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Technical Note

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Characterization of a Porous Nano-electrospray Capillary Emitter at Ultra-low Flow Rates

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Abstract

Biopharmaceuticals, especially therapeutic monoclonal antibodies, have emerged as a very promising new generation of protein-based drugs. However, their comprehensive analysis continues to pose new challenges for the bioanalytical field. Hyphenation of capillary electrophoresis with electrospray ionization (CE-MS) is a promising technique to address these challenges. One of the main advantages of CE-MS is the ability to produce stable electrospray at ultra-low flow rates (5–20 nl/min range). In this short communication we report on the characterization of a porous nano-electrospray capillary emitter focusing on the effects of ultra-low flow rate on ionization efficiency, ion suppression and detection sensitivity. Ion suppression effect of a poorly-ionizable sugar in the presence of an easily-ionized peptide was reduced by almost 2-fold. Intact therapeutic antibody infusion analysis demonstrated that MS detection sensitivity increased by an order of magnitude with the decrease of flow rate from 250 nL/min to 20 nL/min using the nano-electrospray capillary emitter.

Introduction

Regulatory approval of biotherapeutics, such as therapeutic monoclonal antibodies (mAbs), require full structural and functional characterization, but their heterogeneity and complexity in most cases are not easily addressable with the analytical techniques available today (1, 2). Comprehensive characterization is required since unwanted changes in primary amino acid sequences, quality attribute modifications, and post-translational modifications (including glycosylation) could impact therapeutic efficacy, bioavailability and biosafety; thus their in-depth analysis is very important (3). Liquid phase separations (HPLC and capillary electrophoresis) followed by mass spectrometry play a dominant role in the field of biotherapeutics analysis (4–6). Capillary electrophoresis (CE) hyphenated with mass spectrometry (MS) is a powerful combination of a high performance liquid phase separation and a versatile detection method, providing excellent selectivity and high sensitivity (7). Due to the mobility-based separation of CE, results are orthogonal to LC–MS approaches, providing new or confirmatory results.

The CE separation capillary with a porous nano-electrospray capillary emitter (8) holds the promise to fulfill some of the new requirements in the characterization of biotherapeutics at all levels. In particular, ultra-low flow rates (a few nL/min) generally termed nano-electrospray ionization (ESI) (9) offers high ionization efficiency and sensitivity (10, 11). Using such very low flow rates enables the use of highly aqueous solutions for ESI-MS (12), ideally suited to obtain high performance separations by CE. Notably, aqueous buffers are suitable electrolytes for the analysis of hydrophilic molecules like glycopeptides and glycoproteins. Furthermore, nano-ESI has reduced sample requirements, a valuable advantage when sample availability is limited. On the other hand, the regular use of nano-ESI in the bioanalytical field was restricted by its

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relatively poor reproducibility (13, 14) and the lack of the necessary experience with this new technology.

In the pioneering paper of Schmidt *et al.* (15) the authors demonstrated some of the advantageous characteristics of nano-ESI and suggested that ion suppression is essentially eliminated at flow rates below 20 nL/min. At the time, the varying shapes and opening diameters of their pulled sprayer tips did not support good signal reproducibility, but there was no commercially available nano-ESI sprayer, which would have served as robust and reliable technique for their investigations. Later, Moini developed a low flow rate interface design for coupling liquid phase separation with ESI-MS (8) based on a porous etched tip at the capillary outlet. His approach led to the integration of capillary electrophoresis and the electrospray ionization into a single dynamic process (16). A handful of nano-ESI infusion studies have already showed ionization efficiency benefits at ultra-low flow rates. The first of these experiments illustrated a ~18-fold improvement in peptide sensitivity (counts/mole) when comparing flow rates at tens to hundreds of nanoliter per minute (17). Shortly after, infusion of a phosphopeptide mixture at 10 and 100 nL/min demonstrated dramatically-reduced ion suppression for the highly phosphorylated peptides (18), an otherwise common problem in shotgun (phospho)proteomics experiments. The Smith lab also reported on improved MS detection sensitivity of nano-ESI (19) and claimed poor reproducibly with the available emitters (20).

Most recently, a set of intact protein and native MS experiments illustrated the sensitivity improvements when operating in the ultra-low nanoliter per minute flow rate range using the integrated approach (5).

In this paper, we report on the effect of ultra-low flow rates on ionization efficiency, ion suppression and detection sensitivity for molecules of biotherapeutic interest using a porous nano-electrospray capillary emitter. All experiments were carried out in infusion mode, so that experimental variations from electromigration and/or electroosmosis during a CE separation did not need to be considered. The ion suppression phenomenon was studied in depth using a well-defined oligosaccharide–peptide mixture, similar to Schmidt *et al.* (15). The sensitivity of the approach at the intact protein level was investigated by infusion of Humira. While most previous studies focused on characterizing 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 nano-electrospray capillary emitter.

Experimental

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except if noted otherwise. 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). At the intact protein level, Humira (adalimumab) was analyzed in 3 µM concentration in 3% formic acid.

Flow rate experiments were carried out with the online hyphenation of CESI 8000 (Sciex Separations, Brea) and Thermo LTQ mass spectrometer (Bremen, Germany), while a Thermo Q-Exactive mass spectrometer was applied for intact protein analysis. The total capillary length was 91 cm (30 µm i.d./150 µm o.d.) with the porous nano-electrospray emitter. 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, 10 ms max injection time. The Thermo Q-Exactive was set up as: 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.

Infusion flow rates were controlled with a built-in pressure pump of the CESI 8000 instrument and were calculated using the

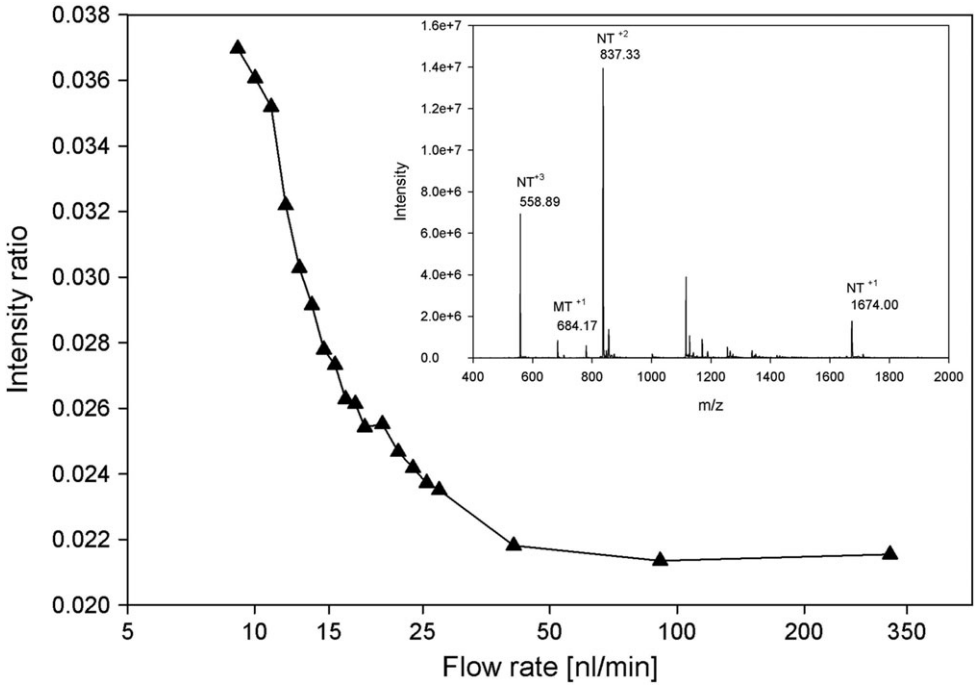


Figure 1. Signal intensity ratios at different flow rates between maltotetraose (MT) and neurotensin (NT) calculated as $MT^{+1}/(NT^{+1} + NT^{+2} + NT^{+3})$. Maltotetraose and neurotensin were infused in an equimolar mixture. The inset depicts a characteristic MS spectrum at 9.12 nL/min flow rate.

Hagen–Poiseuille equation: $\Delta P = (8\,\mu L\dot{V})/(\pi r4)$, 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. Buffer viscosity, 1.7 mPa s at 25°C was determined using Myr V1 (Tarragona, Spain) rotation viscosity meter.

Results

The ion suppression phenomenon in the porous nano-electrospray capillary emitter was characterized with the use of a well-defined oligosaccharide–peptide mixture: maltotetraose, an uncharged oligosaccharide considered to be weakly ionizable and neurotensin, an easily protonated and ionized 13 amino acid peptide. Figure 1 shows the signal intensity ratios as a function of the flow rate. For better visualization, the flow rate axis (X) is in logarithmic scale (consult Supplementary material for non-logarithmic plot). As observed, the lower the flow rate the higher the maltotetraose/neurotensin intensity ratio, generally following an exponential trend. The inset in Figure 1 depicts a characteristic MS spectrum of the 1:1 neurotensin–maltotetraose mixture at 9.12 nL/min flow rate. The higher ratio

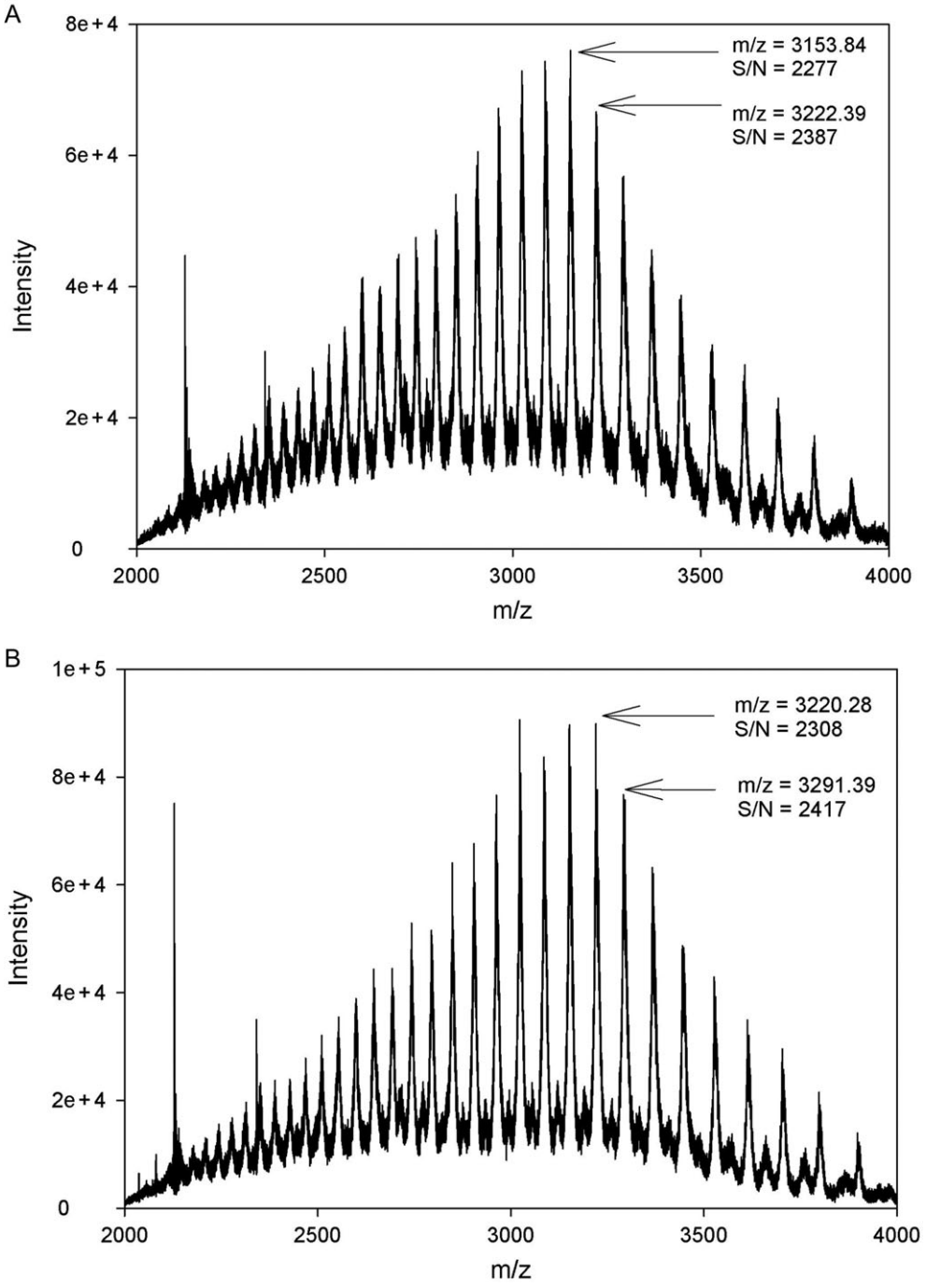


Figure 2. Humira infusions at 20 nL/min (A) and 250 nL/min (B) flow rate. Twenty minutes integration was used to generate MS spectra.

is the result of an increased maltotetraose signal from the reduced ion suppression at the lower flow rates. In practice, using ultra-low flow rates with nano-electrospray capillary emitters, biotherapeutic formulations characterized either as sugars (stabilizers during drug formulation (21)) or containing sugars (e.g., glycoproteins), will be more easily ionized, detected, quantified and their signals more closely represent their actual abundances. Similar observations, i.e., reduced ion suppression of hard to ionize substances at low nano-ESI flow rate was published by Woodab and coworkers (22) further supporting our current observations.

It is known, that lower flow rates produce smaller initial droplet sizes (15). However, it is not straightforward how droplet size influences the ionization efficiency. Both widely accepted models, namely the ion evaporation (23, 24) and charge residual models (25), focus on how the majority of charges are transferred from the droplet to the analyte, but the molecular dynamics of ESI is still not completely understood (26). Our experiments attempted to shed light on the nature of ESI phenomena from a physical–chemical point of view. As a first approximation we consider that specific surface-to-volume ratios play an important role. For a binary system, where aqueous droplets are dispersed in air, the liquid phase (ε_{rl}, relative permittivity ~80) acts as a conductor while air acts as an insulator (ε_{ra}, relative permittivity ~1). In such a system, charge carriers are situated at the interface surface, i.e., an electrosprayed droplet carries a net positive charge at the droplet surface, so molecules with inherent positive charges (like peptides, in our case neurotensin) accumulate in the bulk phase of the droplet due to Coulombic repulsion. Consequently, assuming equal initial concentration of both analytes, neutral molecules will have partially higher concentration at the surface, which can be considered as valuable competitive advantage for capturing charges during droplet fission. Higher surface-to-volume ratios ensure 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.

Discussion

Robust, comprehensive and reproducible characterization of intact protein therapeutics is of high importance for the biopharmaceutical industry. As more complex therapeutic molecules are created, the demands for high performance bioanalytical methods will be pushed to new limits. In addition to molecular complexity, other challenges can arise when sample availability is limited (27, 28), such as in discovery phase, in pharmacokinetic/pharmacodynamics or in clone selection studies. mAbs are subject to co- and post-translational modifications, e.g., glycosylation micro-heterogeneity and degradative events, which result in molecular changes that may affect antigenicity and immunogenicity (29). mAb analysis performed at the peptide level after tryptic digestion lacks information about the intact structure of the molecules, e.g., modification stoichiometry and heterogeneity is lost (2). Analysis of mAbs by nano-ESI can provide information at the intact protein level even from very small amounts of samples. Figure 2 compares the MS spectra from the analysis of an intact protein therapeutic (Humira) by simple infusion using the porous nano-electrospray emitter capillary at different flow rates. Note that the MS conditions were not optimized, as our interest in this work was to demonstrate the capabilities and advantages of nano-ESI-MS analysis with ultra-low flow rates. The spectra in Figure 2A was obtained by infusing a non-desalted 3 µM Humira sample with a flow rate of 20 nL/min. Figure 2B shows the same experiment at 12.5-fold higher flow rate (250 nL/min).

The sensitivity of the porous nano-electrospray capillary emitter setup for intact protein analysis was evaluated by comparing the MS spectra measured at different flow rates. A qualitative comparison of the spectra in Figure 2 revealed no significant differences between 20 and 250 nL/min flow rates. However, upon further consideration a notable advantage in sensitivity is observed that has been previously shown with other proteinaceous molecules (5, 17). By integrating the spectra over the same infusion times, the lower flow rate required less sample amount to achieve the same quality spectra. In this case, the signal intensity was 12.5 times greater at 20 nL/min (6.3×10^{16} counts/mol) than at 250 nL/min (6.04×10^{15} counts/mol). This ratio was proportional to the flow rate and clearly illustrated the benefits of performing nano-ESI mAb analysis by the porous nano-electrospray capillary emitter, in terms of sensitivity, at ultra-low flow rates. While this is not only an interesting attribute of nano-ESI-MS, it is also most applicable when sample availability is limited. For instance, over the 20 min course of the two 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 study in this paper demonstrated the effect of ultra-low flow rate on ionization efficiency, ion suppression and detection sensitivity for molecules of biotherapeutic relevance using a porous nano-electrospray capillary emitter. Of important theoretical consideration, results of the present study reconfirmed that ultra-low flow rates significantly decrease ion suppression. Throughout the flow rate experiments, the porous nano-electrospray emitter enabled stable and reproducible electrospray even within the ultra-low flow rate range (<20 nL/min). The ion suppression effect was reduced by almost 2-fold for a weakly-ionizable sugar in the presence of an easily-ionizable peptide. Infusion experiments of a therapeutic mAb with nano-ESI-MS at flow rates of 20 nL/min (typical electroosmotic flow rate of CE) and 250 nL/min (typical flow rate of nanoLC and sheath-flow CE-MS) demonstrated enhanced sensitivity. The sensitivity improvement was an order of magnitude better and inversely proportional to the flow rate ratio. It was also shown that 20 nL/min flow rate resulted in adequate spectrum quality at the intact protein level as high flow rate (250 nL/min), thus it could be concluded that nano-ESI-MS facilitates the analysis of mass-limited samples. In summary, the ultra-low flow rate infusion experiments using the porous nano-electrospray capillary emitter demonstrated a valuable sensitivity advantage and the setting can be considered as a powerful tool for high sensitivity characterization of biotherapeutics.

Supplementary Data

Supplementary data are available at *Journal of Chromatographic Science* online.

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