Development of New USP Reference Standards:
Characterization of Three Monoclonal Antibodies Using High Resolution Mass Spectrometry

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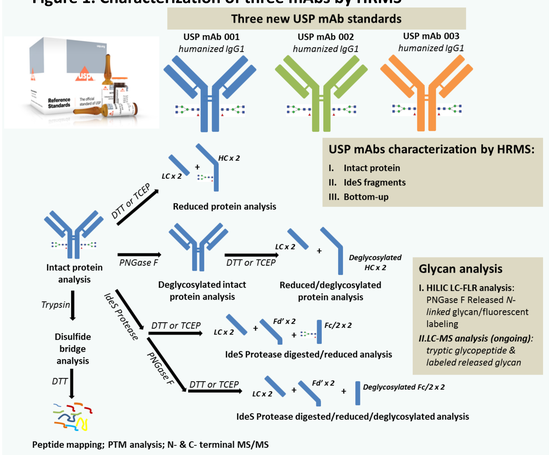
Introduction

- Monoclonal antibodies (mAbs) represent a major share of today's biopharmaceutical medicines. To ensure product safety, efficacy and consistency, biopharmaceutical companies must characterize the mAbs for the presence of variants and impurities throughout the process development and manufacturing lifecycle. Due to their inherent variability, analytical characterization of mAbs is a challenging task and often involves multiple approaches to assess the product quality attributes.
- To support analytical characterization, US Pharmacopeia is developing three new mAb reference standards, **USP mAb 001, 002 and 003**. These reference standards will provide well-characterized mAb materials that can be used to support method development, training, tech transfer, and evaluation of system suitability. In this poster, we present the mass spectrometry-based characterization of these mAb bulk materials.

Methodology

- LC-High resolution mass spectrometry (HRMS) analysis of three USP mAbs included intact and IdeS fragments mass and bottom-up approaches. Released glycan profiling was performed by HILIC LC-FLR.

Figure 1. Characterization of three mAbs by HRMS



Results

A. Intact mass and IdeS fragment analysis of three USP mAb standards

- Confirmed the theoretical sequence. Δmass of intact < 12 Da & < 5 Da for IdeS characterization
- Detected major PTMs: glycosylation, *N*-terminal pyro-Glu and *C*-terminal Lys truncation

B. Glycan analysis

- HILIC LC-FLR analysis of released glycan labeled with fluorescent reagent

Figure 2. Characterization of USP mAb 001 by intact HRMS (deconvoluted MS)

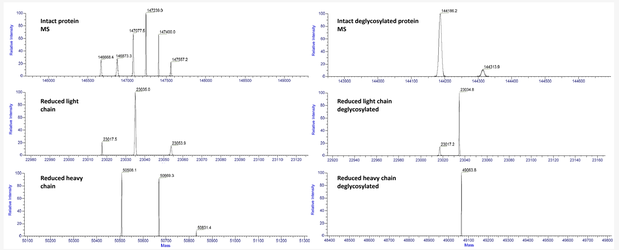


Figure 3. IdeS characterization of USP mAb 001 (deconvoluted MS)

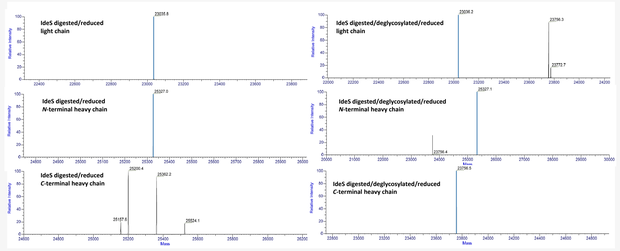


Table 1. Intact MS analysis of three USP mAb RS

USP mAb Standards		USP mAb 001				USP mAb 002				USP mAb 003			
Experiment	Composition	Average MW (theoretical)*	Average MW (measured)	Δ Da	Estimated # of intrachain S-S bond (LC and HC data only)	Average MW (theoretical)	Average MW (measured)	Δ Da	Estimated # of intrachain S-S bond (LC and HC data only)	Average MW (theoretical)*	Average MW (measured)	Δ Da	Estimated # of intrachain S-S bond (LC and HC data only)
Intact protein analysis	G0F/G0F	147,075.2	147077.5	2.3	N/A	149161.5* 149197.5**	149158.0 149192.8	-3.5 -4.7	N/A	145737.4 **	145730.5	-6.9	N/A
	G0F/G1F	147,237.4	147,239.0	1.6	N/A	149323.7* 149359.7**	ND 149348.3	 -11.4	N/A	145899.6**	145895.3	-4.3	N/A
	G1F/G1F or G0F/G2F	147,399.6	147,400.0	0.4	N/A	149485.9* 149521.9**	ND 149510.3	 -11.6	N/A	146061.8**	146054.1	-7.7	N/A
	G1F/G2F	147,561.7	147,557.2	-4.5	N/A	149468.0* 149684.0**	ND ND		N/A	146223.9**	ND		N/A
Deglycosylated intact protein	deglycoform	144,186.6	144,186.2	-0.4	N/A	146272.9* 146308.9**	146270.2 146299.4	-2.7 -9.5	N/A	142812.8* 142848.8**	142809.1 142843.2	-3.7 -5.6	N/A
Reduced protein	Light Chain	23,039.5	23,035.0	-4.5	2	23450.9	23446.4	-4.5	2	22,400.5	22395.9	-4.6	2
	Heavy Chain with G0F	50,514.3	50,508.1	-6.2	3	51164.0**	51156.2	-7.8	4	50484.3**	50476.2	-8.1	4
	Heavy Chain with G1F	50,676.5	50,669.3	-7.2	3 or 4	51326.2**	51318.3	-7.9	4	50646.5**	50638.3	-8.2	4
	Heavy Chain with G2F	50,838.6	50,831.4	-7.2	3 or 4	51488.3**	ND			50808.6**	ND		
Reduced deglycosylated protein	Light Chain	23,039.5	23,034.8	-4.7	2	23450.9	23446.2	-4.7	2	22,400.5	22395.8	-4.7	2
	Heavy Chain	49,069.9	49,063.8	-6.1	3	49719.7**	49711.6	-8.1	4	49040.0**	49032.1	-7.9	4

* Theoretical mass assuming N-term PyroGlu and C-term Lys truncation. ** Theoretical mass assuming C-term Lys truncation.
Mass spec instrument: Orbitrap Elite and QE Plus Orbitrap mass spectrometers

Table 2. HRMS analysis of the three USP mAbs fragments released by IdeS protease

USP mAbs		USP mAb 001			USP mAb 002			USP mAb 003		
Experiment	Composition	Theoretical Mass (Da)	Observed Mass (Da)	Δ Mass (Da)	Theoretical Mass (Da)	Observed Mass (Da)	Δ Mass (Da)	Theoretical Mass (Da)	Observed Mass (Da)	Δ Mass (Da)
IdeS Digested/ Reduced	Light Chain	23039.5	23035.8	3.7	23450.9	23448.3	2.6	22400.5	22399.2	1.3
	HC *N*-terminal Product	25328.3	25327.0	1.3	25946.0	NA	NA	25266.2	NA	NA
	HC C-terminal Product + G0F	25204.0	25200.4	3.6	25236.1	25232.4	2.9	25236.1	25232.5	3.6
	HC *C*-terminal Product + G1F	25366.2	25362.2	4.0	25398.3	25394.1	3.3	25398.3	25394.3	4.0
	HC *C*-terminal Product +G2F	25528.3	25524.1	4.2	25560.4	NA	NA	25560.4	NA	NA
IdeS Digested/ Reduced/ Deglycosylated	Light Chain	23039.5	23036.2	3.3	23450.9	23449.3	1.6	22400.5	22399.8	0.7
	HC *N*-terminal Product	25328.3	25327.1	1.2	25946.0	25942.9	3.1	25266.2	25265.6	0.6
	HC *C*-terminal Product + Deglycoform*	23759.7	23756.5	3.2	23791.8	23789.0	2.8	23791.8	23789.0	2.8

* Deglycosylated mass was calculated with deamidation at the site of deglycosylation by PNGase F

Figure 4. Glycan analysis of USP mAb 001 RS by LC-FLR

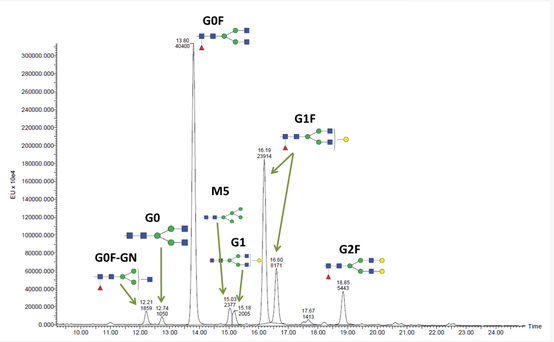
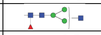
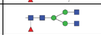
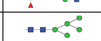
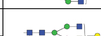
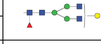


Table 3. HILIC LC-FLD analysis of released glycan

Possible glycan	Structure	Normalized peak area%		
		USP mAb 001	USP mAb 002	USP mAb 003
G0F-GN		2.2	1.8	2.1
G0		1.2	ND	1.3
G0F		46.3	70.7	51.9
Man5		2.8	3.5	3.9
G1		2.4		
G1Fa		27.9	15.5	26.7
G1Fb		9.5	6.3	8.6
G2		1.6	ND	ND
G2F		6.2	2.3	5.7

Conclusion

- Bulk materials for three new USP mAb IgG1 reference standards under development were characterized by high resolution mass spectrometry using multiple approaches.
- Pyro-glutamate formation, C-terminal Lys clipping, and glycosylation patterns were assessed for all three mAbs.

Next Steps

- Optimize reduction protocol to generate more complete reduction of intra-chain disulfides
- Multi-laboratory collaborative study, including intact mass analysis, peptide mapping, and compendial methods from USP General Chapter <129>, <210> and <212> for size variants, oligosaccharides and sialic acid
- Future studies to analyze charge variants and assess other methods for analysis of size variants and glycosylation