ULTRA-LOW ESI-MS FLOW RATES MAKE A DIFFERENCE IN THE ANALYSIS OF BIOThERAPEUTICS

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ABSTRACT
Biopharmaceuticals, especially therapeutic monoclonal antibodies, have emerged as a very promising new generation of protein drugs, but concomitantly represent new analytical challenges for the field. While these large biomolecules require comprehensive structural characterization, their heterogeneity and complexity in most instances are beyond the application domain of the analytical techniques available today. Integration of capillary electrophoresis with electrospray ionization in a single dynamic process (termed CESI) coupled with high resolution mass spectrometry holds the promise to fulfill this gap, even at the intact protein level. Some of the main advantages of CESI-MS are the ability to produce stable electrospray at ultra-low flow rates (5-20 nL/min range) in a robust and reliable manner. In this presentation, the effect of CESI flow rate on ionization efficiency, ion suppression and detection sensitivity will be discussed. Our intact therapeutic antibody analysis results demonstrated that the sensitivity of CESI-MS was increased by an order of magnitude with the decrease of the flow rate from 250 nL/min to 20 nL/min. On the other hand, ultra-low flow rates significantly (2.5x) reduced the ion suppression effect in respect to samples containing both highly and weakly ionizable analytes of biotherapeutic interest.

SAMPLE PREPARATION
Mixtures of maltotetraose (M = 684.12 g/mol) and neurotensin (M = 1674.04 g/mol) were prepared in equimolar concentration of both analytes at 10⁻³ M in a mixture of 10 mM aqueous ammonium acetate and methanol (1:1 by volume). For the intact protein analysis Humira was analyzed at a concentration of 3 µM in 5% formic acid solution.

INSTRUMENTATION

CESI 8000 conditions

Uncoated capillary
Pressure infusion
Capillary i.d.: 30 µm
Capillary length: 91 cm

CESI 8000 was hyphenation with Thermo LTQ and Q-Exactive mass spectrometers, respectively.

ION SUPPRESSION
The liquid flow rate in electrospray ionization (ESI) determines the initial droplet size, thus plays an essential role in the efficiency of the spray process. It has also been reported that genuine nano-ESI, where ion suppression is sufficiently low or even negligible, is only available under a given flow rate limit of 20 nL/min. While previous studies focused on the determination of such a flow rate limit with regular ESI spray settings, this is the first study to investigate this effect with the porous sprayer (CESI setup). CESI is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single dynamic process². In this presentation, flow rates were controlled by the built-in pump of a CESI 8000 instrument and were accurately determined using the Hagen-Poiseuille equation:

\[ \Delta P = \frac{4 \mu L \Delta V}{\pi r^4} \]

where \( \Delta P \) is the pressure difference, \( \mu \) is the dynamic viscosity, \( L \) is the total length of the capillary, \( V \) is the volumetric flow rate and \( r \) is the radius of the capillary.

First the ion suppression phenomenon was systematically studied using a well-defined oligosaccharide – peptide mixture. Maltotetraose represented an uncharged oligosaccharide, which is considered a weakly ionizable analyte. Neurotensin, on the other hand, is an easily protonated, 13 amino acid peptide. Figure 1 shows the signal intensity ratios as the function of the increasing flow rate. As one can observe, the lower the flow rate the lower the neurotensin / maltotetraose intensity ratio, i.e., lower the ion suppression. As a first approximation we consider that lower flow rates produce smaller initial droplet size thus the formed free ions have a more diluted chemical environment where analyte – analyte interactions are less pronounced. Consequently, the inherent charge of neurotensin has no such a significant effect on the individual ionization efficiency of the maltotetraose molecules.

![Figure 1 Signal intensity ratios at different flow rates between neurotensin (NT) and maltotetraose (MT) calculated as (NT)⁺ / (MT)⁺ / (MT)⁻].

![Figure 2 Characteristic MS spectra of Δ²⁺:1 neurotensin and maltotetraose mixture at 9.12 nL/min flow rate.](image)

![Figure 3 Humira infusion at 20 nL/min (A) and 250 nL/min (B) flow rate. 20 min integration was used to generate MS spectra.](image)

![Figure 4 Normalized signal intensities at 20 nL/min (left) and 250 nL/min (right) flow rates. Sample: 3 µM Humira.](image)

Conclusions

**Increased sensitivity.** CESI-MS at a flow rate of 20 nL/min showed increased sensitivity by an order of magnitude compared to higher flow rate (250 nL/min). **Reduced sample requirement.** CESI-MS at a flow rate of 20 nL/min produces the same spectrum quality at the intact protein level as at a flow rate of 250 nL/min. **Decreased ion suppression.** Ultra-low flow rates (< 20 nL/min) significantly (2.5x) reduced the ion suppression effect in respect to samples containing both highly and weakly ionizable analytes.

REFERENCES

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