# Supercharging for Improved ECD/ETD-Based HDX-MS of Biotherapeutics

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## HYDROGEN/DEUTERIUM EXCHANGE (HDX)

Gas-phase fragmentation can provide increased structural resolution in bottom-up protein HDX approaches. Conventional vibrational heating-based activation methods, e.g., collision induced dissociation, suffer from extensive hydrogen scrambling.[1]

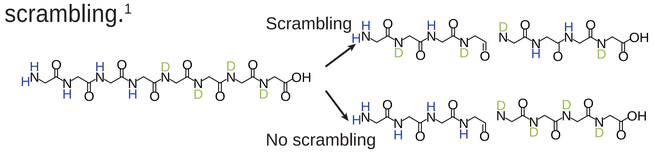



**Figure 1.** Selectively labeled peptide undergoing gas-phase dissociation.

Electron-based activation methods have been shown to allow fragmentation without hydrogen scrambling under certain conditions.[2] However, these methods are incompatible with low charge state precursor ions.[3,4] Digestion with pepsin compounds this issue because this proteolytic enzyme typically generates singly- and doubly- charged peptides. Supercharging reagents have been demonstrated to increase the average charge state[5-7] and thus improve electron-capture and electron-transfer dissociation (ECD/ETD) outcomes. Here, we apply supercharging towards the improvement of bottom-up biotherapeutic protein HDX.

## SUPERCHARGING STRATEGY

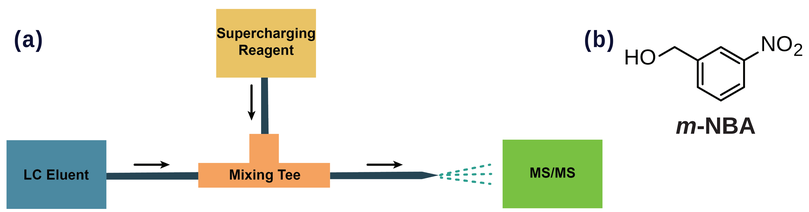



**Figure 2.** (a) Simplified diagram of post-column mixing tee set-up to introduce supercharging reagent. (b) Stucture of supercharging reagent *m*-nitrobenzyl alcohol (*m*-NBA).

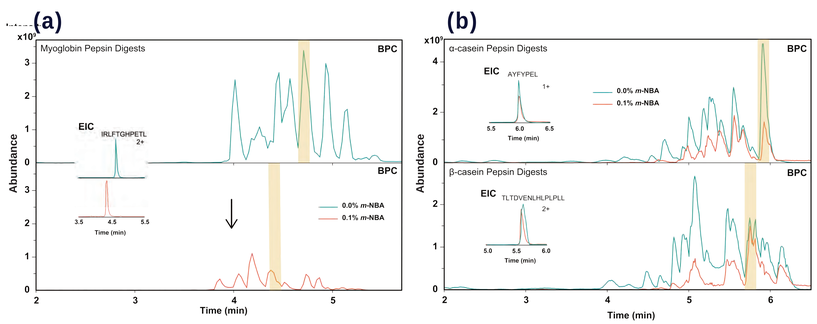



**Figure 3.** Incorporation of supercharging reagents directly into the mobile phase causes retention time shifting for myoglobin peptic peptides (a). Adding *m*-NBA post-column to α- and β-casein digests avoids such shifting (b).

## AVERAGE CHARGE STATE INCREASE UPON SUPERCHARGING

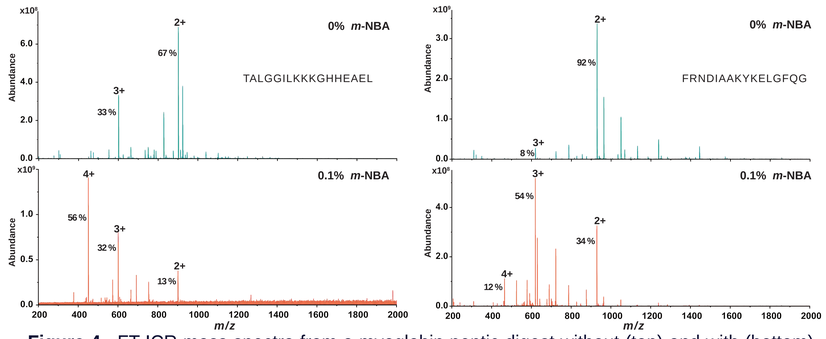



**Figure 4.** FT-ICR mass spectra from a myoglobin peptic digest without (top) and with (bottom) 0.1% *m*-NBA added to the ESI solution post-column.

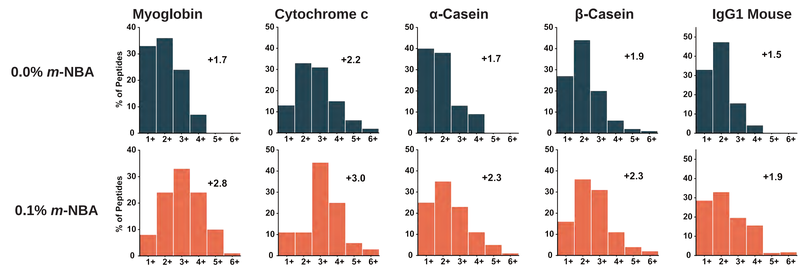



**Figure 5.** Bar graphs indicating increase in higher charge state peptides for both small and large proteins including an antibody. Average calculated charge states shown in the upper right corners.

## COMPATIBILITY OF *m*-NBA-BASED SUPERCHARGING WITH HDX

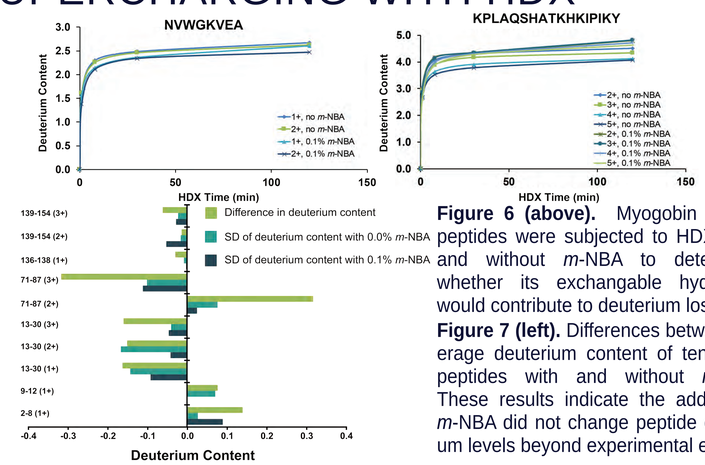
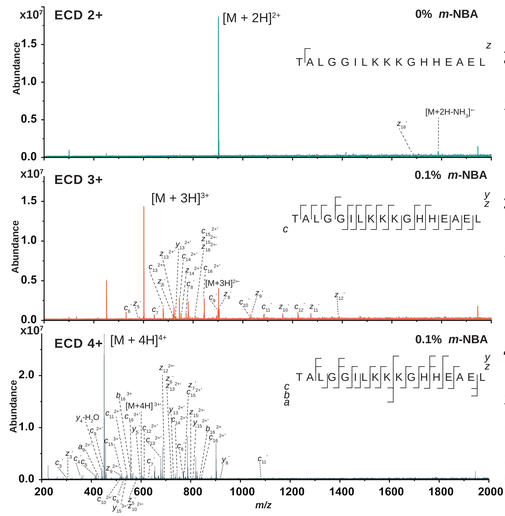



**Figure 6 (above).** Myogobin peptic peptides were subjected to HDX with and without *m*-NBA to determine whether its exchangable hydrogen would contribute to deuterium loss.

**Figure 7 (left).** Differences between average deuterium content of ten peptic peptides with and without *m*-NBA. These results indicate the addition of *m*-NBA did not change peptide deuterium levels beyond experimental error.

## IMPROVED FRAGMENTATION UPON SUPERCHARGING




**2+ precursor ions**
Fragmentation efficiency: 12%
Sequence coverage: 6%

**3+ precursor ions**
Fragmentation efficiency: 72%
Sequence coverage: 100%

**4+ precursor ions**
Fragmentation efficiency: 63%*
Sequence coverage: 94%

*Attributed to the lower abundance of 4+ ions. The 4+ precursor yielded two unique *c*-type ions and two unique *z*-type ions compared with ECD of 3+ ions.

**Figure 8.** ECD MS/MS spectra of a doubly-, triply-, and quadruply-charged peptic peptide. Maximum sequence information was obtained by combining all spectra.

## CONCLUSIONS AND FUTURE WORK

In this work, we demonstrated use of a supercharging reagent to increase the overall charge state of peptic peptides generated from large and small proteins including an antibody. While the particular supercharging reagent studied did not contribute to loss of HDX information, chromatographic retention times shifted significantly. After implementing a mixing tee and additional pump post-column to deliver the supercharging reagent in our set-up, we no longer noticed this issue.

Going forward, we aim to apply this strategy to biotherapeutics aided by a Trajan Leap automated HDX system. It is our goal to use supercharging and HDX-MS to study a variety of antibodies such as comparing biosimiliars under stressing conditions to gain stability information.

## ACKNOWLEDGMENTS

**References:**

[1] Jørgensen, T. J. D. et al. *J. Am. Chem. Soc.* **8**, 2785-2793 (2005)
[2] Rand, K. D. et al. *J. Am. Chem. Soc.* **4**, 1341-1349 (2008)
[3] Kalli, A. et al. *Mol. Biosyst.* **6**, 1668-1681 (2010)
[4] Good, D. M. et al. *Mol. Cell. Proteomics.* **11**,1942-1951 (2007)
[5] Kjeldsen, F. et al. *Anal. Chem.* **79**, 9243-9252 (2007)
[6] Meyer, J. G. et al. *J. Am. Soc. Mass. Spectrom.* **23**, 1390-1399 (2012)
[7] Going, C. C. et al. *Anal. Chem.* **87**, 3973-3980 (2015)

This work was supported by the University of Michigan and an Agilent Technologies Thought Leader Award.