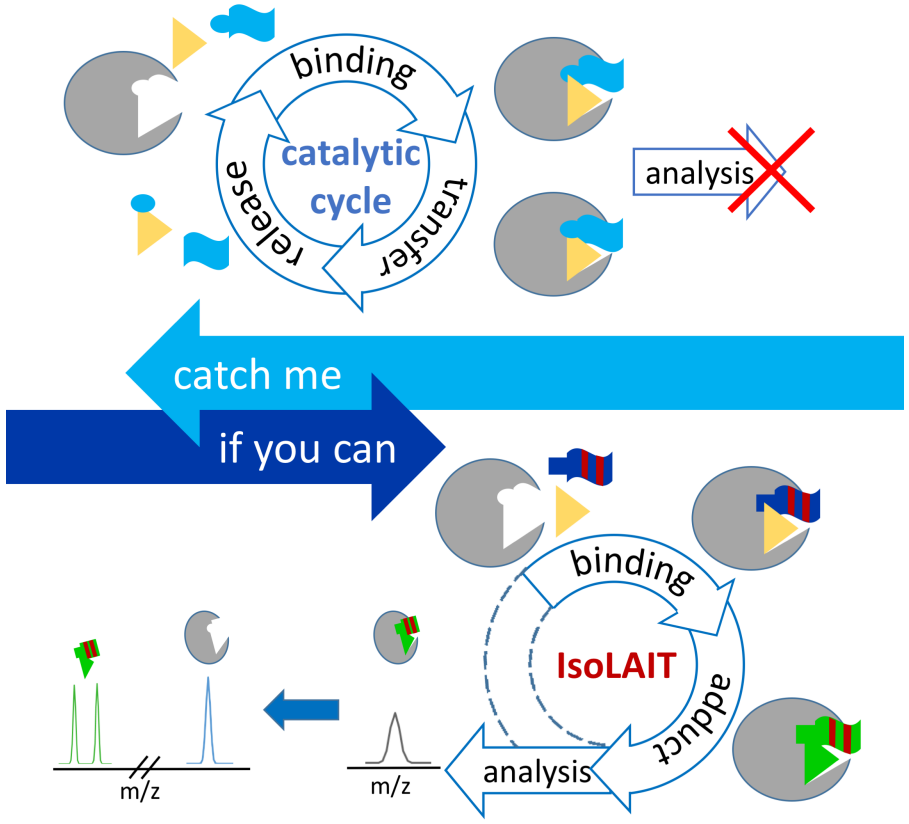
Jing Yan
Vicki Wysocki
Ohio State

specific variant-ligand Interaction

ChemBioChem. 2017, 18, 613
J Am Chem Soc. 2016, 138, 2877

Exciting Opportunities for Analytical Scientists

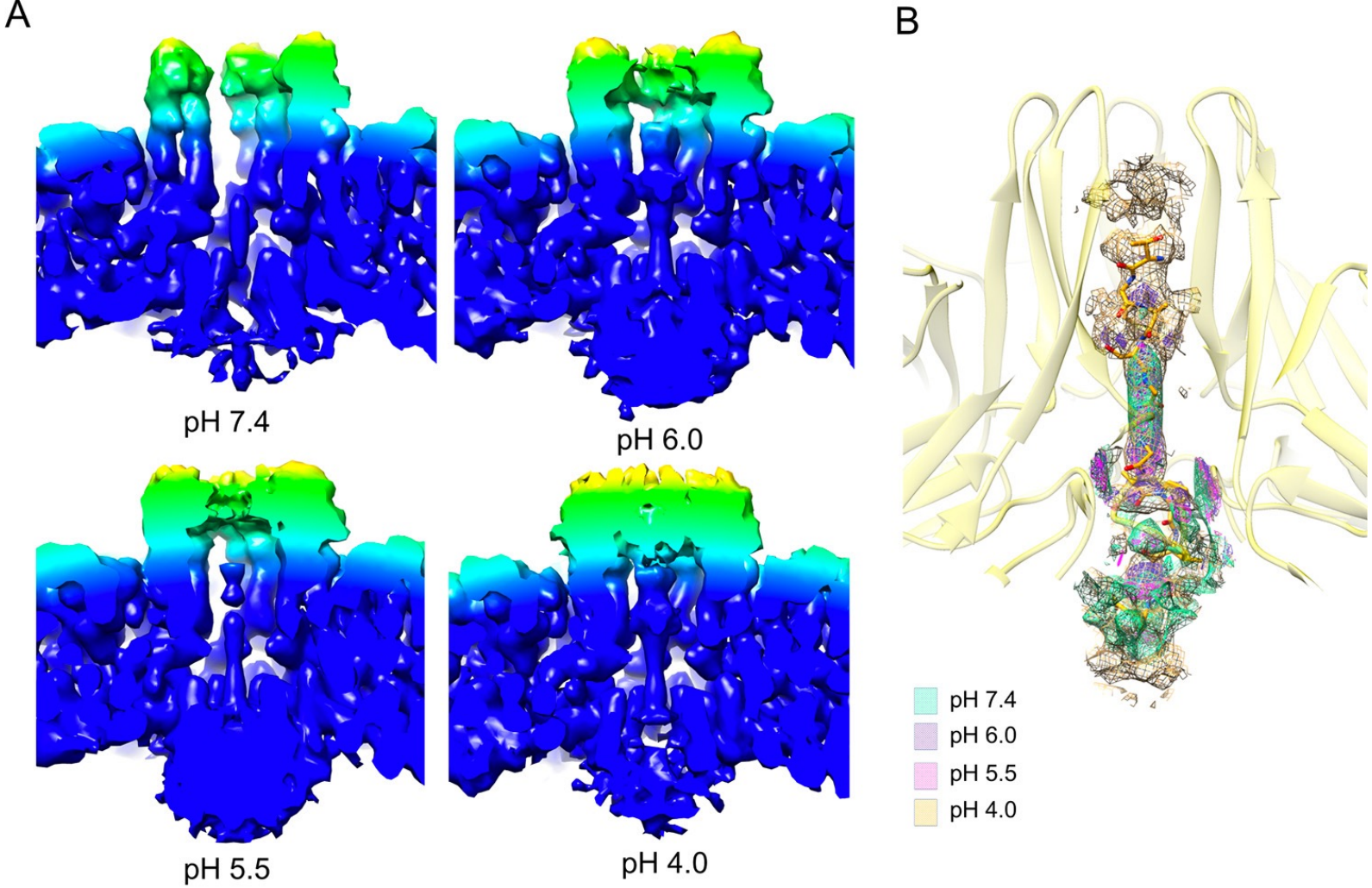


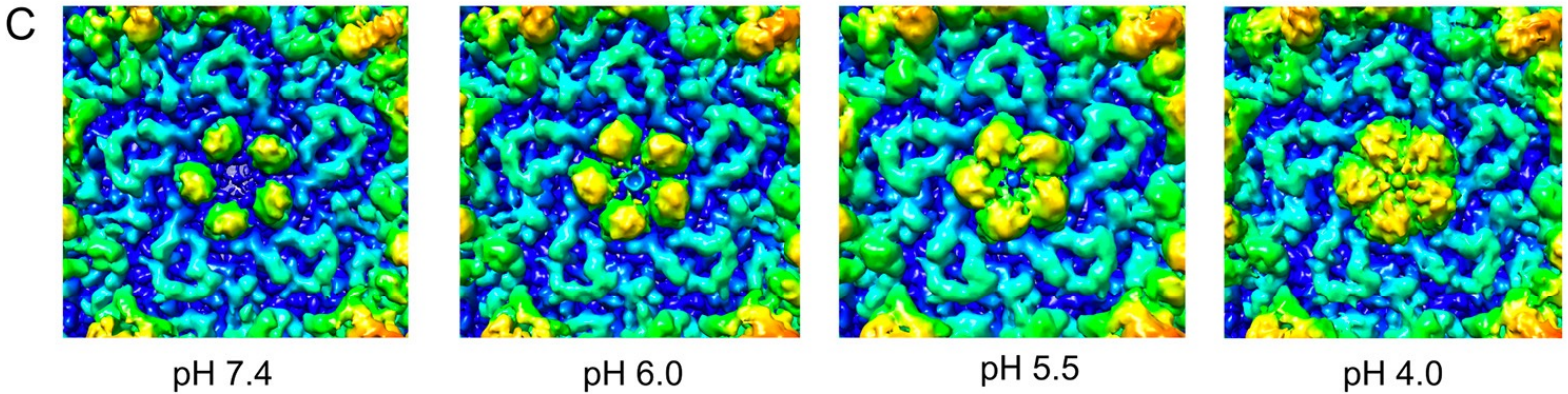


native and ion mobility mass spec: meta-stable species, non-covalent interactions

mass, structures, properties-reactivities

- Mass spectrometry
  - ionization
  - identification: isotopic labeling
- New labeling tool: glutamine (Gln) via transglutaminase (TGase)
- Knowledge: elucidation of structures: new, unknown species (PTM's)
  - reactive species: metabolites, methylglyoxal (MGO)
  - crosslinking
- Sample preparation:
  - deamidation, isoaspartic acid (isoAsp), succinimide
  - meta-stable species: chemical trapping, native mass spec
- New modalities and new opportunities: higher-order structure (HOS)
  - virus-like particles (VLPs): adeno-associated virus (AAV)





Chemo-Enzymatic Selective Labeling of Native Capsid

inside-outside

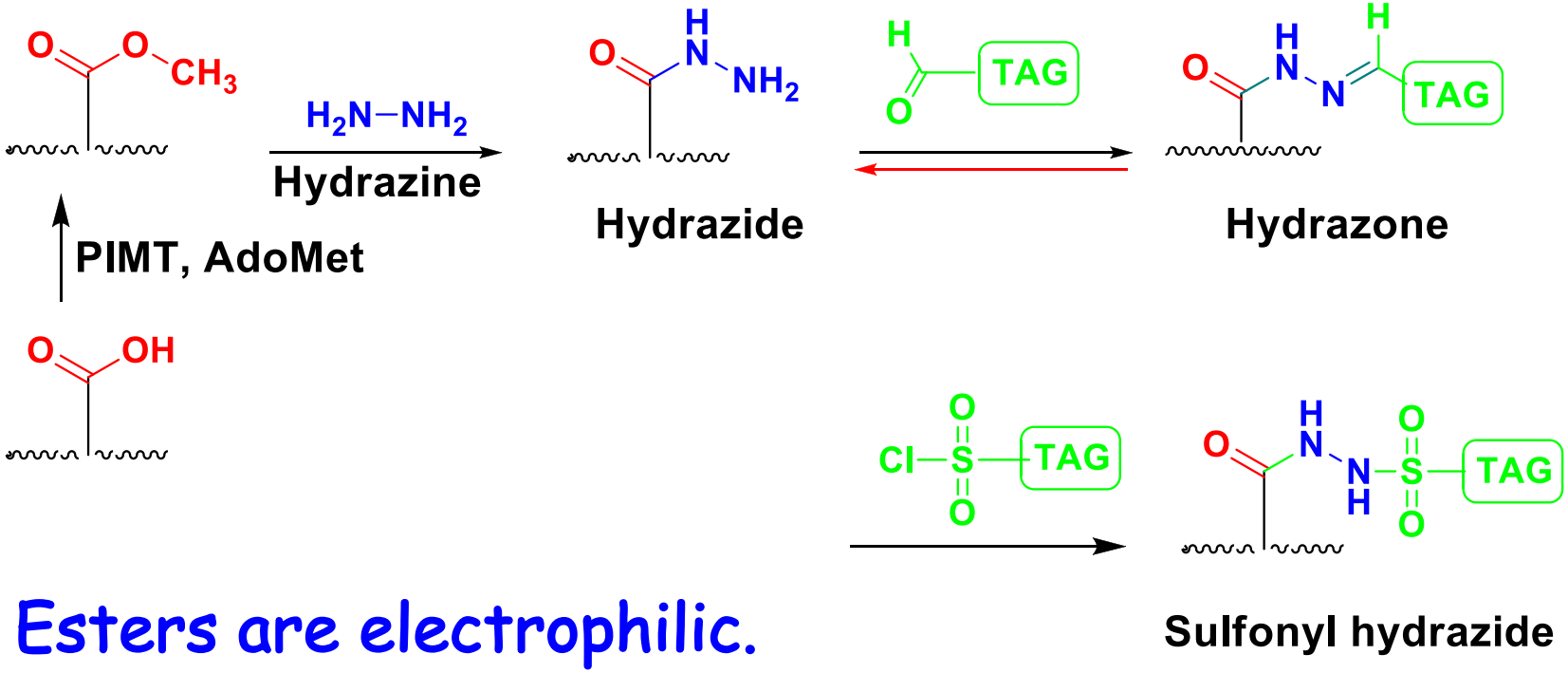
conformational change

charge variants

specific PTM

FIG 2 pH-dependent structural dynamics of the AAV9 5-fold axis.

Hydrazide Chemistry for Dummies



Esters are electrophilic.

Water is not an organic solvent!

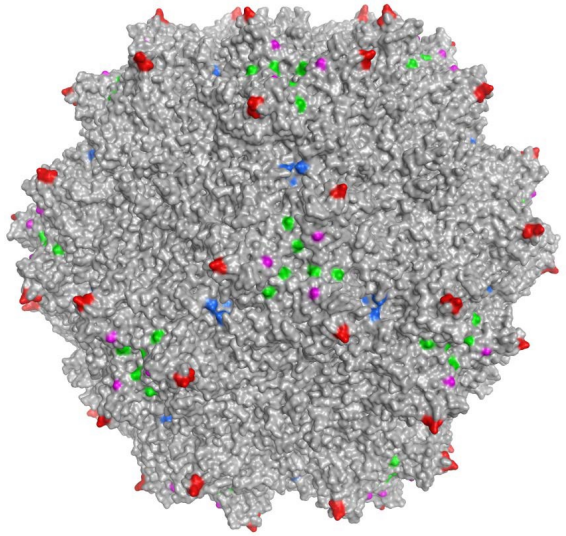
1st Ed., SunnyLand Press, 1999, Ann Arbor, Michigan.

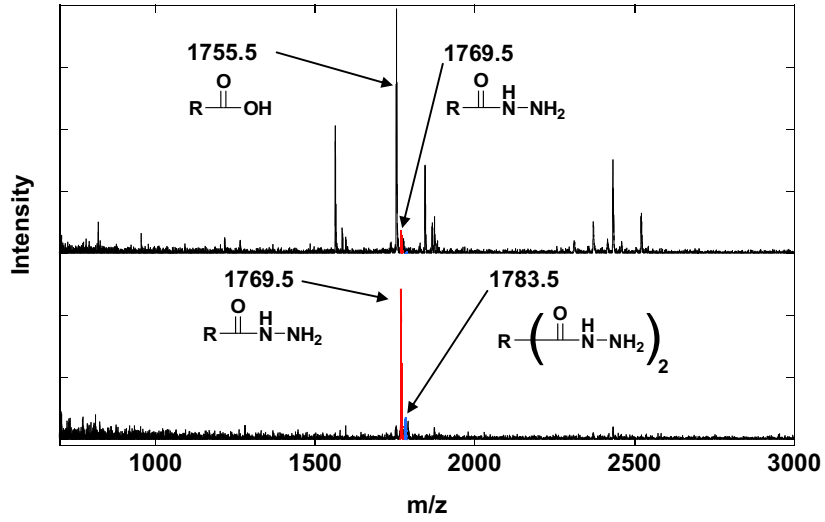
UNIVERSITY OF MICHIGAN

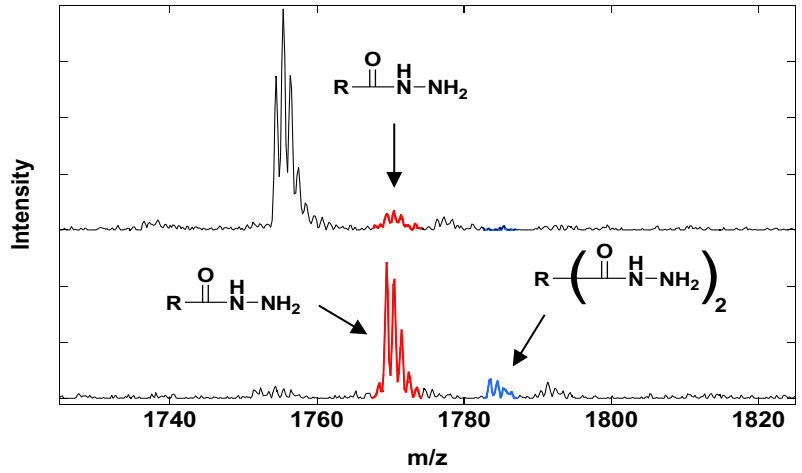
WASHINGTON STATE UNIVERSITY

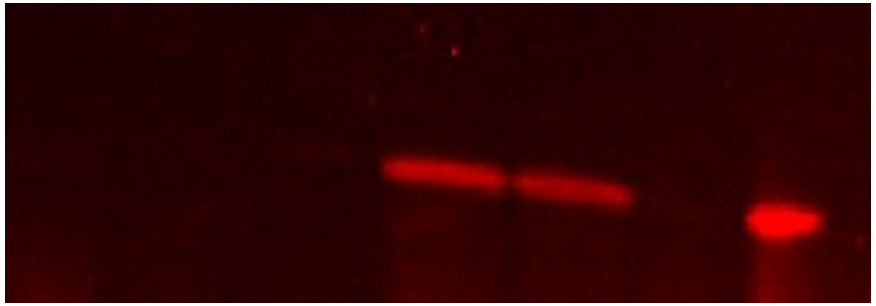
Josh Alfaro

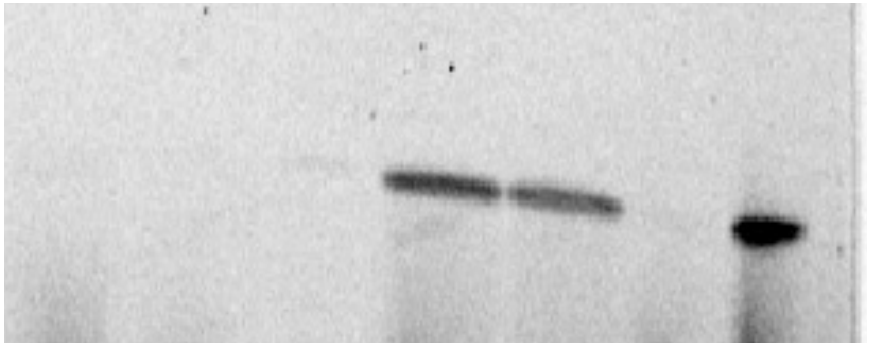
Affinity Enrichment and Labeling of Intact Proteins (Capsid?)

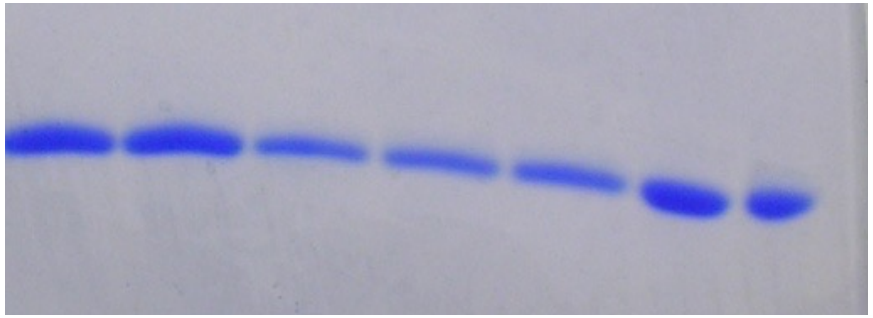












Kunal Daud
Northeastern, Sarepta

Joshua F. Alfaro, et al, Anal. Chem. 2008, 80, 3882.
J Pharm Sci, 2014, 103, 3033, Klaene, Ni, Alfaro, Zhou

industry PhD

Northeastern

- Maintain full-time employee status at the company
- Projects: a range of possibilities
- Intellectual properties (IP): industry controlled
- Publications: faculty are experienced
- Co-advisors: academic and industrial
- Duration: 3 to 5 years

- AbbVie, Alnylam, Amgen, Biogen, Charles River, Dragonfly, GSK, Genzyme, GreenLight, Novartis, Pace, Pfizer, Sarepta, Takeda, ThermoFisher, etc
- Sunny Zhou: z.zhou@northeastern.edu

Da Ren
Director at Amgen

Chris Chumsae
Formerly at AbbVie
Now Director at BMS

mass, structures, properties-reactivities

- Mass spectrometry
  - ionization
  - identification: isotopic labeling
- New labeling tool: glutamine (Gln) via transglutaminase (TGase)
- Knowledge: elucidation of structures: new, unknown species (PTM's)
  - reactive species: metabolites, methylglyoxal (MGO)
  - crosslinking
- Sample preparation:
  - deamidation, isoaspartic acid (isoAsp), succinimide
  - meta-stable species: chemical trapping, native mass spec
- New modalities and new opportunities: higher-order structure (HOS)
  - virus-like particles (VLPs): adeno-associated virus (AAV)

Artifact Anonymous (AA)

Communication:

reviews, white pages and guidelines

Collaboration:

academia

industry (biopharma, instruments)

regulatory agencies: FDA, NIST

Control:

standardization and optimization

unknowns: prediction, first principle

If you would like to participate and contribute, please contact Sunny Zhou at z.zhou@northeastern.edu. Thank you!

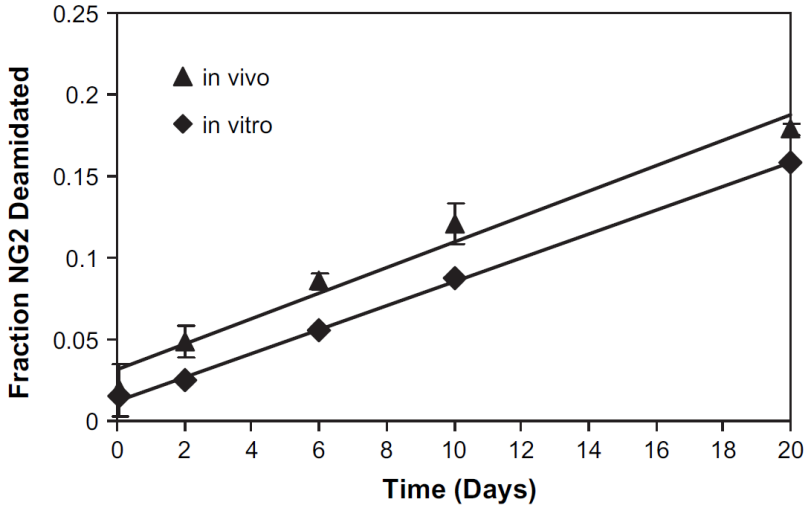
extra slides

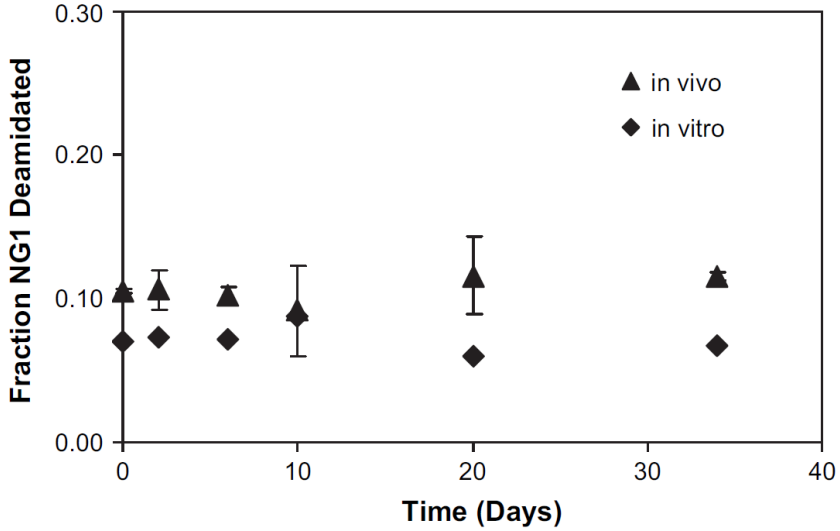
Stone Shi
Amgen
AskGene

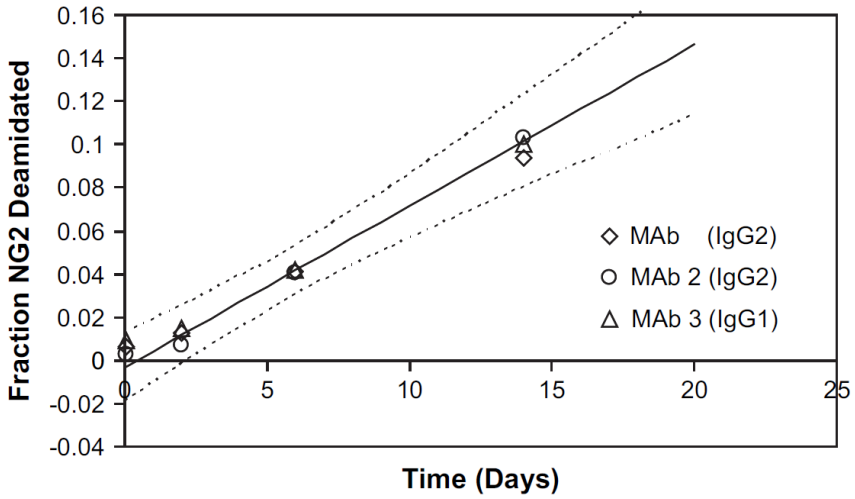
Human antibody Fc deamidation in vivo

Y. Diana Liu [a], Jian Zhang van Enk [b], Gregory C. Flynn [a,*]

Biologicals, 2009, 37, 313.







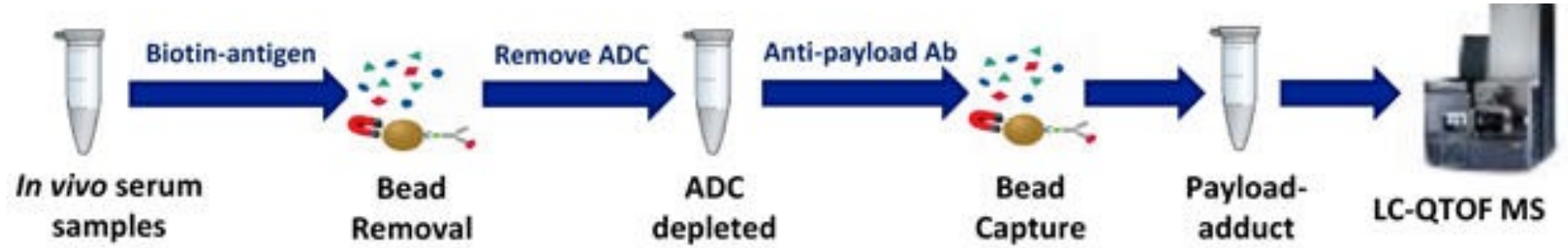
- Biotransformation: in vivo
- Antibodies: long-lasting, in vivo half-lives 2-3 weeks
- Endogenous IgG from human serum: 23% deamidation
- Formulation: ~ pH 5; Serum: pH 7.4 (biotransformation, in vivo)
- Other PTMs: glycation, C-term lysine, Pyro-Glu, disulfide, covalent dimer

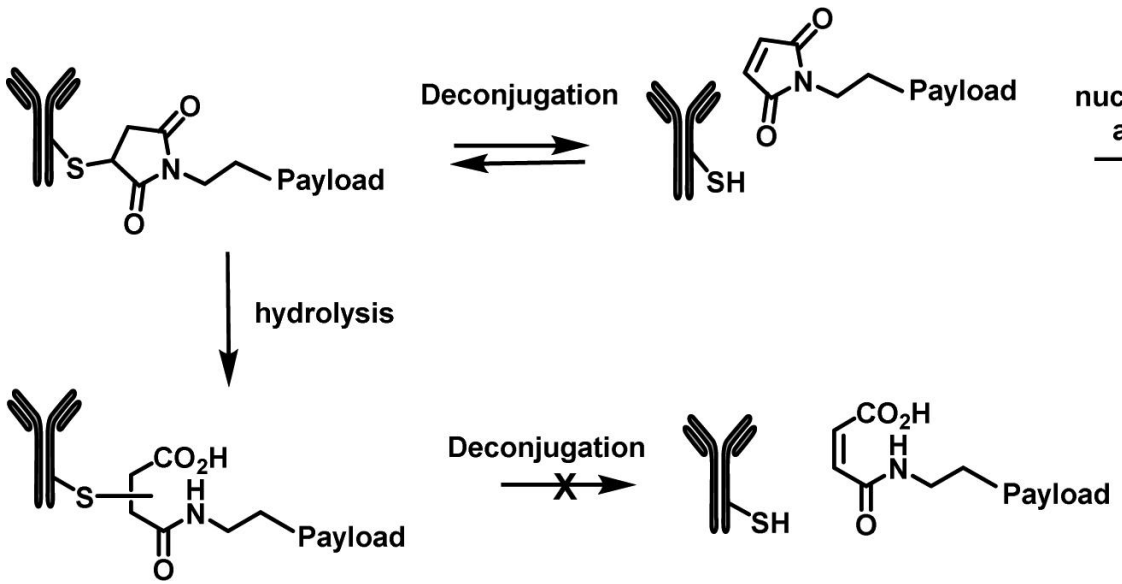
Stability of IgG in serum. mAbs, 2010, 2, 1; Ivan R. Correia, et al. AbbVie

Where Did the Linker-Payload Go? A Quantitative Investigation on the Destination of the Released Linker-Payload from an Antibody-Drug Conjugate with a Maleimide Linker in Plasma

Cong Wei,[*,†] Guodong Zhang,[†,§] Tracey Clark,[†] Frank Barletta,[†] L. Nathan Tumey,[‡] Brian Rago,[†] Steven Hansel,[†] and Xiaogang Han[*,†]

[†]Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc., Eastern Point Road, Groton, Connecticut 06340, United States





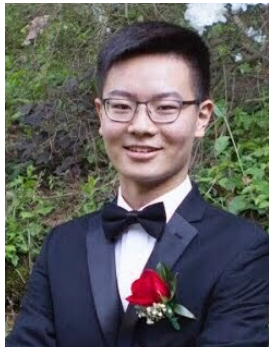
serum albumin

sample preparation
separation
analysis: mass spec
chemistry: first principle

Kinetics: Half-Lives and Progression

Percentage Occurred

	Half-Life (days)								
Time (hours)	1	2	5	10	20	50	100	200	365
0.1	0.29	0.14	0.06	0.03	0.01	0.01	0.00	0.00	0.00
0.2	0.58	0.29	0.12	0.06	0.03	0.01	0.01	0.00	0.00
0.5	1.43	0.72	0.29	0.14	0.07	0.03	0.01	0.01	0.00
1	2.85	1.43	0.58	0.29	0.14	0.06	0.03	0.01	0.01
2	5.61	2.85	1.15	0.58	0.29	0.12	0.06	0.03	0.02
5	13.45	6.97	2.85	1.43	0.72	0.29	0.14	0.07	0.04
10	25.08	13.45	5.61	2.85	1.43	0.58	0.29	0.14	0.08
24	50.00	29.29	12.94	6.70	3.41	1.38	0.69	0.35	0.19



Eddie Zhou

Asn-Gly > Asn-Ser, Asn-His >> Asn-Pro

(NG > NS, NH > NP)

not good (1 d), not sure, not happy (10 d), no problem

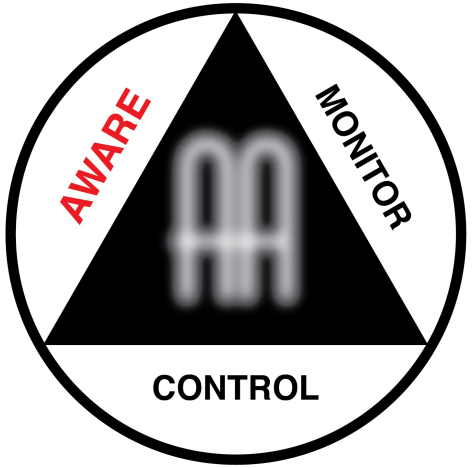
heat sample at 60 ºC for 30 min
50 mM ammonium bicarbonate, pH 8.2
5 mM TCEP
5% of cysteine to alanine

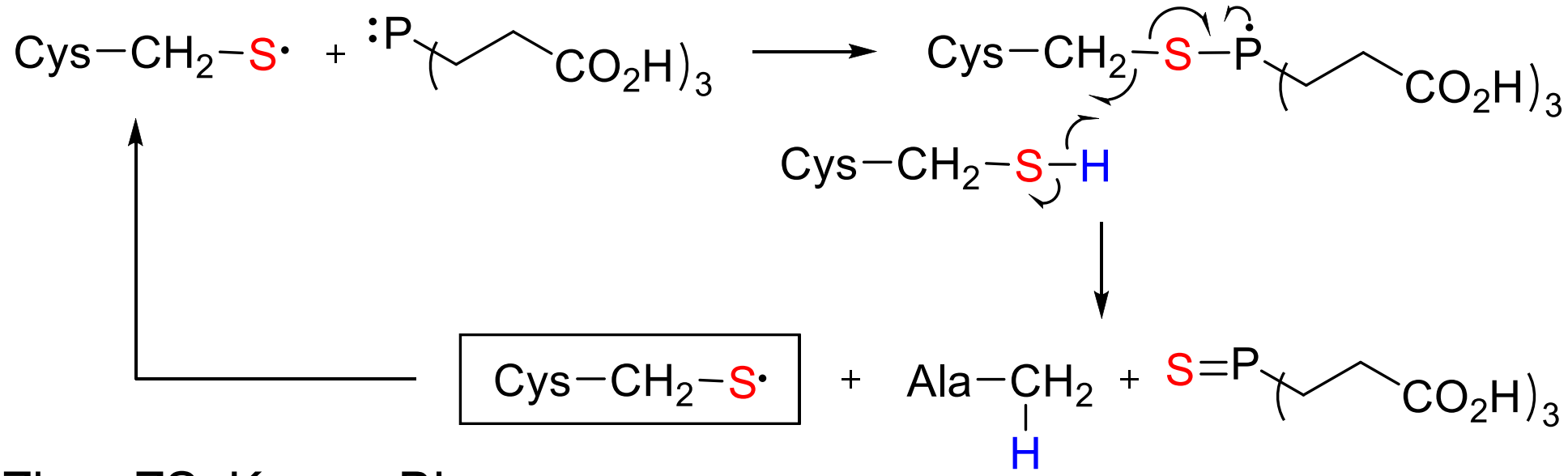
Radical Mechanism for Desulfurization Sample Prep

genetic mutation?

change 2 bases: highly unlikely

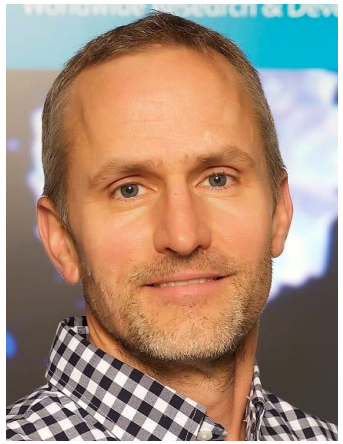
Cys/C	UGU, UGC
Ala/A	GCU, GCC, GCA, GCG





Wang Z, Rejtar T, Zhou ZS, Karger BL.
Rapid Commun Mass Spectrom. 2010, 24, 267.

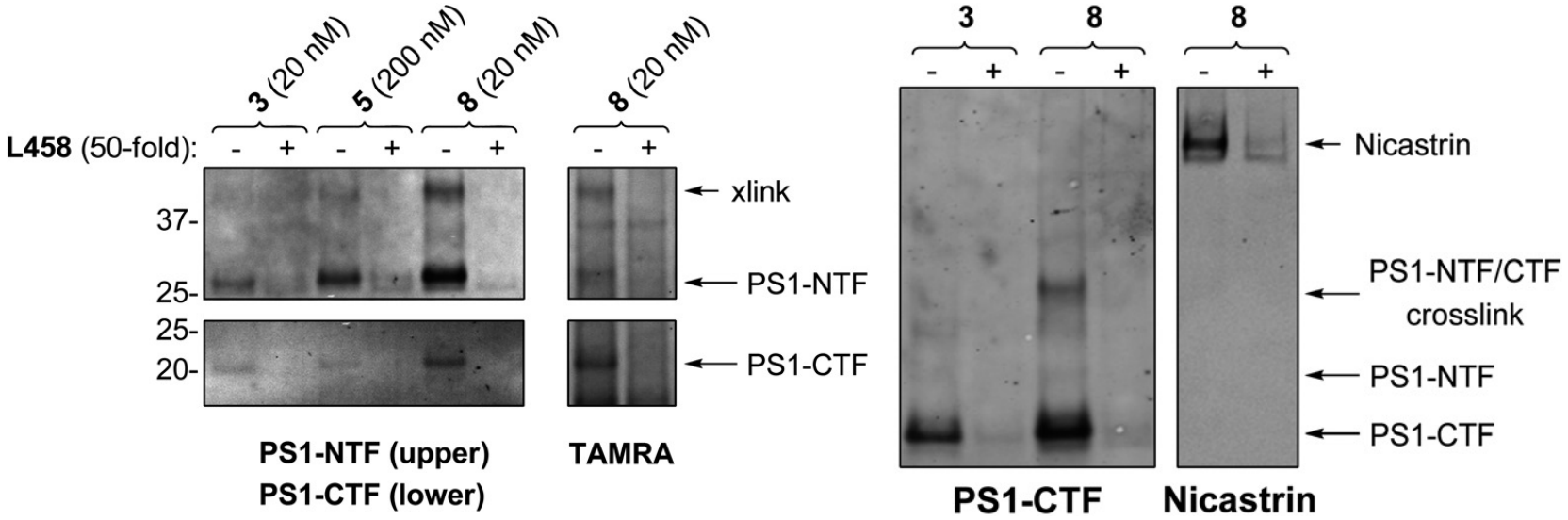
Challenges in Detecting Crosslinked Adducts by Mass Spectrometry



Doug Johnson
Biogen, Pfizer

Investigating γ-secretase protein interactions in live cells using active site-directed clickable dual-photoaffinity probes†

T. Eric Ballard,‡*[ab] Heather E. Murrey,[a] Kieran F. Geoghegan,[c] Christopher W. am Ende[b] and Douglas S. Johnson*[a]



Ballard, Doug Johnson, et al at Pfizer. Med. Chem. Commun., 2014, 5, 321

Problems:
crosslink and site-of-labeling cannot be confirmed w/ mass spec data.

isotopic labeling