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Ionization Efficiency, Ion Suppression and Detection Sensitivity of CESI-MS

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Introduction

Regulatory approval requires full structural and functional characterization, but the heterogeneity and complexity of biologics in most cases are not easily addressable with the analytical techniques available today.[1] Full characterization can reveal changes in primary amino acid sequences, quality attribute modifications and post-translational modifications (PTMs), including glycosylation, that may affect therapeutic efficacy, bioavailability and biosafety. Monoclonal antibodies (mAbs) are widely used in therapies for autoimmune and infectious diseases as well as to combat cancer. With a number of innovator patents expiring, a growing number of biosimilar alternatives are expected to enter the market. Liquid phase separation methods (liquid chromatography and capillary electrophoresis) followed by mass spectrometry will play a crucial role in the field of biotherapeutics analysis.[2] Capillary electrophoresis (CE) coupled with mass spectrometry (MS) is a powerful combination of a high-performance liquid phase separation technique and a versatile detection method, providing excellent selectivity, high sensitivity and structural information.[3] Due to the electrophoretic mobility-based separation of CE, results are orthogonal to LC-MS approaches, providing new or confirmatory results. The unique design of the CE separation capillary with an integrated etched tip4 for electrospray ionization (CESI) provides much promise to fulfill some of the new requirements in biotherapeutics characterization at all levels, including intact (level 1), reduced (level 2), bottom up (level 3) and glycosylation (level 4) analyses. In particular, CESI-MS benefits from the inherent ultra-low flow rate of the method (a few nL/min), generally termed as nano-electrospray ionization (nano-ESI), featuring high ionization efficiency and excellent sensitivity. The use of very low flow rates enables the application of highly aqueous solutions for ESI-MS,[5] ideally suited for high-performance CE separations. Notably, aqueous buffers are suitable electrolytes for the analysis of hydrophilic molecules such as glycopeptides and glycoproteins. Furthermore, nano-ESI has reduced sample requirements, which
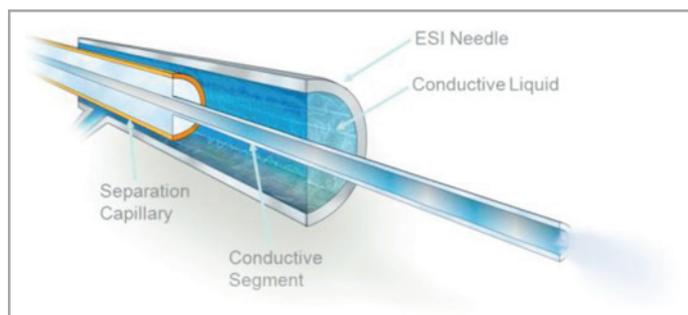
Figure 1. The OptiMS cartridge from SCIEX provides an ultra-low flow ESI interface.

is a valuable advantage when the sample availability is limited, as it is during clone selection.

In the pioneering paper of Schmidt et al.,[6] some of the advantageous characteristics of nano-ESI were demonstrated, including the suggestion that ion suppression is apparently eliminated at flow rates below 20 nL/min. Subsequently, a low flow rate ESI-MS interface was introduced for capillary electrophoresis with ESI-MS.[4] This design included an etched tip at the capillary outlet providing adequate connection to close the high-voltage separation circuit and simultaneously providing the high voltage necessary for the electrospray process. The approach is termed CESI, referring to the integration of capillary electrophoresis and electrospray ionization into a single dynamic process.[7] A handful of CESI infusion studies have already shown the ionization efficiency benefits at ultra-low flow rates. The first of these experiments illustrated an ~18-fold improvement in peptide sensitivity (counts/mole) when comparing flow rates at tens to hundreds of nanoliters per minute.[8] Infusion of a phosphopeptide mixture at 10 and 100 nL/min showed dramatically reduced ion suppression for highly phosphorylated peptides,[9] an otherwise common problem in shotgun (phospho) proteomics. Most recently, a set of intact protein and native MS infusion experiments using CESI illustrated sensitivity improvements when operating in the ultra-low flow range.[2]

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This technical note reports the effect of CESI-MS flow rate on ionization efficiency, ion suppression and detection sensitivity for analytes of biotherapeutic interest. All experiments were carried out in infusion mode, thus no electric field mediated variations (electrophoretic and/or electroosmotic mobility) was considered. The ion suppression phenomenon was systematically studied using a well-defined oligosaccharide and peptide mixture representing molecules of contrasting ionization capability. The sensitivity of the approach at the intact protein level was examined by infusing Humira, a commercially available mAb. While most previous studies focused on the advantageous characteristics of nano-ESI with sprayers pulled from glass capillaries, we report on continued studies to investigate the effect of ultra-low flow rates with a commercial CESI capillary.

Experimental

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) ¬unless otherwise noted. Mixtures of maltotetraose (M = 684.12 g/mol) and neurotensin (M = 1674.04 g/mol) were prepared in equimolar concentration of both analytes at 10–5 mol/L in a 1:1 mixture of 10 mM aqueous ammonium acetate and methanol (J.T. Baker, Center Valley, PA). At the intact protein level, Humira, also called adalimumab (Abbott Laboratories, Abbott Park, IL) was analyzed (3 µM in 3% formic acid).

Flow rate experiments were carried out with online hyphenation of a CESI 8000 Plus High Performance Separation-ESI Module (Beckman Coulter, sold through SCIEX, Brea, CA) capillary electrophoresis unit and an LTQ mass spectrometer (Thermo, Bremen, Germany). The total length of the separation capillary was 91 cm with 150 µm outer and 30 µm inner diameter. Typical instrument parameters of the LTQ system were: positive polarity, 200 °C capillary temperature, 35 V capillary voltage, 1.8 kV spray voltage, 100 V tube lens voltage, 400–2000 m/z scan range, 1 micro scan, 3e5 AGC target and 10 ms max injection time. A Thermo Q Exactive mass spectrometer was used during the intact protein analysis experiments with the following setup: positive polarity, 350 °C capillary temperature, 1.6 kV spray voltage, 25 V S-lens voltage, 100 S-lens RF level, 2000–4000 m/z scan range, 140 K resolution, 10 micro scan, 3e6 AGC target and 60 ms max injection time.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.

- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Theory

Previously, we calculated flow rates based on the time for the sample to fill an entire capillary, and we concluded that neither electrospray nor the MS vacuum had an effect on the flow inside the capillary.[2] Based on these results, flow rates were controlled with the built-in pressure system in the CESI 8000 Plus instrument and were estimated using the Hagen-Poiseuille equation:

$$\Delta P = \frac{8\mu L \dot{V}}{\pi r^4} \qquad (1)$$

where ΔP is the pressure difference, μ 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.
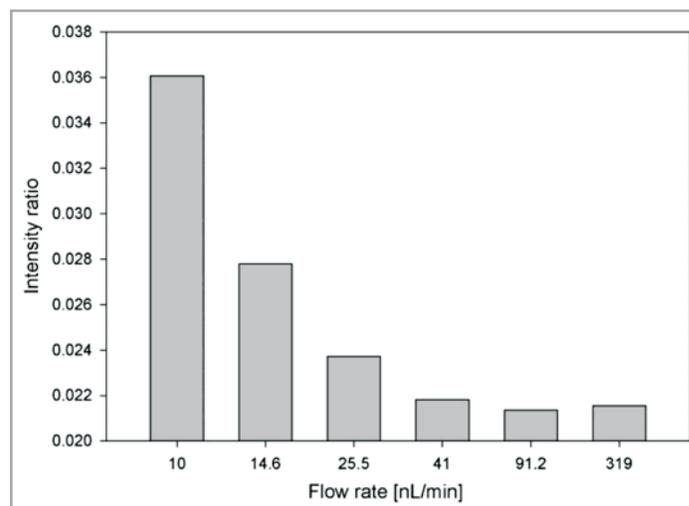

Figure 2. Measured signal intensity ratios at different flow rates between maltotetraose (MT) and neurotensin (NT) calculated as $MT^{+1}/(NT^{+1} + NT^{+2} + NT^{+3})$. Maltotetraose and neurotensin were infused with an equimolar mixture.

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Results and Discussion

Ion suppression: The ion suppression phenomenon was systematically studied using a well-defined oligosaccharide/peptide mixture. Maltotetraose is a hydrophilic oligosaccharide with no charge and is considered weakly ionizable. Neurotensin is a 13 amino acid peptide that is easily protonated and ionized. Figure 2 depicts the calculated maltotetraose/neurotensin signal intensity ratios at increasing flow rates from 10 nL/min to > 300 nL/min. As observed, lower flow rates resulted in a higher-intensity ratio, generally following an exponential trend. The higher ratio is the result of an increased maltotetraose signal from the reduced ion suppression at the lower flow rates. In practice, for biotherapeutic molecules characterized by CESI-MS either as sugars or those containing sugars, they will be more effectively ionized, detected and quantified, and their signals will more closely represent their actual abundance.

We then plotted the normalized signal intensities as a function of flow rates for the maltotetraose and neurotensin (the latter in +1, +2 and +3 charge states), as depicted in Figure 3. Please note that an equimolar mixture of maltotetraose and neurotensin were infused. Figure 3 further verifies that ion suppression was practically negligible at a ~20 nL/min flow rate. Normalized signal intensities (i.e., per mole) converge to a saturation regime starting at around 20 nL/min, comparable to earlier published results.[6] This is notable since they fit their data to 3 different theoretical ESI models based on scaling laws relating to initial droplet size and flow rate. Thus the benefits of using CESI are consistent with those of nano-ESI. Additionally, since the CESI capillary has an etched, electrically conductive tip instead of a metal-coated, pulled tip, it does not suffer from typical clogging issues or loss of conductive, metal coating.
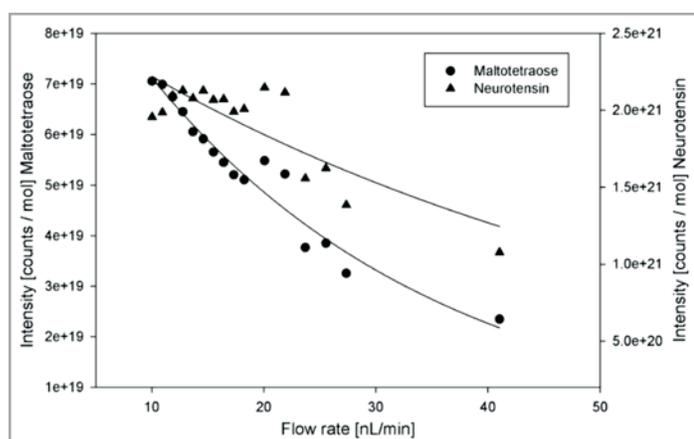
Figure 3. Normalized signal intensities at different flow rates of maltotetraose and neurotensin (+1, +2, and +3 charge states). Maltotetraose and neurotensin were infused with equimolar mixture.

It is known that lower flow rates produce smaller initial droplet sizes.[6] However, it is not known precisely how droplet size influences ionization efficiency. The widely accepted ion evaporation[10] and charge residual models[11] focus on how the majority of the charges are transferred from the droplet to the analyte, but the molecular dynamics of ESI are still not completely understood.[12] Our experiments attempted to shed light on the nature of ESI phenomena from a physical chemistry point of view.

As a first approximation, we considered that specific surface-to-volume ratios play an important role during the ion suppression phenomena. For a binary system, where aqueous droplets are dispersed in the air, the liquid phase (εrl, relative permittivity ~80) acts as a conductor, while air acts as an insulator (εr, relative permittivity ~ 1). Thus charge carriers are situated at the interface surface, i.e., an electrospray droplet carries a net positive charge at the droplet surface, so molecules with inherent positive charges, such as peptides (in our case, neurotensin), accumulate in the bulk phase of the droplet due to repel forces (Coulomb's law). Consequently, assuming equal initial concentration of both analytes, neutral molecules will have a partially higher concentration at the surface since they

do not repel each other. This can be considered a valuable competitive advantage for charges during the droplet fission, as a higher surface-to-volume ratio ensures a higher concentration of neutral compounds in the surface layer. We also considered that the inherent charge of neurotensin had no significant effect on the individual ionization efficiency of the maltotetraose molecules.

The intact mAb analysis showed that robust, comprehensive and reproducible characterization of intact protein therapeutics is very important for the biopharmaceutical industry. As more complex therapeutic molecules are created, the demand for high-performance and sensitive bioanalytical methods is pushed to its limits. In addition to molecular complexity, other challenges may arise when sample availability is limited,[13] such as during the discovery phase or in pharmacokinetics/pharmacodynamics studies. mAbs are subject to co- and post-translational modifications, such as glycosylation site occupancy and micro-heterogeneity as well as degradative PTMs, resulting in changes that may affect effector function, antigenicity and immunogenicity.[14] One type of mAb analysis is performed at the peptide level after tryptic digestion (level 3 analysis), but information about the intact structure of the molecules, such as modification stoichiometry and heterogeneity, is lost.[15] CESI provides mAb analysis at the intact protein level (level 1) even

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from very small amounts of samples. The sensitivity of the CESI-MS setup for intact protein analysis was evaluated by comparing the MS spectra measured at different flow rates. Figure 4 compares the MS spectra from the analysis of an intact therapeutic monoclonal antibody (Humira) by simple infusion using the CESI sprayer at ultra-low (A: 20 nL/min) and low (B: 250 nL/min) flow rates. Please note that the MS conditions were not optimized, as our interest in this work was to demonstrate the capabilities and advantages of CESI-MS analysis with ultra-low flow rates. The spectra in Figure 4A were obtained by infusing a non-desalted Humira sample (3 μM in 3% formic acid) with a 20 nL/min flow rate. Figure 4B shows the same experiment with a higher flow rate (250 nL/min). Although qualitative comparison of the spectra in Figure 4 revealed no significant differences between 20 and 250 nL/min flow rates, upon further
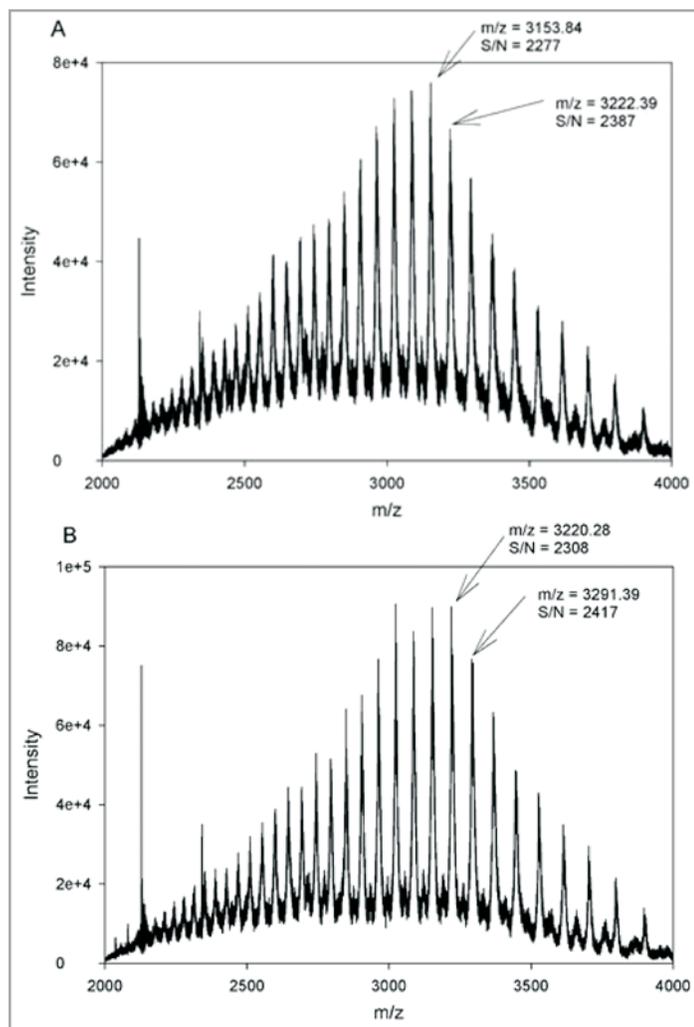
consideration, a notable advantage in sensitivity was observed that was not previously shown with other protein molecules [2, 8]. By integrating both spectra over the same infusion times, a lower flow rate required significantly less sample to obtain the same information. In this case, the signal intensity (in counts per mole) was 12.5 times greater at 20 nL/min than at 250 nL/min. This ratio was proportional to the flow rate and clearly illustrated the benefits of performing nano-ESI mAb analysis using CESI-MS, in terms of sensitivity at ultra-low flow rates. While this is not only an interesting attribute of CESI-MS, it is also most applicable when sample availability is limited. For instance, over the 20 min course of the 2 different flow rate analyses, only 173 ng of Humira was used at 20 nL/min, while 2.16 μg was used at 250 nL/min.

Conclusions

The systematic study of this work demonstrated the effect of low flow rate on ionization efficiency, ion suppression and detection sensitivity using CESI-MS for molecules of biotherapeutic relevance. The results of this technical information bulletin reconfirm that there is an exponential drop in ion suppression at ultra-low flow rates. Throughout the flow rate experiments, CESI enabled stable and reproducible electrospray even at < 20 nL/min. The ion suppression effect was reduced by 2.5-fold for the weakly ionizable oligosaccharide in the presence of an easily ionizable peptide at the ultra-low flow level.

Infusion experiments of a therapeutic mAb with CESI-MS at the flow rates of 20 nL/min (typical electroosmotic flow in CE) and 250 nL/min (typical flow rate in nanoLC and sheath-flow CE-MS) demonstrated enhanced sensitivity, i.e., an order of magnitude better and proportional to the flow rate ratio. It was also demonstrated that using a 20 nL/min flow rate in CESI resulted in apparently the same spectrum quality at the intact protein level as the higher flow rate of 250 nL/min, and thus facilitated the analysis of mass-limited samples. In summary, our infusion experiments illustrated a valuable advantage in CESI-MS sensitivity over sheath-flow CE-MS approaches, which typically operate at similar flow rates as nanoLC. By combining the capabilities of a CE separation with ultra-low flow rate nano-ESI conditions, CESI-MS provides a powerful tool for sensitive and comprehensive characterization biotherapeutics.

Figure 4. Humira infusion at 20 nL/min (A) and 250 nL/min (B) flow rates. 20 min integration was used to generate the MS spectra.

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