Biologics Biotransformation by LC-MS in the Discovery Space: Methods and Case Study

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Outline

- Biotransformation overview and LC-MS platforms
- Deamidation w/ Case Studies
- Oxidation w/ Case Study
- Sialic Acid w/ Case Study
- Clipping w/ Fc Fusion Case Study

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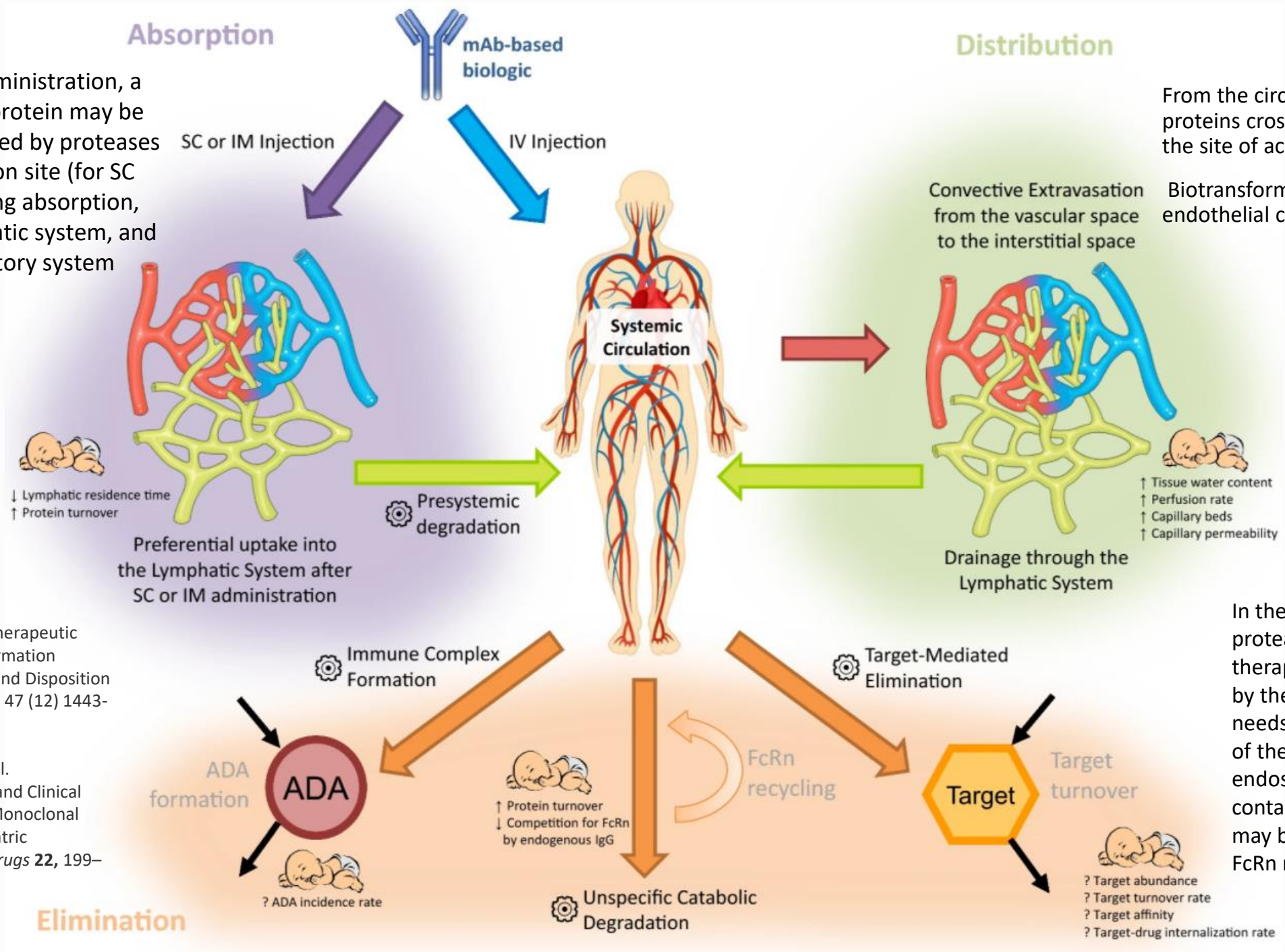
Biotransformation Definition:

Alteration of a drug molecule *in vivo.* For biologics it mainly includes:

1. Amide and glycosidic bond hydrolysis: clipping of amino acid sequence or glycan. Or any other clipping event.
2. Amino acid modification: deamidation, oxidation, isomerization, sulfation,
3. Disulfide bond reduction or shuffling (IgG2)

4. ADC biotransformation is mostly focused on linker cleavage and payload metabolism (not the subject of this ppt).

Following administration, a therapeutic protein may be biotransformed by proteases at the injection site (for SC dosing), during absorption, in the lymphatic system, and in the circulatory system



From the circulation, therapeutic proteins cross the vascular wall to reach the site of action in the target tissue.

Biotransformation might also occur by endothelial cells of different organs.

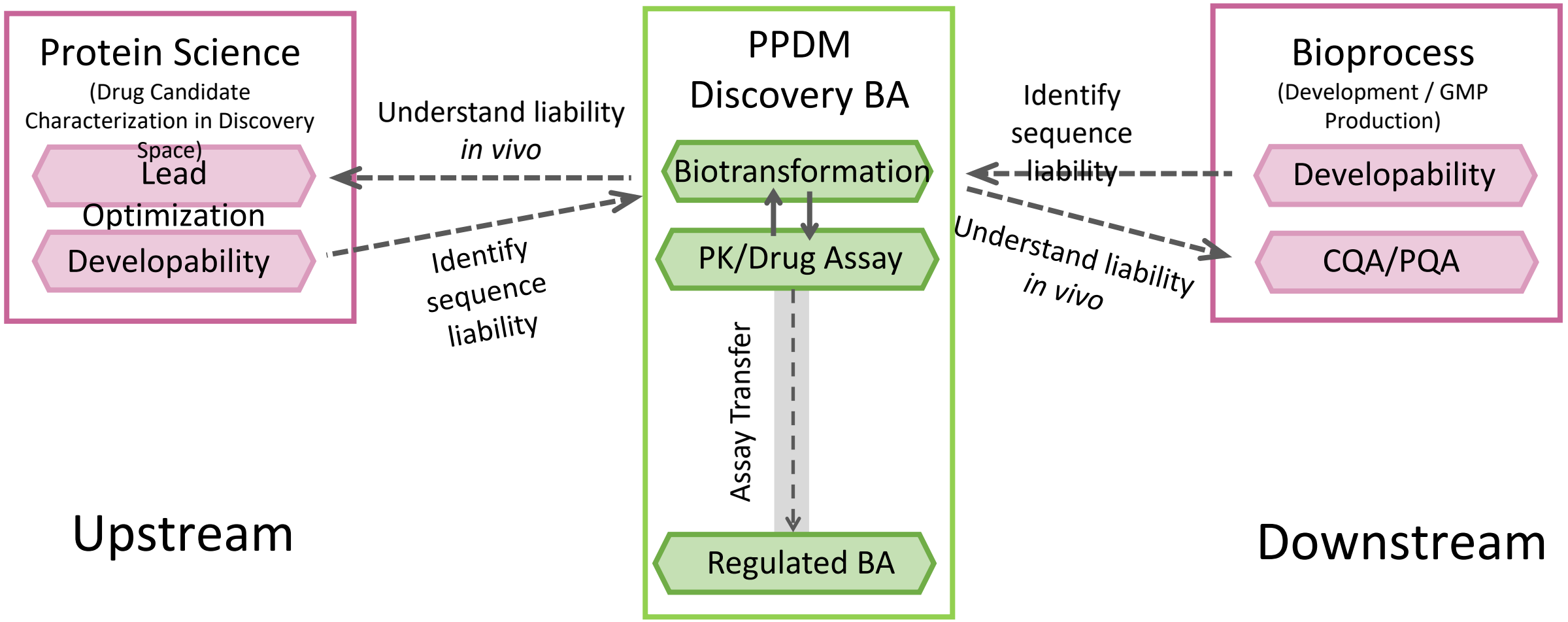
The most likely location for proteases contributing to the formation of circulating biotransformation products is the extracellular space, and the proteases are either soluble (e.g., in blood and lymph) or membrane-associated.

In the case of intracellular proteases, the protein therapeutic needs to be taken up by the cell, and the product needs to be transferred back out of the cell. From early endosomes, biotransformed Fc-containing protein therapeutics may be released into blood by FcRn recycling.

Schadt, S., et. al. Therapeutic Protein Biotransformation Drug Metabolism and Disposition December 1, 2019, 47 (12) 1443-1456;

Temrikar, Z.H., et.al. Pharmacokinetics and Clinical Pharmacology of Monoclonal Antibodies in Pediatric Patients. *Pediatr Drugs* **22,** 199–216 (2020).

How Biotransformation Connects To Other Activities?



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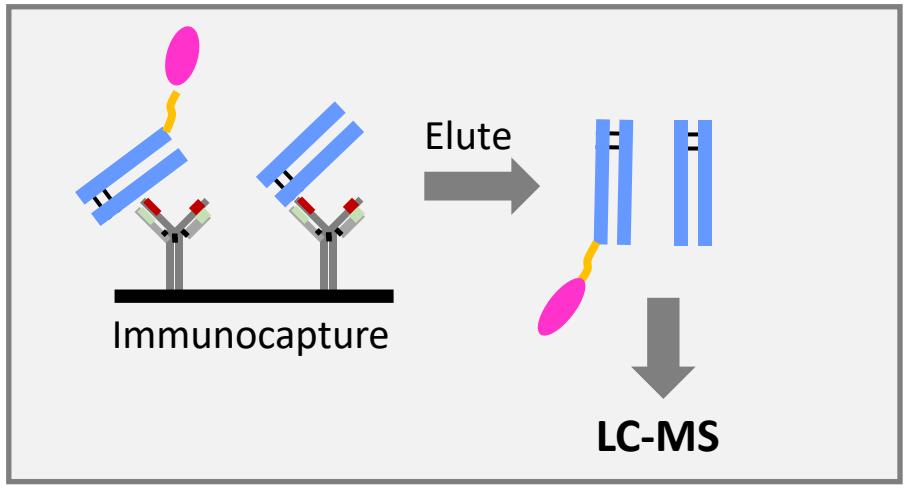
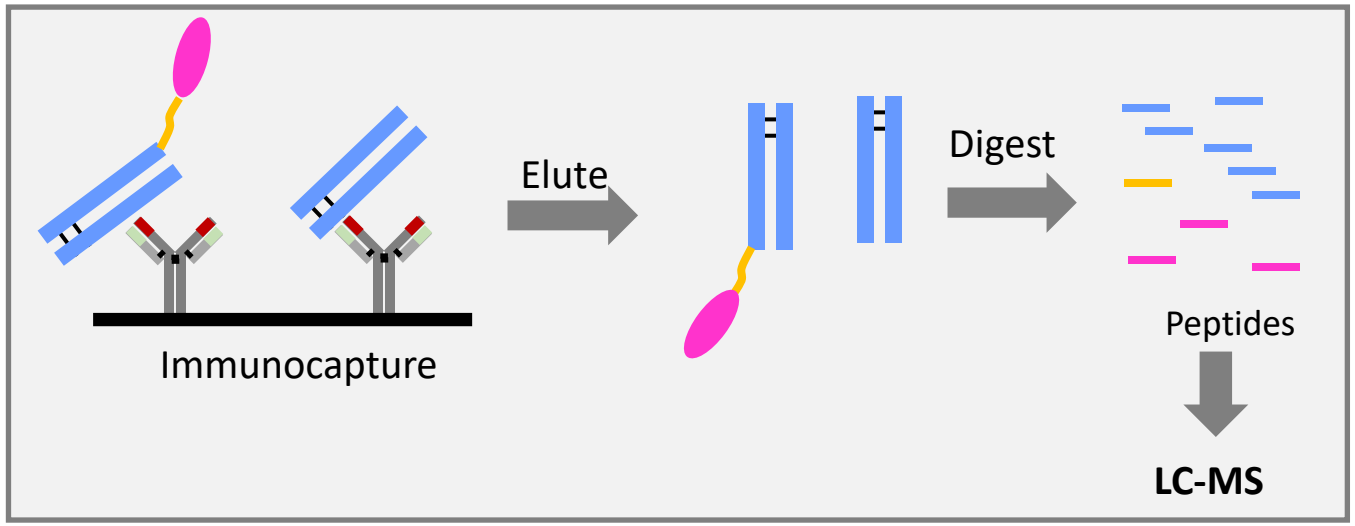
LC-MS Assay Formats

Intact LC-MS



Peptide Based LC-MS



Assay Name	Application			Platform	Throughput	Typical LLOQ (ug/mL)	Note
	Clipping	Small PTM (deamidation)	Large PTM (oxidation etc)				
Intact Mass	✓		Maybe (MS resolution Subunit/Reduced vs Intact)	LC-MS (TOF)	15min/sample	0.5-5	Semi-quantitative Subject to heterogeneity
Peptide based PRM Quant	✓	✓	✓	LC-MS PRM (QE+)	1 hr/sample	0.02-0.2	Subject to digestion issue

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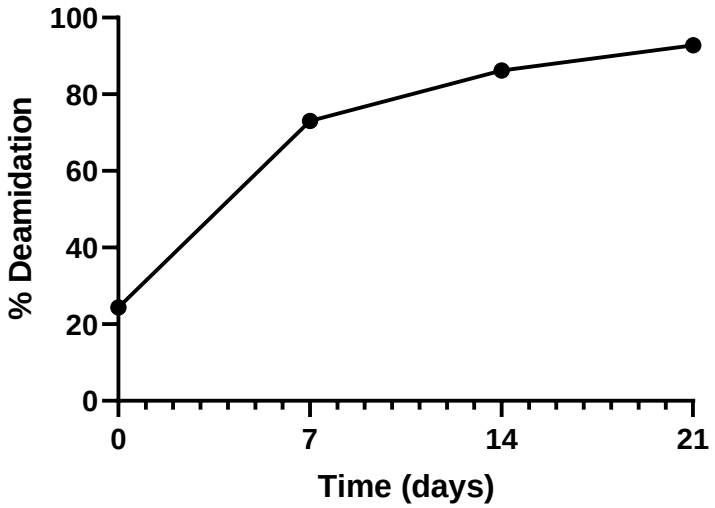
Deamidation Case Study: Monoclonal Antibody A1

Potency and N33 deamidation of Antibody A1 by target binding assessment and LC-MS in Bioprocess

Mab Isotype	Temperature	Potency 1 month (%)	N33 deamidation 1 month (%)	Potency 3 months (%)	N33 deamidation 3 month (%)
IgG2	5°C	108	33.6	96	30.1
	25°C	101	49.2	28	62.6
	40°C	**20**	**76.9**	**5**	**92.6**
	-80°C	173	32.6	101	N/A

Antibody A1
N33 Deamidation in NHP serum



Monoclonal Antibody A1

Developability Study:

- N33 deamidation was identified in Bioprocess. N33 deamidation levels increased from 30% to 93 % in stress studies at 40 °C for 3 month.

Impact on Function

- Deamidation causes loss of potency.

De-risking: In Vitro Stability in NHP

- N33 deamidation increased to ~90% after incubation in NHP serum for 21 days.

Follow Up:

- N33 was subsequently substituted to S and/or T

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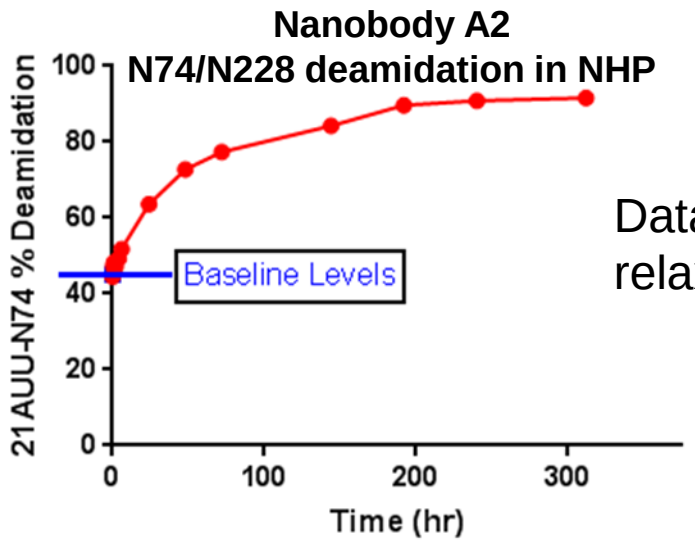
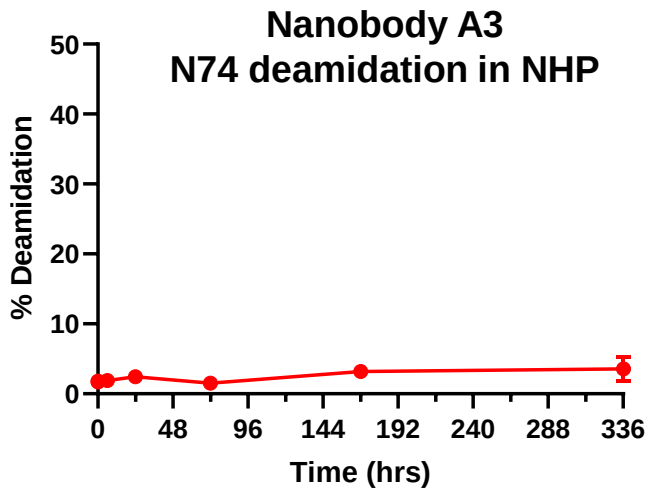
Deamidation Case Study: Nanobody A2 and A3

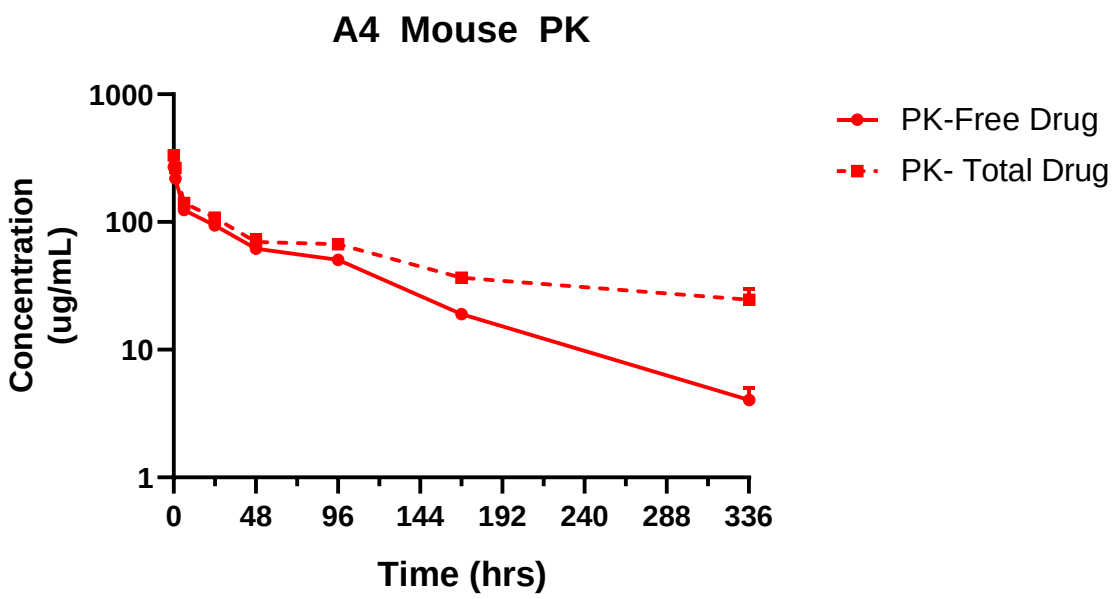
Nanobody A2	Nanobody A3
Developability Study:	**Developability Study:**
• N74/N228 deamidation levels increased from 38% to 78% in stress studies with high pH for 7 days	• N74 deamidation levels increased from 0% to 35% in stress studies with high pH for 7 days.
Impact on Function:	**Impact on Function:**
• **NO** decrease in binding or function	• 3X decrease in binding
De-risking: In vivo Study in NHP	**De-risking: In vivo Study in NHP**
• N47/N228 deamidation level **increased** from 42% to ~90% over 14 days.	• N74 deamidation level **unchanged** over 14 days.
Follow Up:	**Follow Up:**
• Construct moved on	• Construct moved on

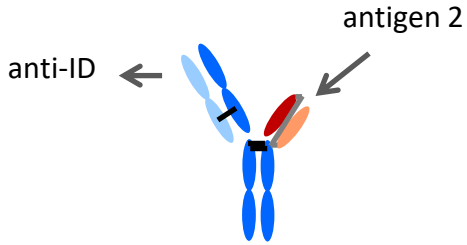
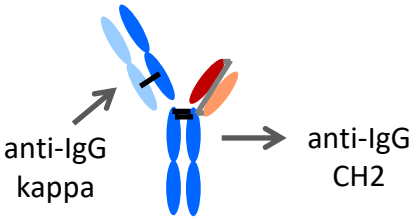


Data supports relaxation of PQA



Deamidation Case Study: Bi-specific Antibody A4



Name	Free Drug Assay	Total Drug Assay
		
Format	MSD	MSD
Capture	Antigen 2	Anti-IgG Kappa
Detection	Anti-idiotype	Anti-IgG CH2
Note	Measure intact / active drug	

Developability Data from Protein Science

Lot	Modification	Location	High pH 10 RT	low pH 3.5 (Acetic Acid) RT	50C	4C
A4	N197 Deamidation	VH-CDR2	19.02	6.04	7.76	5.87
(BIACore Binding to antigen 2)			51%	76%	72%	100%

- Deamidation impacts A4's binding to the antigen 2 and would have reduced readout in Free Drug assay.
- What causes the difference between Free and Total concentration? Deamidation or clipping?

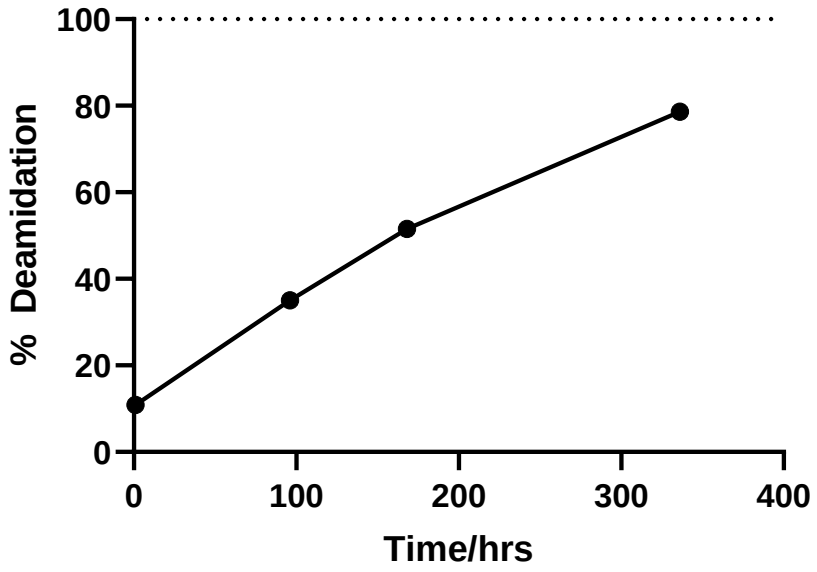
Deamidation Case Study: Bi-specific Antibody A4

PK and Biotransformation of A4 in mouse

Time	LC-MS Conc. (ug/mL)				LBA Conc. (ug/mL)		N197 Deamidation			
	scFv	Shared.Fc	HC	LC	Total	Free	%D	%IsoD	%N	Total * %N (ug/mL)
1	151.5	164.8	170.0	167.3	255	199	3.1%	7.8%	89.1%	226.90
96	54.6	52.5	69.6	65.6	69.0	53.9	9.9%	25.1%	65.0%	44.86
168	50.6	51.6	56.0	59.0	39.9	20.6	12.8%	38.7%	48.5%	19.38
336	38.9	39.3	45.7	50.3	30.5	5.06	16.9%	61.7%	21.4%	6.55

- Drug concentration calculated from peptides of the 4 regions were comparable, indicating no major clipping of the construct.
- Difference between Total and Free assay is most likely to be caused by deamidation in the CDR region
- This deamidation site was later corrected with mutation.

N197 Deamidation of A4 in vivo



Sequence Liabilities: Deamidation Summary

Table 1. Deamidation liability data from stress studies and follow up *in vivo/in vitro* investigation.

Molecule	Sequence Liability	Stress Condition	PTM level in buffer	PTM level in vivo	Other Data/Comments
Monoclonal antibody A1	Deamidation of N33	40 °C	30% (baseline) 77% (1 month) 93% (3 months)	**increase from 25% to 90% at day 21 in ex vivo NHP serum.**	Deamidation led to loss of potency. N33 was subsequently substituted to S and/or T
Nanobody A2	N74 and N228 Deamidation of Framework	High pH	38% (baseline) 98% (high pH)	**increase from 45% to 91% by day 14 in NHP**	High pH stressed samples retained binding and function. However, high baseline deamidation levels was a CMC issues due to the high product heterogeneity.
Nanobody A3	N74 Deamidation of Framework	High pH	35% (high pH)	Deamidation levels mostly unchanged in-vivo (NHP)	
Bi-specific antibody A4	N197 Deamidation VH-CDR2	High pH	6% (baseline) ~20% (high pH)	**Increase from 10% to 90% by day 14 in mouse.**	Deamidation causes the loss of binding to the target. It impacts both the molecule's efficacy/potency and its read out in the free PK assay. Deamidation was later corrected.

Conclusion:

- Out of 4 molecules with high deamidation liabilities in forced degradation studies, 3 showed high deamidation rate in-vivo/in vitro

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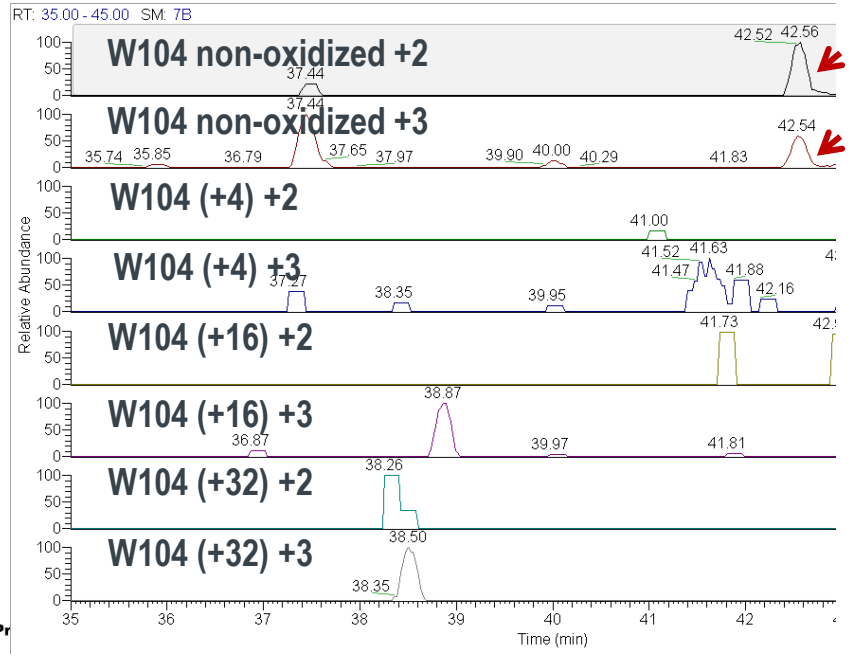
Oxidation Case Study: Monoclonal Antibody B1

Developability data from Bioprocess and Protein Science

STRESS STUDIES Antibody B2	Development Batch ~8 mg/mL				Discovery Batch 7.5 mg/mL			Discovery Batch 1 mg/mL		
	stable pool	Dark control	1X light	2X light	control	1X light	2X light	control	1X light	2X light
	26AJW	27AJW	28AJW	29AJW	36AJW	37AJW	38AJW	39AJW	40AJW	41AJW
W104 Oxidation	**0.3**	**0.7**	**30.2**	**59.7**	**0.1**	**17.9**	**32.9**	**0.2**	**16.9**	**27.7**

Oxidation of W104 in NHP, 3 mpk dose

NHP 1877: Day 8 EIC



- Stressed samples loses binding and function.
- No W104 oxidation detected at all time points in all animals. Time points analyzed were 2 hrs, 6 hrs, 24 hrs, 96 hrs (predose), 98 hrs, and 192 hrs
- Due to project stage, liability information was communicated to Bioprocess and team decided to move molecule forward. Later, a mutation was identified that retained binding (W104F); however, the molecule presented poor/unacceptable colloidal properties.

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Sequence Liabilities: Oxidation

Table 2. Oxidation liability data from stress studies and follow up *in vivo* investigation.

Molecule	Sequence Liability	Stress Condition	PTM level in buffer	PTM level in vivo	Other Data/Comments
Monoclonal Antibody B1	W104 oxidation in the CDR3	Light Stress	0% to ~18-30% (Light stress 1X) 0% to ~30-60% (Light stress 2X)	oxidation undetectable in-vivo (NHP)	**Stressed samples lost binding and function**.
Monoclonal Antibody B2	W50 Oxidation in HC CDR2 W90 oxidation in LC CDR3	AAPH and Light stress	**W50:** 1% to 15% (AAPH); 2% to 10% (Light) **W90:** 2% to 95% (AAPH); 2% to 20% (Light)	No oxidation observed in vivo (mouse)	CDR Ws are generally involved in binding and are difficult to mutate with effect on binding and function. All mutations tested were not acceptable for this molecule.

Conclusion:

- Out of 2 molecules with oxidation liabilities in forced degradation studies, **NEITHER** showed oxidation in-vivo

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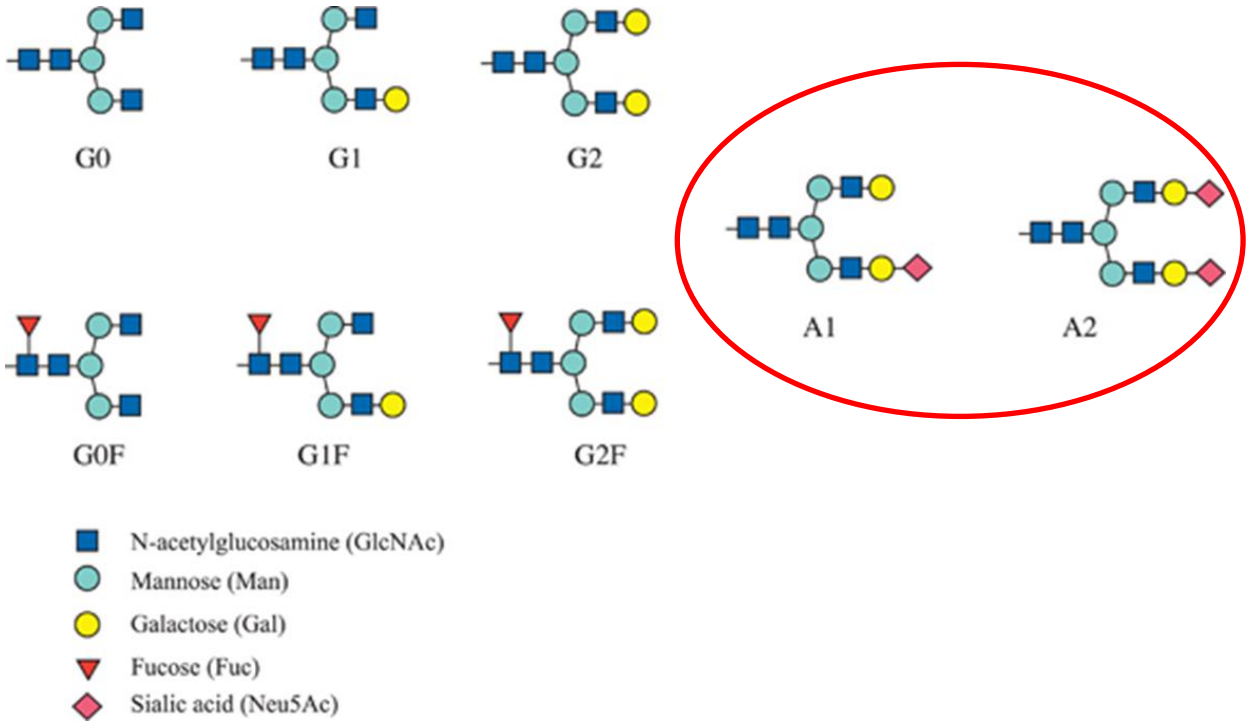
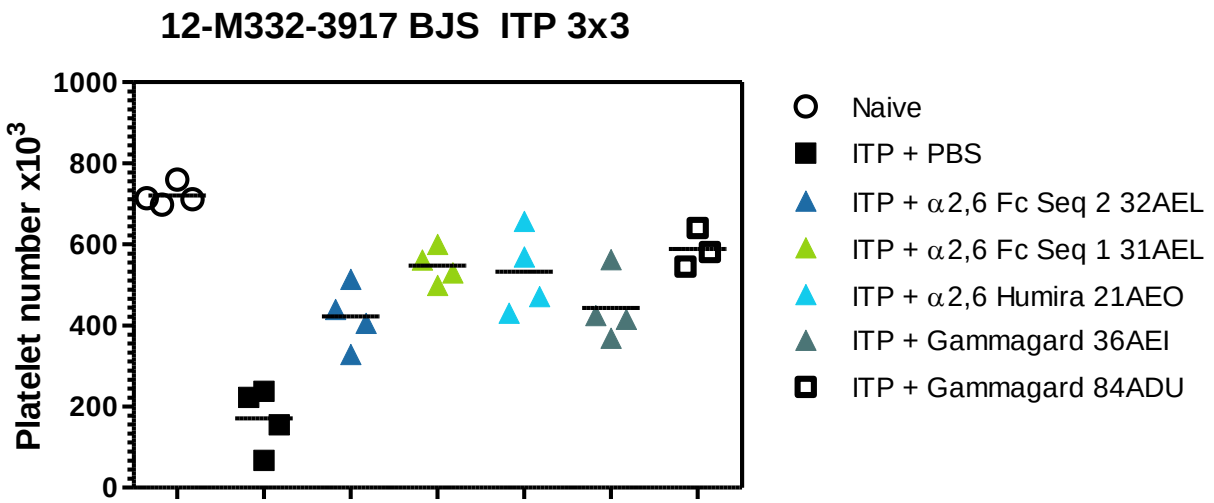
Sequence Liabilities: Sialic Acid

Fc Construct C1 Case Study

Background: α-2,6 sialylated Fc molecules (as IgGs or Fc fragments) have been shown to have anti-inflammatory and anti-"autoimmune" properties in several preclinical mouse models of arthritis and thrombocytopenia.





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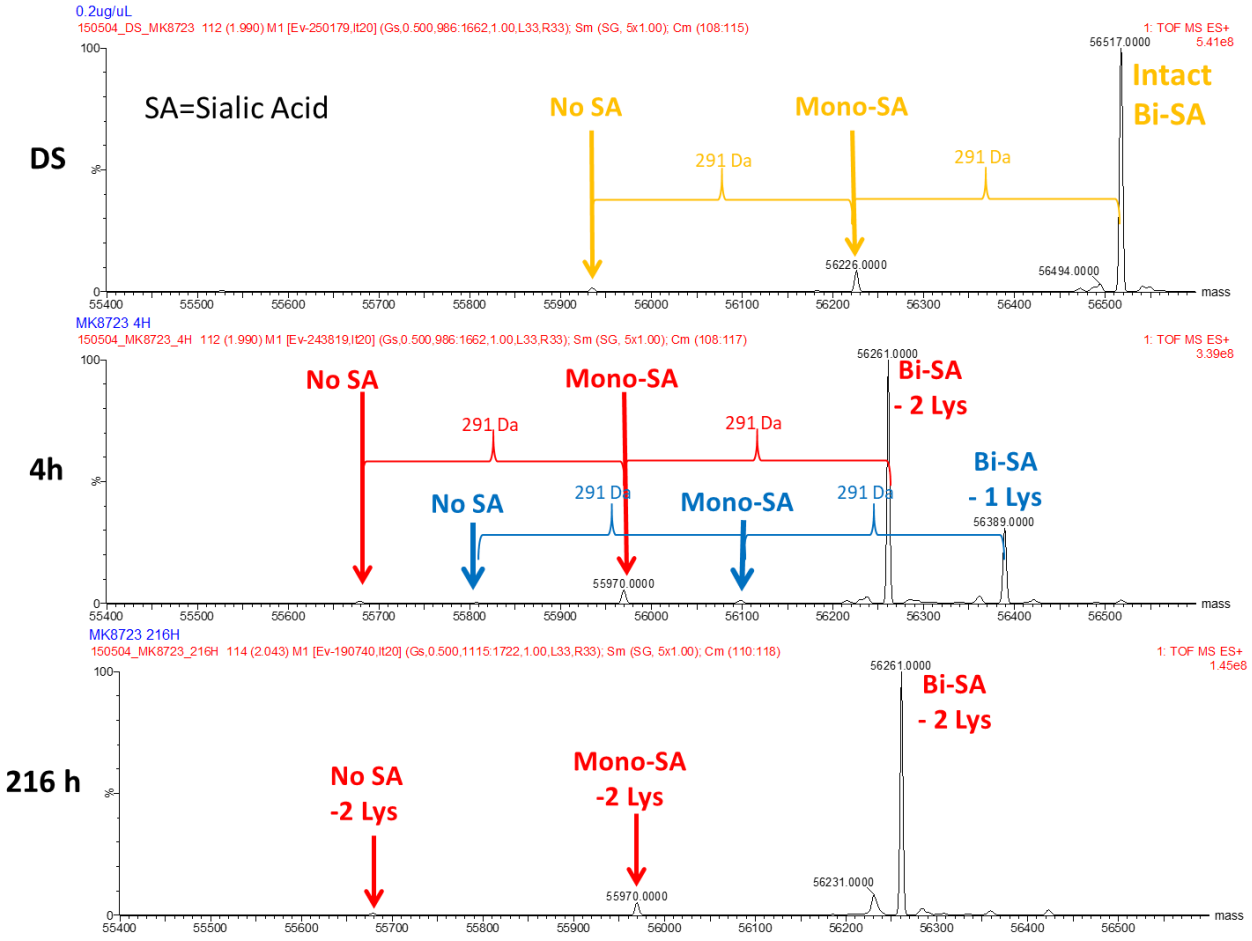
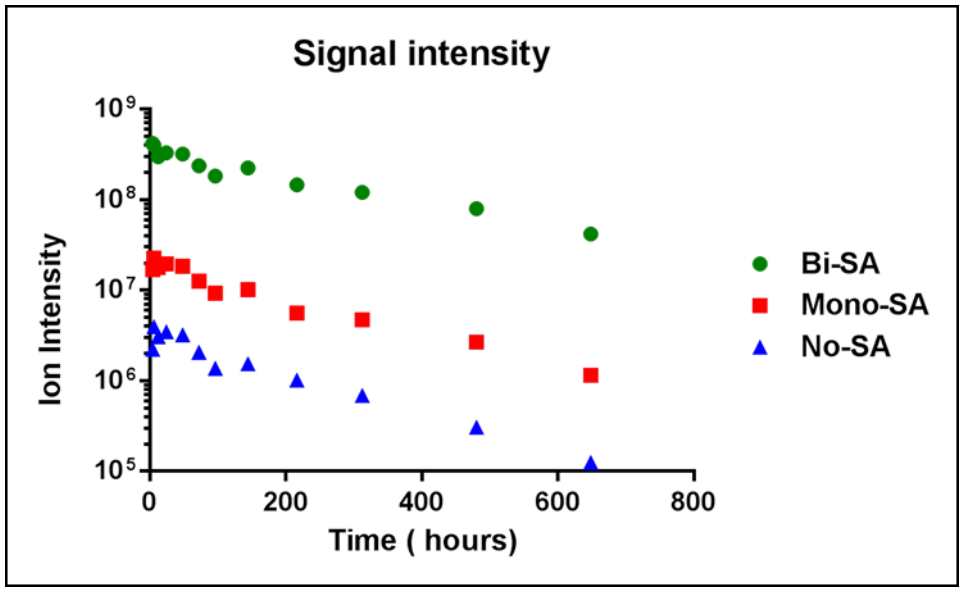
Sequence Liabilities: Sialic Acid

Fc Construct C1 Case Study



Deconvoluted Intact Mass Spectra

Monoclonal Antibody C1 – a2,6 sialylated Fc



Clinical Samples

- Issue: No efficacy in POC study in human
- POC Human samples analyzed in the discovery space to understand the intactness of the drug
- Human POC samples showed no clipping of sialic acid. Lack of potency is not due to sialic acid loss.
- However, fast loss of C-term lysine was identified

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Sequence Liabilities: Clipping (Fc Fusion Case Study)

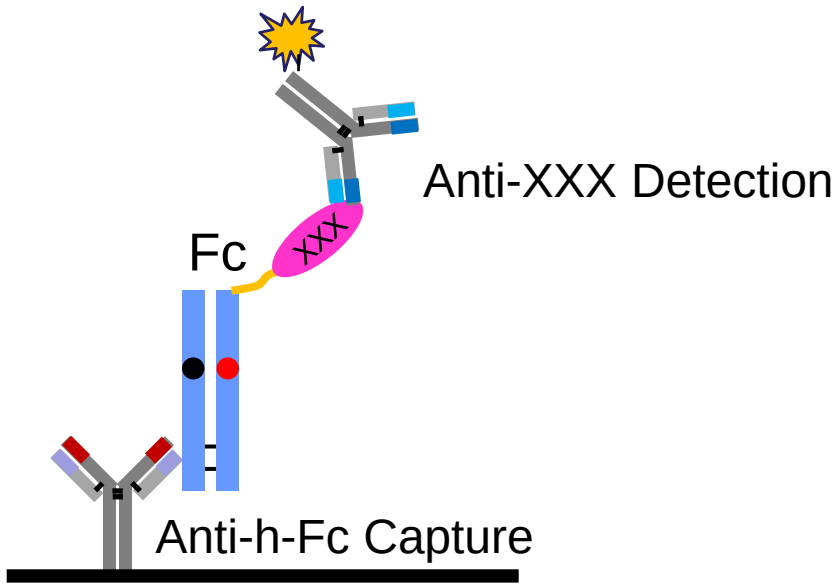
It's all from the fast clearance of a benchmark D1 Fc fusion in mouse

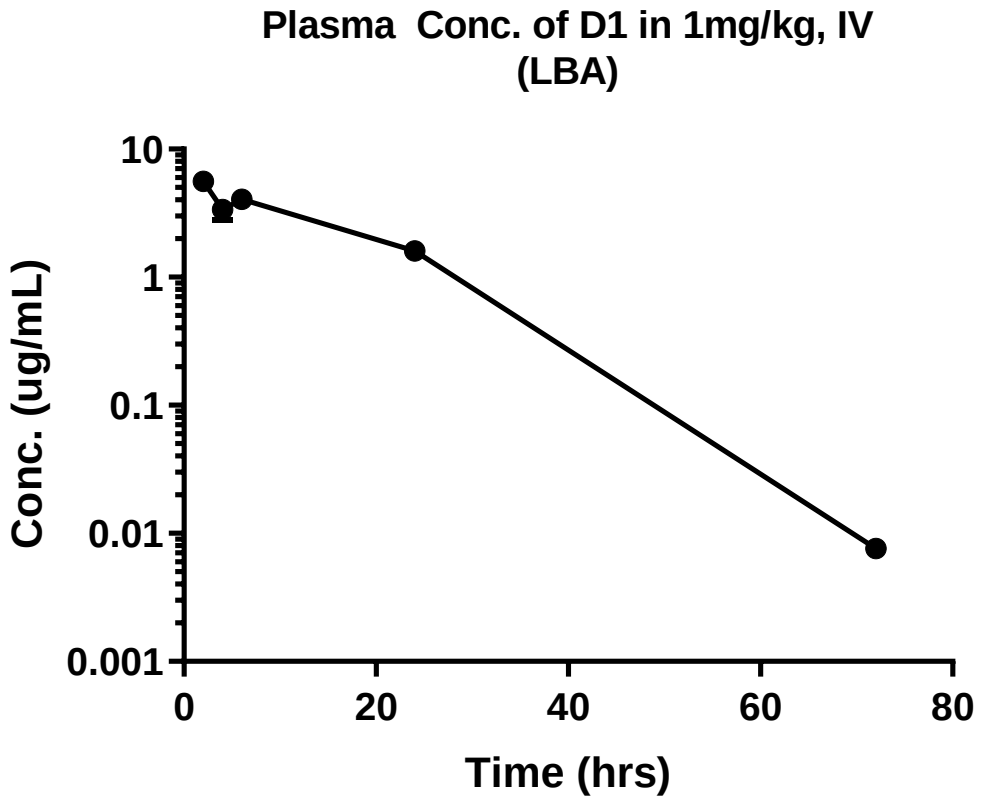
- Method of LBA (Intact assay)

anti-FC capture

anti-XXX detection

measure intact molecule only





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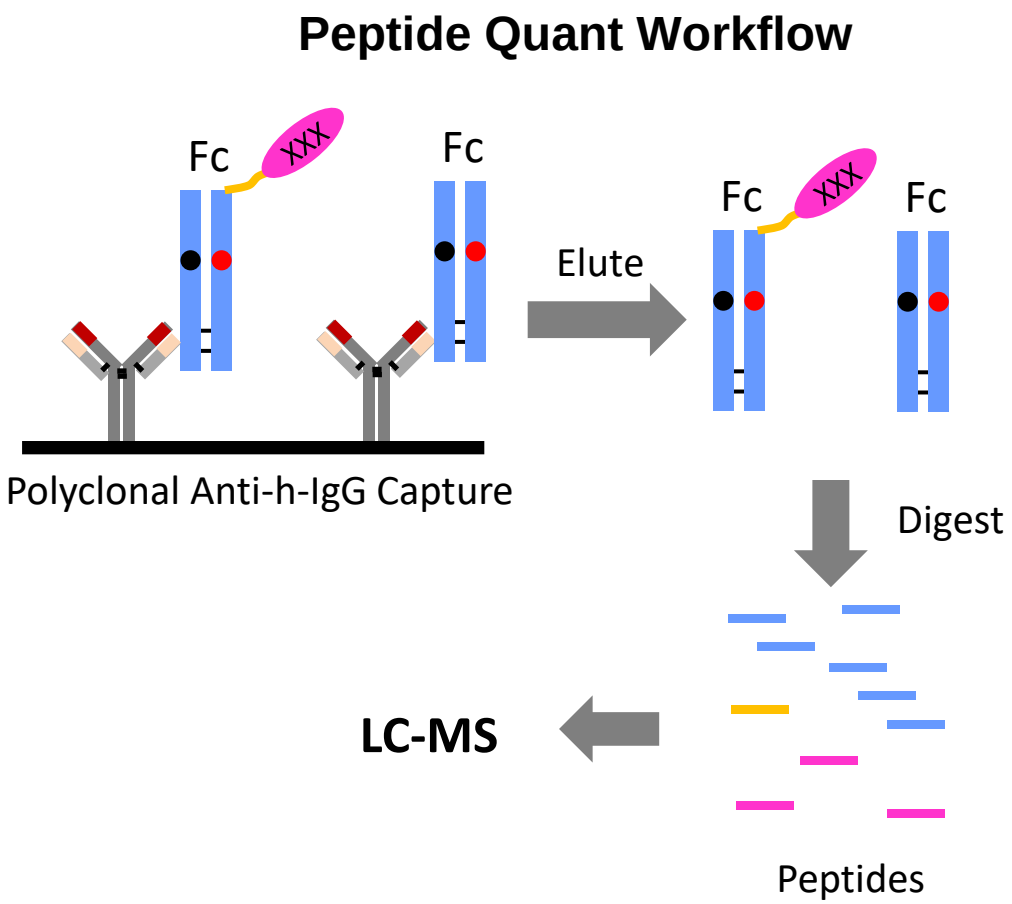
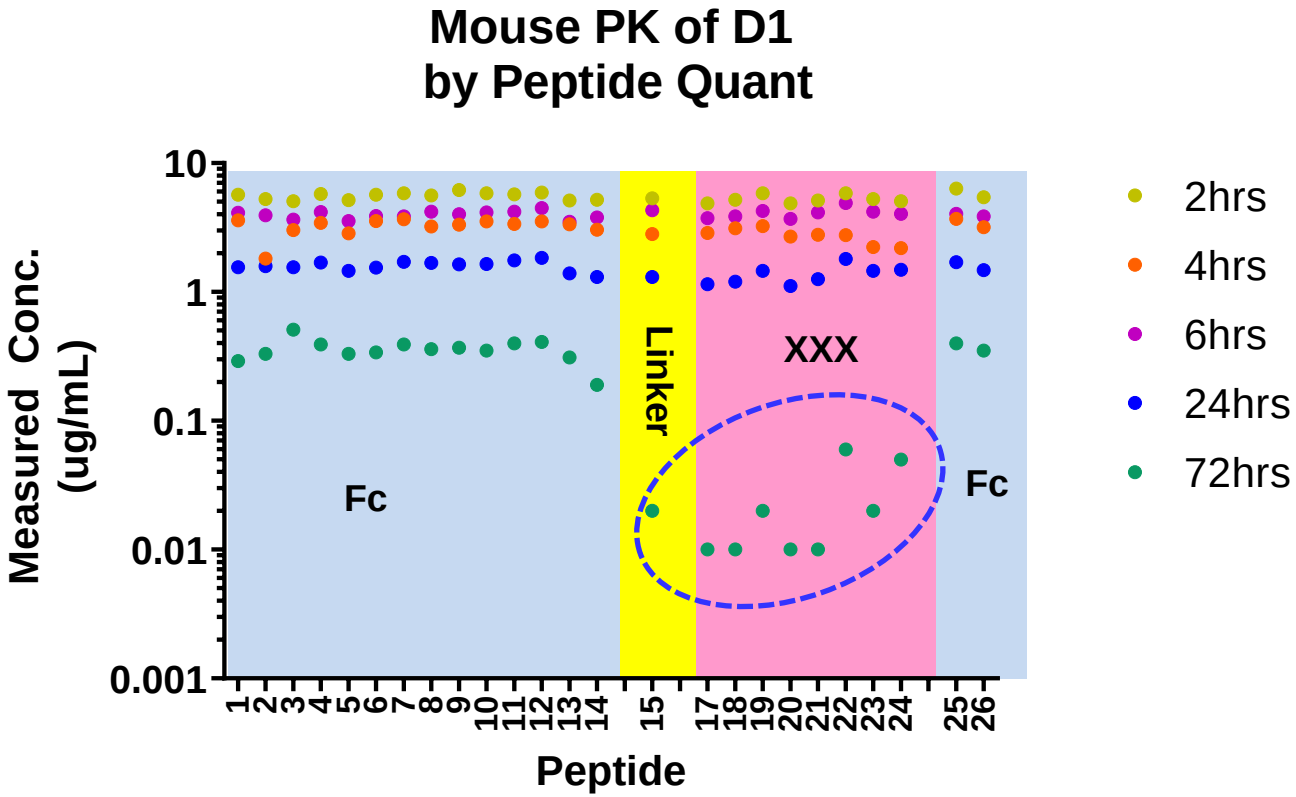
Sequence Liabilities: Clipping (Fc Fusion Case Study)





- Concentration of all peptides of the XXX and the linker region dropped at 72 hrs, indication clipping happened around the linker between 24-72 hrs.

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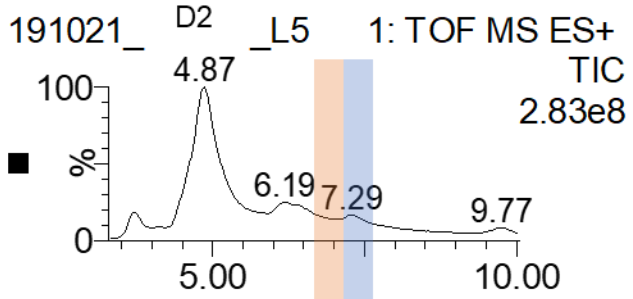
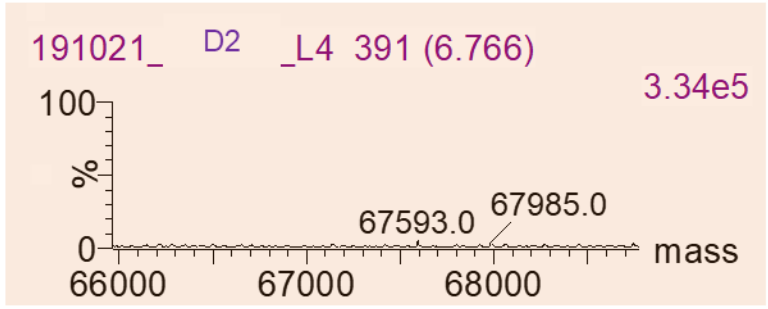
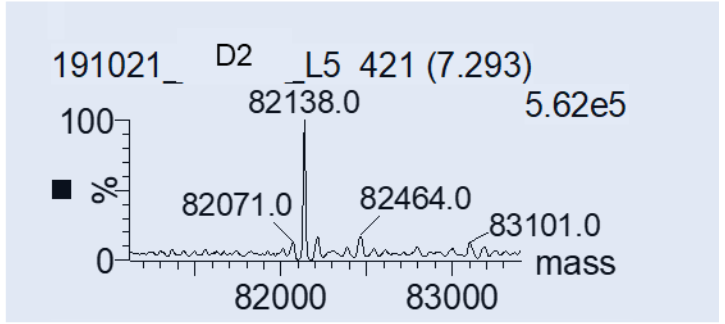
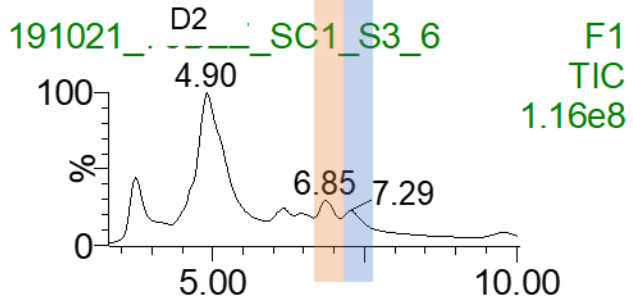
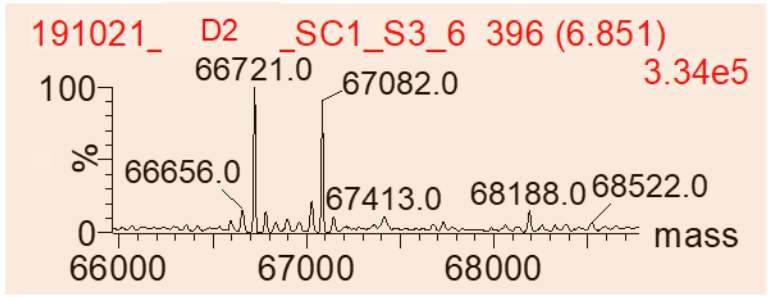
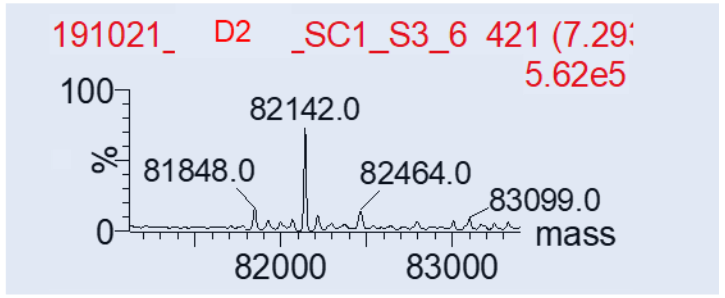
Sequence Liabilities: Clipping (Cytokine-Fc Fusion Case Study)

	Chromatogram	Clipped Fc Fusion	Intact Fc Fusion
0.2 ug/mL Spiked			
0.5mpk 6hr			

1. Accurate capture of clipping site.
2. Less sensitive and not quantitative- clipped product only detected in selected samples

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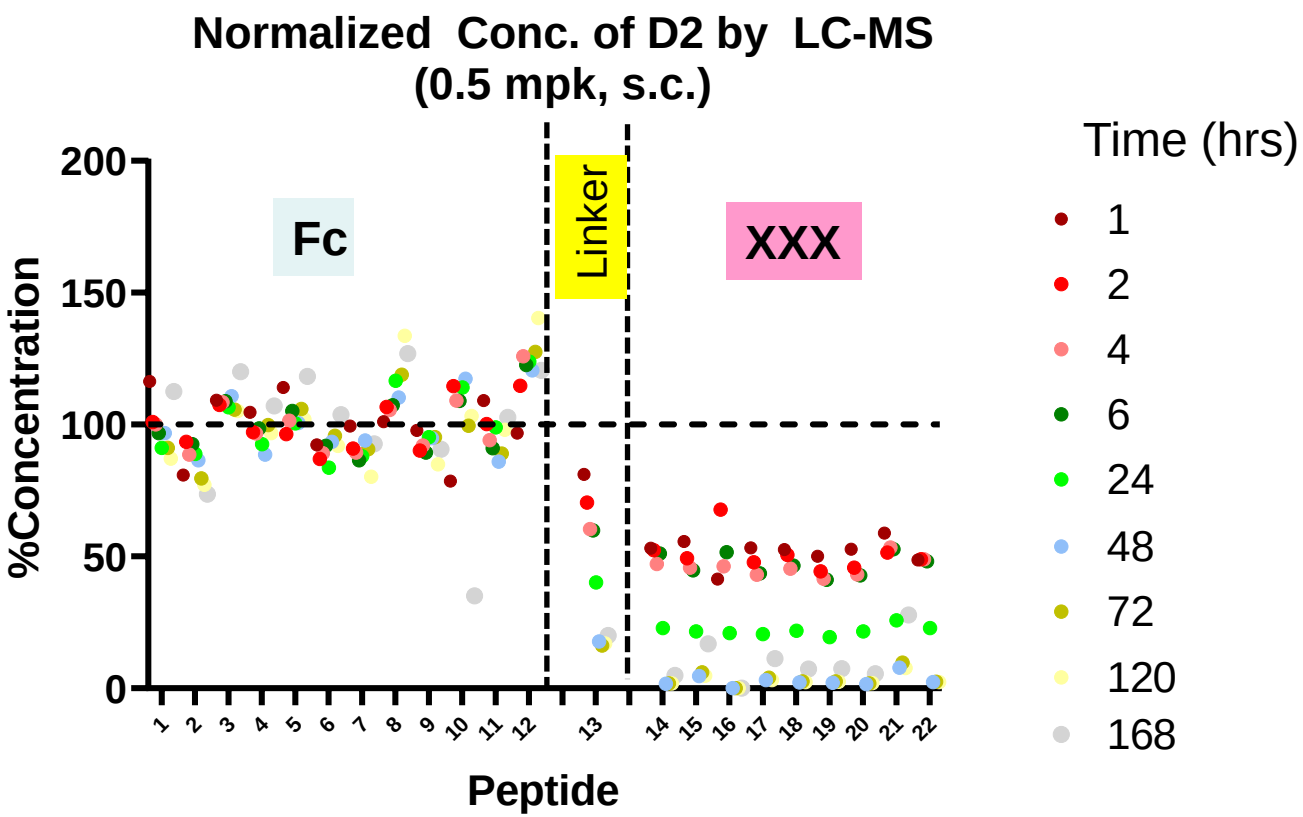
Sequence Liabilities: Clipping (Fc Fusion Case Study)



*Concentration averaged from Fc peptides are considered 100%

- Parallel Reaction Monitoring (PRM) based peptide quant could reveal *in vivo* clipping with much more sensitive than intact mass.
- By 48 hours, Fc Fusion D2 was almost completely clipped and there is only naked Fc remained.
- The fact that linker peptide and all peptide from XXX region shared similar % concentration indicates that the clipping was around the linker. Also, there could be multiple clipping sites.

Take Home Messages

- We are building the data set on PTMs:
  - Deamidation observed in stress study in buffers would likely to happen in-vivo (4 data sets).
  - Oxidation in dev study did not translate in-vivo (2 data sets).
  - Industry trend to study amino acid modifications in-vivo to help relax PQA specs in BioProcess.
- We are building the data set on clipping:
  - PRM based peptide quant assay could reveal clipping with higher sensitivity compared to intact mass. Clipping could be format dependent and species dependent.
  - PRM based peptide quant assay has the potential to reveal unexpected PTM liability.

Acknowledgement

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Thank you!

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