# Evaluating the Addition of Supercharging Reagents During CESI-MS for Identification & Characterization of Biopharmaceuticals

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## OVERVIEW

**Purpose**: Our purpose of this study is to evaluate the effect of supercharging reagents on peptide ionization during CESI-MS. CESI refers to the integration of capillary electrophoresis (CE) with electrospray ionization (ESI) into one dynamic process within the same devise.

**Methods**: Trypsin digested monoclonal antibody (mAb) was analyzed by tandem MS. Supercharging reagents were added to the CE background electrolyte (BGE).

**Results:** Supercharging during CESI-MS showed a significant increase in the number of higher charge states attained by the peptides. In addition, for glycopeptides, the intensity of the precursor ions as well as fragment ions increased with supercharging.

## INTRODUCTION

- Supercharging reagents have been demonstrated to enhance the multiple charging of proteins and peptides during ESI. This improves the mass measurement accuracy and fragmentation efficiency.
- Recently, Bonvin et al., have added the supercharging reagents to the sheath liquid interface during CE-MS to modulate charge state distribution of proteins.[1] But, the sheath flow dilutes the analytes migrating from the capillary as well as increasing the flow. Alternatively, supercharging reagents can be directly added to the BGE during CESI-MS, a ultra-low flow technique, to further maximize ionization.
- Palivizumab, a humanized therapeutic antibody, targeted against respiratory syncytial virus, is an ideal representation of IgG1 type monoclonal antibody. Tryptic peptides generated from this antibody were analyzed by CESI-MS and supercharging reagents were added to evaluate the efficiency of charge enhancement during CESI-MS.

## MATERIALS AND METHODS

**Sample preparation:** 100 µg of palivizumab was solubilized, reduced with DTT, alkylated with IAM, and digested overnight with trypsin. The sample was then dried down and resuspended in 100 µl of leading electrolyte (100 mM ammonium acetate at pH 4).

**CESI-MS conditions:** 50 nL of the sample was injected into the separation capillary and transient-isotachophoresis focuses the sample during electrophoretic separation. 10% acetic acid served as the BGE and a separation voltage of 20 kV at normal polarity was applied. For supercharging experiments, reagents such as m-NBA and sulfolane were added to the BGE.

Beckman Coulter CESI 8000 was connected on-line to a TripleTOF® 5600+ MS (SCIEX, Framingham, MA). Information dependent acquisition (IDA) mode consisting of 250 msec TOF MS survey scan and 50 msec IDA on the top 30 ions which exceed 150 cps was utilized for data acquisition. Rolling collision energy was used to induce fragmentation and the dynamic exclusion time was set to 5 sec. The total run time was set to 60 min. Auto-calibration was enabled once every 3 runs.

**Data analysis:** Data analysis was performed using SCIEX BioPharmaView™ and ProteinPilot™ software. Glycopeptides were extracted manually and MS/MS spectra were checked for diagnostic ions.

## RESULTS

### I. Identification of small hydrophilic and large hydrophobic peptides

- Small hydrophilic peptides, which are often lost in the void volume in RP-LC can be identified well by CESI-MS. Shown here are the peptides with 2-5 residues identified from both heavy and light chains (HC & LC) of palivizumab.
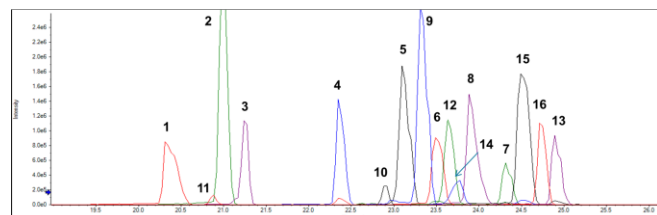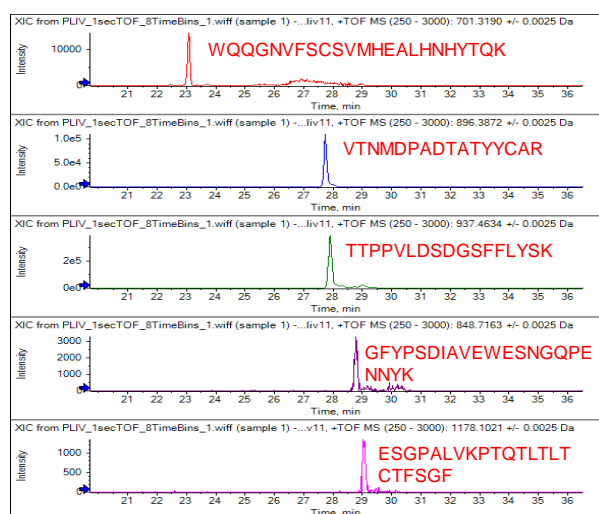


**Extracted ion electropherogram (EIE) of tryptic peptides from palivizumab ranging from 2 to 5 amino acids. The table provides more information about each peptide in the EIE.**

## REFERENCES

Bonvin, G., Rudaz, S., Schappler, J. Analytica Chimica Acta 813 (2014) 97-105.

| No. | Sequence | m/z observed | Charge | Ab chain |
|---|---|---|---|---|
| 1 | AK | 218.1503 | 1 | HC |
| 2 | SR | 262.152 | 1 | HC |
| 3 | APK | 315.2031 | 1 | LC |
| 4 | EAK | 347.1935 | 1 | LC |
| 5 | EYK | 439.2193 | 1 | HC |
| 6 | VEPK | 236.6423 | 2 | HC |
| 7 | DTSK | 225.6136 | 2 | HC |
| 8 | VQWK | 280.6635 | 2 | LC |
| 9 | TISK | 448.2772 | 1 | HC |
| 10 | GQPR | 457.2513 | 1 | HC |
| 11 | VDKR | 517.3084 | 1 | HC |
| 12 | SCDK | 255.1054 | 2 | HC |
| 13 | ADYEK | 313.1451 | 2 | LC |
| 14 | QPPGK | 263.6529 | 2 | HC |
| 15 | LTISK | 281.1845 | 2 | HC |
| 16 | LTVDK | 288.1741 | 2 | HC |

- Electrophoretic separation is based on the charge-to-hydrodynamic volume ratio of the peptides and not based on hydrophilic / hydrophobic interactions with the separation capillary. Large hydrophobic peptides can also be identified equally well, resulting in the high sequence coverage obtainable by this technique.



**Extracted ion electropherogram (EIE) of peptides from palivizumab HC ranging from 16 to 23 amino acids. The table provides more information about each peptide in the EIE.**

| Sequence | m/z observed | Charge | # of aa |
|---|---|---|---|
| [420]WQQGNVFSCSVMHEALHNHYTQK[442] | 701.319 | 4 | 23 |
| [84]VTNMDPADTATYYCAR[99] | 896.3872 | 2 | 16 |
| [396]TTPPVLDSDGSFFLYSK[412] | 937.4634 | 2 | 17 |
| [374]GFYPSDIAVEWESNGQPENNYK[395] | 848.7163 | 3 | 22 |
| [6]ESGPALVKPTQTLTLTCTFSGF[27] | 1178.1021 | 2 | 22 |

### II. Charge state distribution of peptides during supercharging

- Sulfolane and m-NBA were added to the BGE (10% acetic acid) at 1% concentration (v/v).
- Number of spectra obtained at 95% confidence was plotted as a function of charge states for each instance.
- With 1% sulfolane, the % of confident spectra at 3+ and 4+ charges increased compared to without any reagent.
- With 1% m-NBA, 50.4% of confident spectra were obtained from 3+ charge state.
- Small % of confident spectra at 5+ charges were present only when m-NBA or sulfolane was added.
- Addition of supercharging reagents results in an increase in the average and maximum charges attained by the peptides. Relative intensity of the highest observed charge state also increases. For instance, in palivizumab HC, a 63 amino acid tryptic peptide without any missed cleavage had 5 fold more intensity with 1% m-NBA.
- Consequently, this peptide was identified by MS/MS with a total of 22 b & y fragment ions only when m-NBA was present.
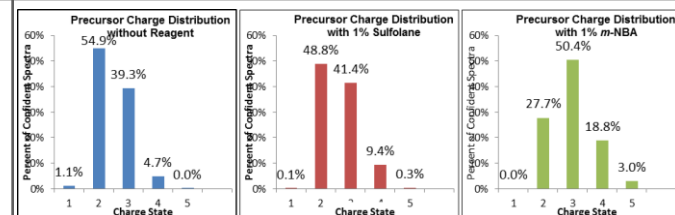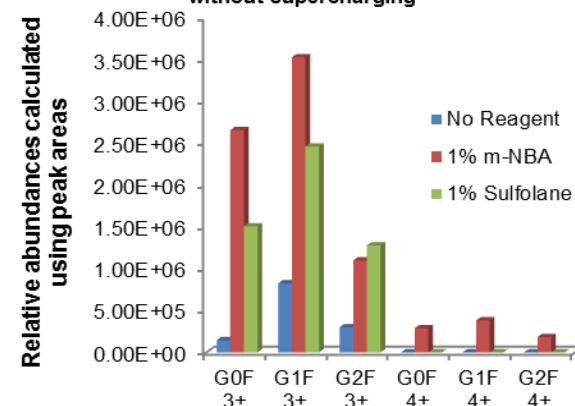
**EIE of 63-mer tryptic peptide without any reagent (A) and with 1% m-NBA (C). (B) and (D) show the mass spectrum without and with 1% m-NBA respectively.**
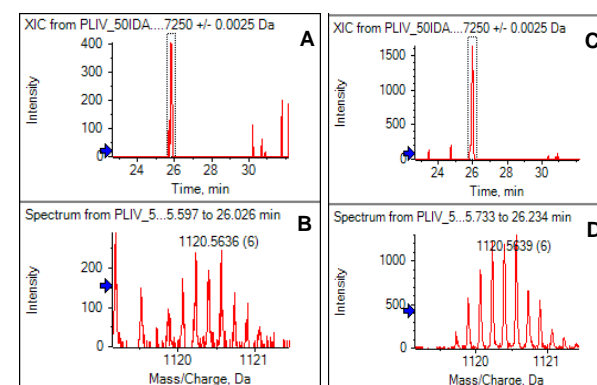


### III. Glycopeptide identification and supercharging

- Relative intensities of glycopeptides (G0F, G1F, & G2F) from palivizumab with/without supercharging were compared. Addition of either reagent showed a significant increase in the relative abundance of all 3 glycopeptides at 3+ charge state.
- Increased intensity of glycopeptide precursor ions obtained from supercharging resulted in highly intense fragment ions during tandem MS. The intensity of [peptide+glycan] fragments increased by approximately 5 to 10 fold for all three glycopeptides.



**Tandem MS spectra of G0F glycopeptide at 3+ charge state compared with and without supercharging reagents. The inset shows the relative abundance of fragment ion at 1392 m/z.**
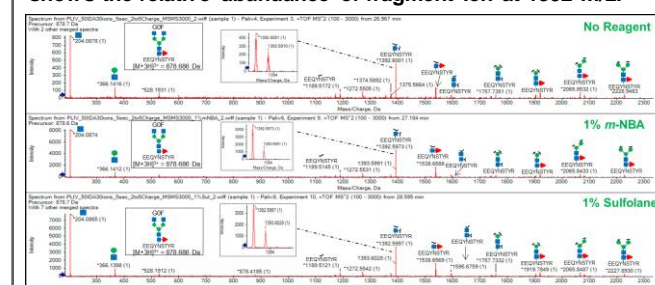


## CONCLUSIONS

- CESI-MS allowed efficient analysis of both short hydrophilic and long hydrophobic peptides, thereby providing high sequence coverage for antibody identification.
- Charge state enhancement was observed for tryptic peptides during CESI-MS. Higher charge states fall in the lower m/z region and thus, can be fragmented without any limits for precursor selection during MS/MS.
- Addition of supercharging reagents during CESI-MS enhances glycopeptide identification. The relative abundances of precursor & fragment ions significantly improved for different glycopeptides. Furthermore, greater S/N ratio, improved peak shape, and mass resolution can potentially benefit automated glycopeptide analysis.