Systematic optimization of electron activated dissociation for top-down targeted protein sequencing

Jason Causon, Ihor Batruch
SCIEX, Concord, Canada

INTRODUCTION

Recent developments in mass spectrometry hardware and software have significantly improved top-down and middle-down analysis of proteins and protein sub-units in the case of monoclonal antibodies (mAbs). Acquisition methodology, chromatography separation and data processing are all factors in maximizing performance and sequence coverage.[1-3]

In this work, we detail the systematic optimization of electron activated dissociation (EAD) with MRM^{HR} for top-down / middle-down protein sequence confirmation, and we will investigate the dependencies between acquisition and processing strategies.

MATERIALS AND METHODS

Sample:
Equine myoglobin was obtained from Sigma-Aldrich, reconstituted and diluted down to a concentration of 1 µg/µL and 100 ng/µL.

HPLC:
The separation was achieved using a Waters ACQUITY UPLC BEH C4 column (2.1 × 50 mm, 1.7 µm, 300 Å) at a flow rate of 0.4 mL/min. The column was kept at 60°C in the column oven of an ExionLC AD system.

MS/MS:
MRM^{HR} EAD experiments were performed in SCIEX OS software using the ZenoTOF 7600 system. Multiple experiment setups were evaluated for determining the dependencies. The data were analyzed using a new middle-down workflow template in the Biologics Explorer software.[4-6]

ZenoTOF 7600 system

Biologics Explorer software

RESULTS

Figure 1 shows an overview of the EAD-based workflow, and Figure 2 shows the fragmentation provided by EAD when the Zeno trap is activated in the ZenoTOF 7600 system.

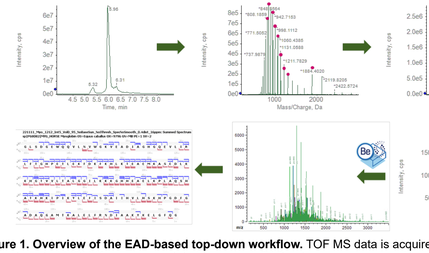


Figure 1. Overview of the EAD-based top-down workflow. TOF MS data is acquired, charge states identified and then a targeted MRM^{HR} per charge state is acquired. EAD data are analyzed and annotated using a workflow template offered by Biologics Explorer software.

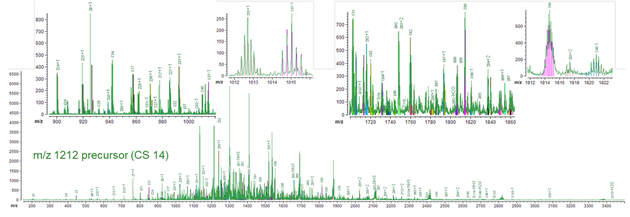


Figure 2. A representative EAD spectrum. EAD using the Zeno trap provided excellent fragmentation of myoglobin and permitted the detection of low-abundant fragments. Insets are regions of the MS/MS data that show the matching of isotope abundances across charge states.

Figure 3 shows the impact on both the number of mapped fragments and the total sequence coverage for the selected charge states. The general trend is that the lower charge states generate the highest sequence coverage versus the most abundant higher charge states.

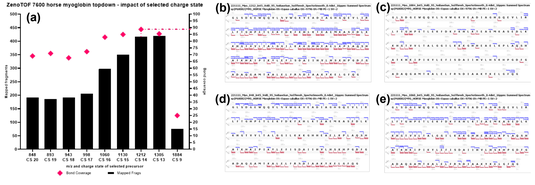


Figure 3. The impact of charge state on sequence coverage. (a) shows the bond coverage and the number of mapped fragments per charge state. A range of 15% to 90% is achieved. (b) – (e) fragment maps for charge states 14, 9, 20 and 16.

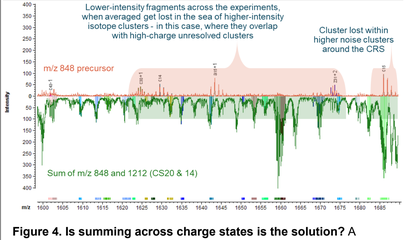
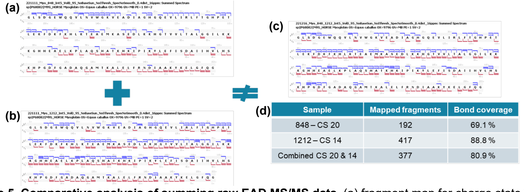


Figure 4. Is summing across charge states is the solution? A comparison of single charge state versus summed.

Figure 4 shows the most abundant charge in comparison to a summation with the highest coverage charge state.

The issue observed with trying to sum charge states is the very different fragment ion profiles generated, which means that any fragments, especially lower charge fragments, are lost in the sea of highly charged fragments from the lower charge state precursor.

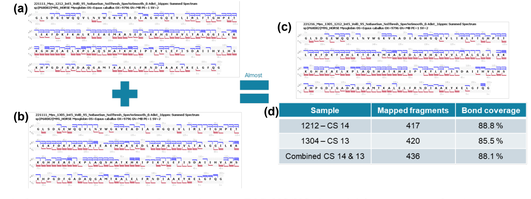
Furthermore, Figures 5 and 6 show the impact on the sequence coverage maps when different charge states are summed.



Sample	Mapped fragments	Bond coverage
848 – CS 20	192	69.1 %
1212 – CS 14	417	88.8 %
Combined CS 20 & 14	377	80.9 %

Figure 5. Comparative analysis of summing raw EAD MS/MS data. (a) fragment map for charge state 20, (b) fragment map for charge state 14 (c) combined fragment map d) tabulates bond coverage and number of mapped fragments.

So, is the glass half full or half empty? A game of two halves… Figure 5 shows the individual maps for charge state 20 and 14 and the sum. If you start with 20 and sum with 14, you would say there was an improvement. If you start with 14, you see a reduction. In essence, there will be a charge state that generates maximum coverage versus the most abundant charge state under these reverse phase conditions. Figure 6 shows that the summation of the 2 highest coverage states almost equals the individuals.



Sample	Mapped fragments	Bond coverage
1212 – CS 14	417	88.8 %
1304 – CS 13	420	85.5 %
Combined CS 14 & 13	436	88.1 %

Figure 6. Comparative analysis of summing raw EAD MS/MS data. (a) fragment map for charge state 14, (b) fragment map for charge state 13 (c) combined fragment map (d) tabulates bond coverage and number of mapped fragments.

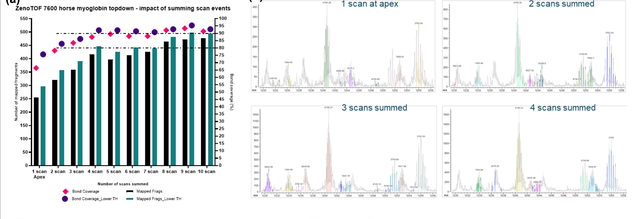


Figure 7. Sequence coverage per scans summed in Biologics Explorer software (a) coverage and mapped fragments versus scans summed with two different intensity thresholds, (b) matching of theoretical isotope envelopes per fragment with scans summed.

Figure 7 shows the trend in coverage per sequential summing of EAD MS/MS scans across the LC peak. The graph shows that just 2 scans are required to reach 80% coverage, which equates to a total of 800 ms of scan time.

In Figure 8, the impact of the electron beam current is shown. The beam current controls the number of electrons within the EAD cell. As shown, coverage increases up to 3,500 nA, after which, the coverage plateaus. Interestingly, the intensity of the high-mass fragments continues to increase while the intensity of the low-mass fragments decreases.

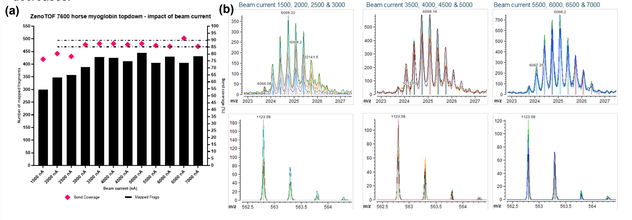


Figure 8. Electron beam current versus bond coverage. (a) sequence coverage and mapped fragments versus beam current. (b) low and high mass fragments at increasing beam current.

CONCLUSIONS

- When optimized for the best charge state, high sequence coverages can reach the 90% mark for myoglobin
- Biologics Explorer software offers an easy-to-use workflow that can help rapidly assess the quality of the total acquisition strategy
- In general, the selected charge states have the highest impact on sequence coverage, followed by how many MS/MS scans are summed and then the beam current and summing of charge state data
- The best option is to sum individual sequence coverage maps after processing each of them individually
- Potential optimization of instrument control acquisition strategies - while the most abundant species are typically chosen for MS/MS, the data from this study indicate a preference for low charge states at lower abundance under reverse phase conditions

REFERENCES

1 Milos Cejkov *et al.* (2021) Electron transfer dissociation parameter optimization using design of experiments increases sequence coverage of monoclonal. *J. Am. Soc. Mass Spectrom.* **32**(3): 762-771.
2 Luca Fornelli *et al.* (2018) Accurate sequence analysis of a monoclonal antibody by top-down and middle-down Orbitrap mass spectrometry applying multiple ion activation techniques. *Anal. Chem.* **90**(14): 8421-8429.
3 Richard D Smith *et al.* (1990) Collisional activation and collision-activated dissociation of large multiply charged polypeptides and proteins produced by electrospray ionization. *J. Am. Soc. Mass Spectrom.* **1**(1): 53-65.
4 A streamlined single-injection middle-down workflow using electron activated dissociation (EAD) for biotherapeutics characterization. SCIEX technical note, MKT-26997-A.
5 Obtaining high sequence coverage and confident post-translational modification (PTM) analysis of biotherapeutics using an electron activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27223-A.
6 Comparative analysis of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27427-A.

TRADEMARKS/LICENSING

To receive a copy of this poster:

- Scan the code with your phone camera
- Complete the form