Improved Middle-Down Characterization of Antibodies Using Multiple Ion Activation Techniques and Ion-Ion Proton Transfer Reactions on a Modified Orbitrap Mass Spectrometer

John E.P. Syka, Christopher Mullen, Stephane Houel, Romain Huguet, Joshua A. Silveira, Helene L. Cardasis, and Vlad Zabrouskov; Thermo Fisher Scientific 355 River Oaks Parkway, San Jose, CA 95134

ABSTRACT

This is a preliminary investigation of utilizing ion-ion proton transfer reactions (IIPT) subsequent to the different modes of fragmentation used in middle-down MS/MS analysis of monoclonal antibodies (mAbs). This study demonstrates that use of IIPT reactions for *m/z* selected ranges of product ions from short electron transfer dissociation (ETD) reactions enables observation of long mAb subunit product ions.

INTRODUCTION

The sub-unit mass analysis of monoclonal antibodies is a common assay. However obtaining critical sequence information via "middle-down" MS/MS analyses wherein intact mAb sub-units Fc/2, LC and Fd are directly dissociated in an MS/MS experiment is still considered a challenge. Last year, we described LC-MS/MS approaches utilizing a broad precursor *m/z* range to *m/z* select several charge states of eluting mAb subunits for collective MS/MS analysis. The described assays involved a multiplicity of ion dissociation types: ETD and Ultra Violet Photo-dissociation (UVPD) and ETD reaction followed by collision cell type collisional dissociation (EThcD)[1]. The best result was obtained from the aggregation of data from twelve LC MS/MS (15 minutes) analyses covering 6 different reaction/activation times for both ETD and UVPD. Sequence coverages ranging from 83% to 92% for the subunits of NIST mAb standards where obtained. However, study of the sequence maps indicated that the sequence coverage was obtained with few long sequence ions and not many complementary N- and C-terminal pairs of product ions were observed.

Previous reports indicated that IIPT reactions subsequent to MS^2 ETD activation leads to increased sequence coverage.[2] At this conference we and one of our collaborators in this area have presentations demonstrating similar advantage in applying IIPT reactions subsequent to UVPD[3,4].

As the molecular weight of apolypeptide precursor ions increases, so too does the number of possible product ions, and considerable spectral congestion occurs in the vicinity of the precursor ion *m/z* and intact charge reduced precursor ions (ETnoD products) preventing the observation of any discrete product ion isotopic clusters from large product ions species. For ETD and UVPD, if the reaction/activation times are extended, multiple generations of product ions are generated, leading to a population of relatively short and low charge sequence ions of that are more widely distributed in the *m/z* domain. IIPT reactions differentially spread product ions across the *m/z* range without the necessity of over reacting/activating, thus conserving the large product fragments. Highly charged product ions undergo multiple sequential IIPT reactions (ion-ion reaction rate constants vary as z^2) and "diffuse" away in *m/z* space from the adjacent lower *m/z* product ions. Here we present results from a preliminary investigation demonstrating the potential for IIPT of selected *m/z* windows of mAb subunit product ions to increase mAb sequence coverage of mAb subunits and allow observation of large sequence ion.

MATERIALS AND METHODS

Sample Preparation: The NIST mAb standard (RM 8671) was used for all analyses. It was first digested with IdeS protease and then reduced under denaturing conditions (Guanidine/DTT) to generate three ~25 kDa subunits (Fc/2, LC and Fd).

LC Separations: A Thermo Scientific™ UltiMate™ 3000 HPLC system was used for all separations (Solvent A: Water with 0.1% Formic Acid (v/v); Solvent B: Acetonitrile with 0.1% Formic Acid (v/v); flow rate: 1.5µl/min: Column 25 cm ×100 µm ID RP-4H; Sample load: 100 ng). The LC column was deliberately overloaded to increase analyte signal abundance and extend the time for acquisition of MS^2 and MS^3 spectra.

MS Instrumentation and Methods: Targeted LC-MS experiments were performed using a modified Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™ MS with ETD capability. A second reagent inlet was added to the ETD source so that the IIPT reagent, perfluoroperhydrophenanthrene (623 *m/z*, $C_{14}F_{24}$), as well as the standard ETD reagent, flouranthene, (*m/z* 202, $C_{16}H_{12}$) could be introduced simultaneously. The reaction q (reagent) during the ion-ion reaction was 0.4 for all experiments. The high pressure cell of the dual cell linear ion trap (where the ion-ion reactions are performed) was an experimental device with a front section length extended to 35 mm from 12.5 mm. This gives the device a ~3 fold higher reagent ion capacity. The MS^3 Orbitrap spectra were acquired in full profile mode (no thresholding data compression) with Precursor Ion Target: 1E6, Precursor Max Injection Time: 800 ms, ETD Reagent Target: 2E6, IIPT Reagent Target: 2E6. See Figures 1–3 and Table 1 for scheduling and definitions of the MS^3 scans and overall LC-MS3 experiments.

Data Analysis: All LC-MS^3 spectra in each time window for the three mAb subunits were averaged in Thermo Scientific™ QualBrowser™ software and single (averaged) spectra were generated. These averaged LC-MS raw files were processed through Thermo Scientific™ BioPharma Finder™ 3.0 software and the MS^3 spectra were automatically *m/z* to mass "deconvoluted" using the Xtract algorithm. The signal-to-noise ratio (SNR) threshold for fragment peak picking was set to 3. The resulting neutral monoisotopic mass peak lists MS^3 were exported to Excel where the mass lists (5 for ETD and 2 for CID and HCD) for each subunit where summed, then fed to ProSight Lite software (the mass tolerance of fragment ions:10 ppm) to produce sequence coverage maps. To account for the possibility that Xtract may error in monoisotopic mass assignments by exactly ±1 Da for very large and low signal-to-noise isotopic peak clusters, mass lists shifted by ±1 Da were, in certain instances, summed into the aggregate mass lists and re-searched.

Figure 1. Preparation of NIST mAb into LC, Fd and Fc/2 Subunits Suitable for "Middle Down" LC/MS^2 and LC/MS^3 Analyses.

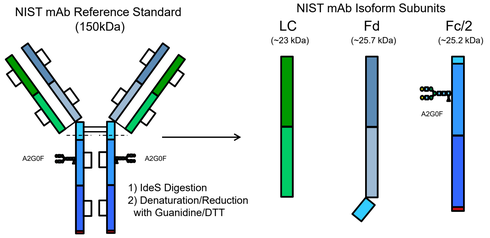


Figure 2. LC/MS Selected Ion Chromatograms of Charge States of the NIST mAb GOF Isoform LC, Fd and Fc Subunits Selected for MS^2 and MS^3 Analyses. The Liquid Chromatography Column was Deliberately Overloaded to Maximize Precursor Ion Abundances and to Extend the Time for Acquisition of MS^2 and MS^3 Spectra.

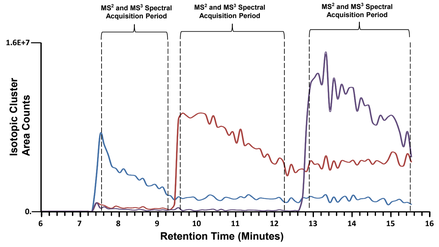


Figure 3. Averaged MS Spectra Showing the Precursor Ion Charge States and *m/z* Selection Window Width of the NIST mAb GOF Isoform LC, Fd and Fc Subunits Selected for MS^2 and MS^3 Analyses.

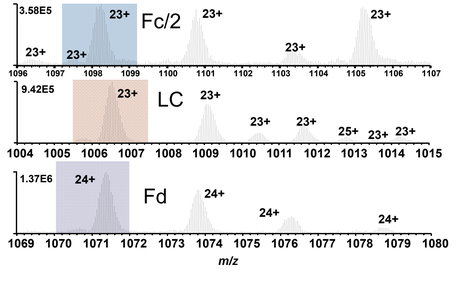


Table 1. Description Analyzer MS Selection and Dissociation/Reaction Settings for the "Middle-Down" LC-MS^3 Experiments Targeting the Fc/2 (23+), LC (23+) and Fd (24+) Sub Units of the NIST mAb Standard (5 ea. MS/ETD/MS/IIPT/MS, 2 ea. MS/CID/MS/IIPT/MS and 2 ea. MS/HCD/MS/IIPT/MS).

LC-MS^3 Experiment No.	NIST mAb Sub Unit Targeted	MS^2 Precursor *m/z* (Th)	MS^2 Precursor *m/z* Isolation Width (Th)	MS^2 Precursor Activation or Reaction Type	MS^2 Precursor Activation Normalized Energy Setting or Reaction Time (ms)	Targeted MS^2 Precursor (MS² Product) *m/z* Selection Widow	MS^3 Precursor Window (Th)	MS^3 Precursor Isolation Width (Th)	Targeted MS^3 *m/z* (Th)	MS^3 Precursor Activation or Reaction Type	MS^3 Precursor Reaction Time (ms)
1	Fc/2	1098.1	2	ETD	3	Between Low *m/z* and Intact MS^2 Precursor *m/z*	780-1080	300	930	IIPT	6
	LC	1006.5	2	ETD	3		700-1000	300	850	IIPT	6
	Fd	1071	2	ETD	3		780-1060	300	910	IIPT	6
2	Fc/2	1098.1	2	ETD	3	Between Precursor *m/z* and 1st Charge Reduced Intact Precursor *m/z*	1106.6-1140.6	40	1120.6	IIPT	6
	LC	1006.5	2	ETD	3		1009-1049	40	1029	IIPT	6
	Fd	1071	2	ETD	3		1073.5-1113.5	40	1093.5	IIPT	6
3	Fc/2	1098.1	2	ETD	3	Between 1st Charge Reduced Intact Precursor *m/z* and 2nd Charge Reduced Intact Precursor *m/z*	1150-1195	45	1172.5	IIPT	6
	LC	1006.5	2	ETD	3		1055-1100	45	1077.5	IIPT	6
	Fd	1071	2	ETD	3		1120-1165	45	1142.5	IIPT	6
4	Fc/2	1098.1	2	ETD	3	Between 2nd Charge Reduced Intact Precursor *m/z* and 3rd Charge Reduced Intact Precursor *m/z*	1200-1250	50	1225	IIPT	6
	LC	1006.5	2	ETD	3		1105-1155	50	1130	IIPT	6
	Fd	1071	2	ETD	3		1170-1220	50	1195	IIPT	6
5	Fc/2	1098.1	2	ETD	3	Between 3rd Charge Reduced Intact Precursor *m/z* and High *m/z*	1270-1570	300	1420	IIPT	6
	LC	1006.5	2	ETD	3		1160-1460	300	1310	IIPT	6
	Fd	1071	2	ETD	3		1230-1530	300	1380	IIPT	6
6	Fc/2	1098.1	2	CID (Ion Trap)	35	Between Low *m/z* and MS^2 Precursor *m/z*	1085-1089	300	938	IIPT	6
	LC	1006.5	2	CID (Ion Trap)	35		696.5-996.5	300	846.5	IIPT	6
	Fd	1071	2	CID (Ion Trap)	35		761-1061	300	911	IIPT	6
7	Fc/2	1098.1	2	CID (Ion Trap)	35	Between MS^2 Precursor *m/z* and High *m/z*	1108-1408	300	1258	IIPT	6
	LC	1006.5	2	CID (Ion Trap)	35		1016.5-1316.5	300	1166.5	IIPT	6
	Fd	1071	2	CID (Ion Trap)	35		1081-1381	300	1231	IIPT	6
8	Fc/2	1098.1	2	HCD (Cell, Cell)	10	Between Low *m/z* and MS^2 Precursor *m/z*	1085-1089	300	938	IIPT	6
	LC	1006.5	2	HCD (Cell, Cell)	10		696.5-996.5	300	846.5	IIPT	6
	Fd	1071	2	HCD (Cell, Cell)	10		761-1061	300	911	IIPT	6
9	Fc/2	1098.1	2	HCD (Cell, Cell)	10	Between MS^2 Precursor *m/z* and High *m/z*	1108-1408	300	1258	IIPT	6
	LC	1006.5	2	HCD (Cell, Cell)	10		1016.5-1316.5	300	1166.5	IIPT	6
	Fd	1071	2	HCD (Cell, Cell)	10		1081-1381	300	1231	IIPT	6

RESULTS

The aggregated results from experiments 1-5 (MS/ETD/MS/PTR) were by far the most promising with many long sequence ions observed (see Figures 5-8) for all the NIST mAb subunits. The results from experiments 6-7 (MS/CID/MS/IIPT/MS) and Experiments 8-9 (MS/HCD/MS/IIPT/MS) were less promising. The pair of CID experiments provided the following aggregate sequence coverages: 19% (Fd), 31% (LC) and 31% (Fc/2). The pair of HCD experiments provided the following aggregate sequence coverages: 13% (Fd), 17% (LC) and 17% (Fc/2). Very few sequence ions having lengths more than 50% of the subunits where observed.

Figure 5. Sequence Coverage Map NIST mAb Fc/2 Subunit from the Product Ion Mass List From Experiments 1-5 (LC- MS/ETD/MS/IIPT/MS Analyses).

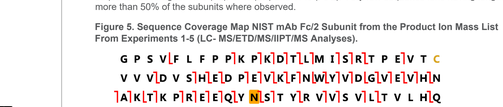


Figure 6. Sequence Coverage Map NIST mAb Fd Subunit from the Collective Product Ion Mass List From Experiments 1-5 (LC- MS/ETD/MS/IIPT/MS Analyses).

Figure 4. Example MS/ETD/MS Spectrum of the Targeted Fd Precursor Ion. A) Full Vertical Scale: Illustrating the 5 ea. MS3 IIPT Precursor Ion *m/z* Selection Windows for the 5 Targeted MS/ETD/MS/IIPT/MS Analyses. B) 20×Expanded Vertical Scale: Illustrating the *m/z* Distribution of ETD and ETnoD Product Ions.

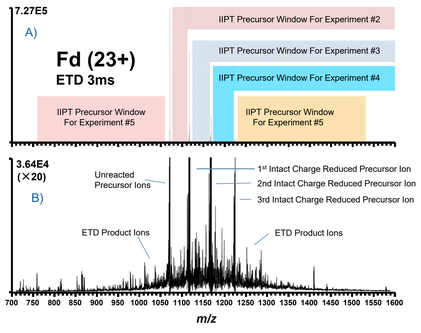


Figure 7. Sequence Coverage Map NIST mAb LC Subunit from the Collective Product Ion Mass List From Experiments 1-5 (LC- MS/ETD/MS/IIPT/MS Analyses).

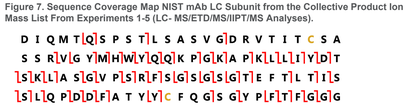
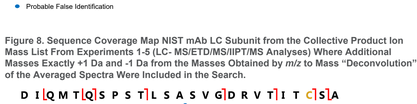
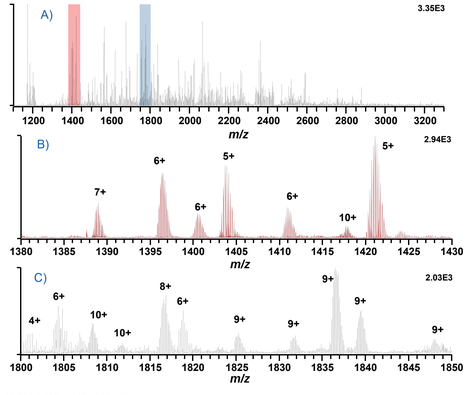


Figure 8. Sequence Coverage Map NIST mAb LC Subunit from the Collective Product Ion Mass List From Experiments 1-5 (LC- MS/ETD/MS/IIPT/MS Analyses) Where Additional Masses Exactly +1 Da and -1 Da from the Masses Obtained by *m/z* to Mass "Deconvolution" of the Averaged Spectra Were Included in the Search.



Figured 9. Averaged MS/ETD/MS/IIPT Spectrum of Targeted Fd Precursor Ion Obtained in LC-MS3 Experiment #4 A) With *m/z* Range Chosen to Entire Range of *m/z* of Product Ions. B) 50 Th Window (Highlighted in Red) With Mostly Lower Charge State (5+ to 7+) Product Ions. B) 50 Th Window (Highlighted in Blue) With Mostly Higher Charge State (8+ to 10+) Product Ions.



CONCLUSIONS

This preliminary study demonstrates that IIPT of selected *m/z* ranges of ETD product ions greatly enhances the observation of large sequence ions for all of the NIST mAb sub units. A similar increase of large sequence ions for the IIPT subsequent to CID and HCD was not demonstrated. We believe this was due to the relatively large (300 Th) *m/z* windows of CID and HCD product ions selected for IIPT. We anticipate that observation of large UVPD product ions of mAb sub units will also be enhanced by a subsequent IIPT of selected m/z windows as was done in the ETD experiments.

REFERENCES

1. Houel, S.; Fornelli, L.; Srzentic, K.; et. al. Middle-down analyses of monoclonal antibody on Orbitrap Fusion Lumos Tribrid *Proceedings of the16th Annual World Congress of the Human Proteome Organization (HUPO) in Dublin, Ireland*, 2017.
2. Anderson, L. C.; English, M.A.; Wang, W. H.; et. al. Protein derivatization and sequential ion/ion reactions to enhance sequence coverage produced by electron transfer dissociation mass spectrometry *IJMS* **2015,** *377*, 717–624.
3. Mullen, C.; Schwartz, J. C.; Syka, J. E. P.; et al. *Improved top-down sequence coverage on a Tribrid mass spectrometer by ion-ion proton transfer (IIPT) reactions subsequent to ETD and UVPD.* Proc. 66th **ASMS Conf**. Mass Spectrometry and Allied Topics, San Diego, VA **[TP741]**.
4. Sanders J, Holden D, et al. Reducing Congestion in Utraviolet Photodissociation spectra of large protein ions using Proton Transfer Reactions. Proc. 66th **ASMS Conf**. Mass Spectrometry and Allied Topics, San Diego, VA **[TOH pm 02:30]**.

TRADEMARKS/LICENSING

PO65302-EN 0518S

Thermo Fisher SCIENTIFIC