Utilizing an easily tunable declustering potential for the removal of adduct ions on intact biotherapeutics

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ABSTRACT

An easy-to-use and robust sub-atmospheric pressure technique that is capable of declustering trifluoroacidic acid (TFA) adducts, improving sensitivity and clarifying mass spectral quality for complex antibody subunit separations is demonstrated. Without declustering, intact antibody subunits that are separated with ion pairing agents will form high degrees of adduct formation, that will obscure and make spectral deconvolution difficult. However, with declustering, it is possible to greatly improve spectral clarity of intact protein subunits and greatly improve sensitivity. A sensitivity improvement of over 10-fold was observed for the declustering approach outlined.

INTRODUCTION

Optimizing chromatography for intact protein separations often requires the use of solvents that are not ideal for electrospray ionization (ESI) and mass spectrometry (MS). Intact proteins commonly observe improved chromatographic separation efficiencies (N) with ion pairing agents, such as, TFA and difluoroacetic acid (DFA). Although TFA yields good peak shape and chromatographic resolution for intact proteoforms on a variety of reversed-phase stationary phase columns, it also pairs with exposed ionizable surface charges on a protein and negatively impacts ESI-MS analyses. Increasing complexity of biotherapeutic proteins drives the need for better separation techniques, while enabling high-quality MS analyses. Here, a method for declustering intact proteins that greatly reduces the adduct formation present on MS spectra for proteins separated with ion pairing agents on front end chromatography is demonstrated.

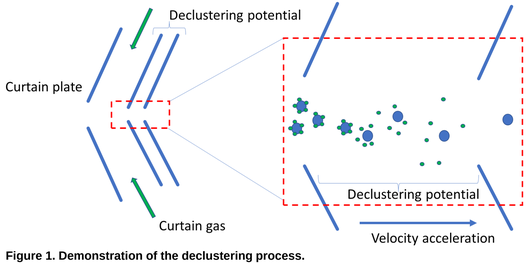

Figure 1. Demonstration of the declustering process.

MATERIALS AND METHODS

Sample preparation: Stock solution of Waters Intact Mass check standard (IgG) were created. A reduction buffer containing 25mM NaCl, 25mM Tris and 1mM EDTA with pH = 8 was created. Twenty µL of original solution of monoclonal antibody (mAb) were mixed with 180 µL of the reduction buffer. 2.1 µL of 100mM DTT were added to the protein-reduction buffer mixture. The samples were incubated at 37ºC for 20 min. 100 µL of the sample was transferred and mixed with equal volumes of 3% acetonitrile and 0.1% formic acid. Add 2.1 µL of 300mM iodoacetamide to the mixture to alkylate the reduced mAb and incubate in the dark for 45 min at room temperature. Samples can be diluted with 3% acetonitrile and 0.1% formic acid and injected into the LC-MS for analysis.

Chromatography: Antibody subunits were separated using a Bioresolve tri-phenyl RP column (3.0 x 150 mm, 2.7 µm, Waters), which was kept at 80ºC in the column oven of an ExionLC system (SCIEX). Table 1 shows the LC gradient used for antibody separation at a flow rate of 1.0 mL/min. Mobile phases consisted of 0.05% TFA in both water and acetonitrile.

Mass spectrometry: LC-MS data were acquired with a TOF MS method in SCIEX OS software using the X500B QTOF system. The key TOF MS parameters are listed in Table 2.

Data processing: SCIEX OS software was utilized to deconvolute and display charge state spectra of the proteins.

Table 1. Chromatographic conditions.

Time [min]	A [%]	B [%]
Initial	*95*	*5*
1	*95*	*5*
2	*77*	*23*
5.5	*52*	*48*
8.0	*5*	*95*
10	*5*	*95*
10.5	*95*	*5*
15	*95*	*5*

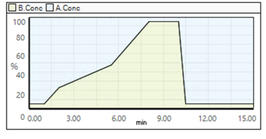

Table 2. TOF MS parameters.

Parameter	Value
Ion source gas 1	*60 psi*
Ion source gas 2	*70 psi*
Curtain gas	*35 psi*
Source temperature	*450ºC*
Spray voltage	*5500 V*
TOF start mass	*600 m/z*
TOF stop mass	*6000 m/z*
Accumulation time	*0.5 s*
Declustering potential	*20–250 V*

RESULTS

Improvements in sensitivity and spectral clarity due to declustering of the TFA adducts allow accurate characterization of separated, complex subunit protein species. Declustering can be achieved in multiple ways. Here, it was accomplished by optimizing the declustering potential (DP) in the jet-free region of the ion optics, before the low-pressure region of the ion optics.

TFA adducts were observed on both the light chain (Figure 2) and heavy chain (Figure 3) of the IgG check standard. The difference between low DP <100 V and DP >200 V is compared for the charge state distributions of the light and heavy chains.

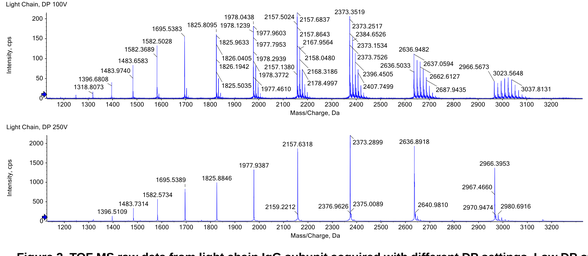

Figure 2. TOF MS raw data from light chain IgG subunit acquired with different DP settings. Low DP of 100 V demonstrated subunit with intense adduct formation (top). Light chain subunit with DP = 250 V has been declustered (bottom), greatly improving signal-to-noise.

As the DP is increased from 100 to 250 V the declustering of TFA adducts significantly improved spectra simplicity and significantly increased the sensitivity by 10-fold. As the intact subunits are adducted the signal is effectively reduced as the charge states are split between multiple adducts, in this case it can be as many as 3–9 TFA adducts for both the light chain and heavy chain of the IgG, depending on the charge state. Using a declustering mechanism we were able to reduce the amount of charge state splitting present with TFA adduction, shown in both Figures 2 and 3.

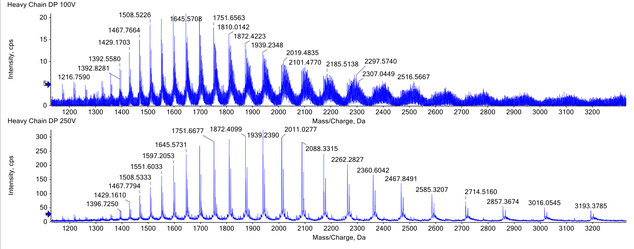

Figure 3. TOF MS raw data from heavy chain IgG subunit acquired with different DP settings. Low DP of 100 V demonstrated subunit with intense adduct formation (top). Heavy chain subunit with DP = 250 V has been declustered (bottom), greatly improving signal-to-noise.

There was significantly more TFA adduction of the heavy chain IgG as compared to the light chain, enabling declustering to yield approximately 15-fold improvement in sensitivity. As the cluster equilibrium was shifted to a lower value, within the dry curtain gas region (a jet-free region), a shift in the charge state distribution of the proteins can be observed as they are declustered. The reasons for this shift is likely a better solvent declustering of lower charge states, rather than mass-dependent ion focusing, collision-activated proton stripping, or collision-activated protonated solvent cluster stripping.[1]

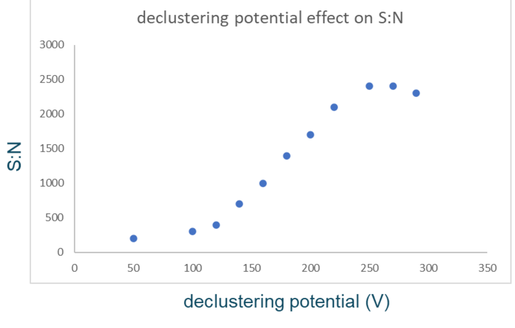

Figure 4: Effect of DP on S/N of IgG mAb light chain. The collapse of charge state splitting from adduct formation due to TFA is shown as DP is applied from 50 V to 300 V.

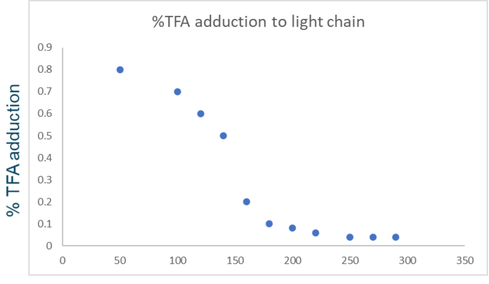

Figure 5. Percent adduct of TFA versus DP of IgG mAb light chain. A reduction in adduct formation could be observed as declustering potential is applied from 50 V to 300 V.

CONCLUSIONS

- Optimal chromatographic separations for intact proteins on reversed phase require the use of ion pairing agents. These ion pairing agents form adducts to intact antibody subunits. Left alone, these adducts greatly reduce sensitivity and increase spectral complexity.
- Sub-atmospheric declustering in the jet-free region prior to the Q-Jet yields excellent declustering and TFA adduct removal from antibody subunits. This declustering greatly improved spectral simplicity.
- In addition to improved spectral simplicity, there is also significantly improved sensitivity for both the heavy chain and light chain. This improved sensitivity, greatly enhanced the signal-to-noise-using purely TFA-modified mobile phases in the chromatography.
- SCIEX OS software was utilized to process the data and generate statistical comparisons

REFERENCES

1. Bruce Thompson, Declustering and fragmentation of protein ions from an electrospray ion source. JASMS, 8(10), 1053-1058.

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