

In-Spray Supercharging of Peptides and Proteins in Electrospray Ionization Mass Spectrometry

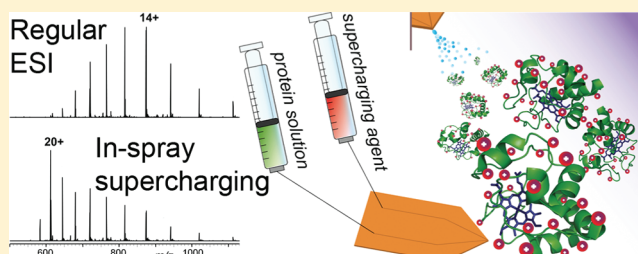
Saša M. Miladinović,[†] Luca Fornelli,[†] Yu Lu,[‡] Krzysztof M. Piech,[†] Hubert H. Girault,[‡] and Yury O. Tsybin^{*,†}

[†]Biomolecular Mass Spectrometry Laboratory, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

[‡]Laboratoire d'Electrochimie Physique et Analytique, Station 6, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

S Supporting Information

ABSTRACT: Enhanced charging, or supercharging, of analytes in electrospray ionization mass spectrometry (ESI MS) facilitates high resolution MS by reducing an ion mass-to-charge (m/z) ratio, increasing tandem mass spectrometry (MS/MS) efficiency. ESI MS supercharging is usually achieved by adding a supercharging reagent to the electrospray solution. Addition of these supercharging reagents to the mobile phase in liquid chromatography (LC)-MS/MS increases the average charge of enzymatically derived peptides and improves peptide and protein identification in large-scale bottom-up proteomics applications but disrupts chromatographic separation. Here, we demonstrate the average charge state of selected peptides and proteins increases by introducing the supercharging reagents directly into the ESI Taylor cone (in-spray supercharging) using a dual-sprayer ESI microchip. The results are comparable to those obtained by the addition of supercharging reagents directly into the analyte solution or LC mobile phase. Therefore, supercharging reaction can be accomplished on a time-scale of ion liberation from a droplet in the ESI ion source.



Electrospray ionization mass spectrometry (ESI MS) allows structural analysis of large intact biomolecules, primarily due to its ability to generate multiply charged ions.¹ Specifically, multiple charging facilitates the observation of large molecular ions with narrow m/z range mass analyzers. The commonly employed low-to-medium resolution ion trap mass spectrometers (IT MS) are typically limited by 4000 m/z upper m/z threshold. High resolution mass spectrometers, for example, Fourier transform mass spectrometers (FTMS), can operate at substantially higher m/z range. However, FTMS resolution rapidly reduces with increasing m/z and, additionally, modern FTMS platforms are often hybridized with IT MS, including quadrupole MS, that further limit their working m/z range.²

Enhanced charging, or supercharging, of biomolecules increases the charge (z) of the ions observed in ESI MS.^{3–9} Furthermore, higher charge states of selected precursor ions increase efficiency of tandem mass spectrometry (MS/MS), especially electron-based MS/MS, including electron capture dissociation (ECD) and electron transfer dissociation (ETD).^{10–13} Typically, the supercharging in ESI MS is achieved by adding a small amount of a supercharging reagent to the analyte solution.^{3–7,9} The most commonly employed supercharging reagents are *m*-nitro benzyl alcohol (*m*-NBA),^{4–7,9,14–17} dimethyl sulfoxide (DMSO),^{5,18,19} and tetramethylene sulfone (sulfolane).^{7,20–22} The addition of small amounts of supercharging reagents to the mobile phase in liquid chromatography tandem mass spectrometry (LC-MS/

MS) has been shown to increase the average charge state distribution of enzymatically derived peptides, leading to improved peptide and protein identification in large-scale applications.²³ However, the presence of supercharging reagents in the LC mobile phase influences the retention time of the analytes and decreases chromatographic resolution.^{18,23}

The complete mechanism of the supercharging phenomenon remains uncertain. Initially, it was proposed that an increase in ESI droplet surface tension, as the result of the supercharging reagent, played the main role in the supercharging process.⁵ Due to their low volatility, supercharging reagents enrich the mature ESI droplet, thereby increasing surface tension. According to Rayleigh charge limit theory, the surface tension is directly proportional to charge availability of the droplet.⁵ However, other works on supercharging mechanism proposed that, in the absence of conformational changes, supercharging is independent of surface tension.^{24,25} A study on supercharging of proteins from their native solution by the Loo group claimed that supercharging does not depend on the conformational changes of proteins in the ESI droplet.²⁵ On the contrary, the Williams group suggested that the conformational changes of proteins are important in the supercharging process.^{6,15–17}

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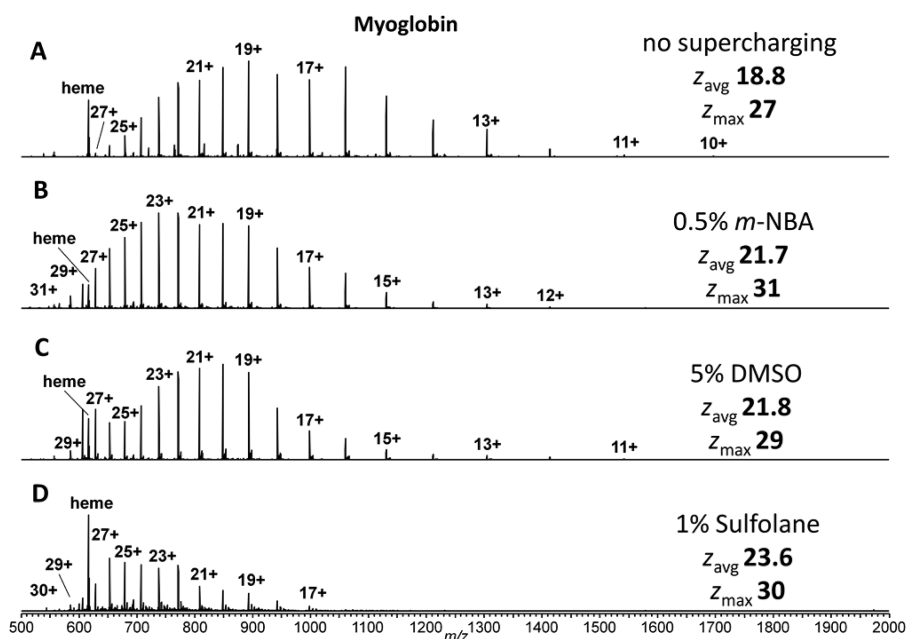


Figure 1. LTQ FT-ICR MS of myoglobin when its solution was electrosprayed via one channel of the dual-sprayer ESI microchip whereas (A) water/methanol, (B) 0.5% *m*-NBA, (C) 5% DMSO, and (D) 1% sulfolane were sprayed through the second microchannel.

Here, we demonstrate the possibility of increasing the average charge state distribution of peptides and proteins by introducing supercharging reagents directly into the electrospray's Taylor cone (referred to as in-spray supercharging). The presented methodology has a strong potential for application in bottom-up, middle-down, and top-down LC-MS/MS experiments, where it can be employed to increase the charge states of the observed peptides and proteins without influencing chromatographic separation.

METHODS

Cytochrome C, myoglobin, and substance P were obtained from Sigma-Aldrich (Buchs, Switzerland). For direct sample injection, analytes were dissolved in water/methanol (50/50, V/V) with addition of 0.1% formic acid to make 5 μ M solutions. The composition of the solvent mimics the composition of the mobile phase that could be used to elute the analytes in LC-MS experiments. The supercharging reagents, including *m*-NBA, DMSO, and sulfolane were purchased from Sigma-Aldrich (Busch, Switzerland) and dissolved in water/methanol (50/50, V/V) to make the commonly employed in supercharging ESI MS 0.5% *m*-NBA, 5% DMSO, and 1% and 5% sulfolane solutions.

To accomplish peptide and protein in-spray supercharging, a dual-sprayer ESI microchip was employed.^{26–28} The microchip consists of two independent microchannels located on the opposite sides of a chip. The microchannels are crossed at the emitter tip on the top of each other but do not have a direct connection. Therefore, the two solutions infused through these channels can only mix in the Taylor cone upon the applied voltage.²⁶ The dual-sprayer ESI microchip was coupled to a hybrid linear ion trap Fourier transform ion cyclotron resonance MS (LTQ FT-ICR MS, Thermo Scientific, Bremen, Germany) equipped with a 10 T superconducting magnet (Oxford Nanosciences, Oxon, UK).²⁹ The MS experiments were performed with initial ion detection in the medium resolution LTQ MS and validated with high resolution FT-ICR MS ion detection. Electron capture dissociation (ECD) and

infrared multiphoton dissociation (IRMPD) were performed in the FT-ICR MS,²⁹ whereas collision induced dissociation (CID) was performed in the LTQ MS.

Analyte solutions were electrosprayed via one microchannel of the dual-sprayer ESI microchip at a 200–500 nL/min flow rate, whereas either water/methanol solution (50/50, V/V; for nonsupercharging experiments) or the supercharging reagent-containing solution was cosprayed through the second microchannel with identical flow rate unless otherwise stated. The ESI potential was applied directly only to the analyte solution through the dedicated electrode, whereas the supercharging reagent solution would experience the applied potential only after mixing with the analyte solution in the Taylor cone. We noted that the lifetime of a dual-sprayer ESI microchip was longer when methanol was used as opposed to acetonitrile. Therefore, the microchip materials should be optimized to provide a longer life operation when acetonitrile is used as a solvent in LC-MS experiment. The mass spectrometer capillary temperature was kept at 200 °C, and the electrospray voltage was set to 1.9 kV. The parameters used to describe analyte charge states include the average charge state distribution (z_{avg}), the charge state of highest abundance peak in the mass spectrum (z_{base}), and the maximum observed charge state (z_{max}).⁵

RESULTS AND DISCUSSION

Myoglobin and cytochrome C proteins contain 154 and 105 amino acids, respectively. Proteins of similar lengths are usually targeted by top-down and middle-down proteomics.³⁰ Previously, myoglobin and cytochrome C were successfully in-solution supercharged with various reagents and methods.^{3–8,14,17–19,22} The ESI mass spectrum of myoglobin ions formed from a regular ESI solution (0.1% formic acid in methanol/water (50/50, V/V) solution) showed a z_{avg} of 18.8, a z_{base} of 19+, and a z_{max} of 27+, Figure 1A. During this control experiment, the supercharging solution sprayed from the second channel was water/methanol (50/50, V/V). In-spray supercharged myoglobin using 0.5% solution of *m*-NBA had a

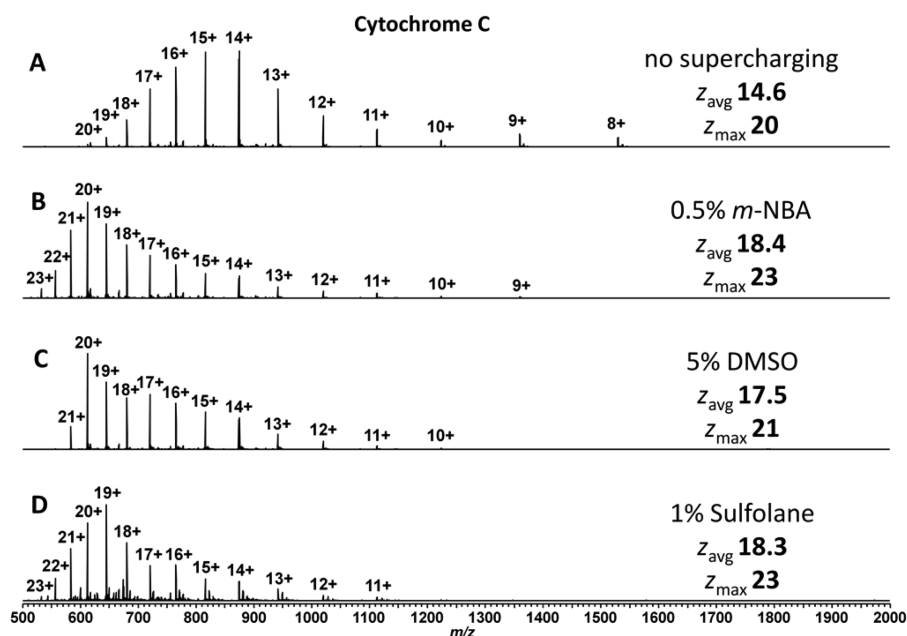


Figure 2. LTQ FT-ICR MS of cytochrome C when its solution was electrosprayed via one channel of the dual-sprayer ESI microchip whereas (A) water/methanol, (B) 0.5% *m*-NBA, (C) 5% DMSO, and (D) 1% sulfolane were sprayed through the second microchannel.

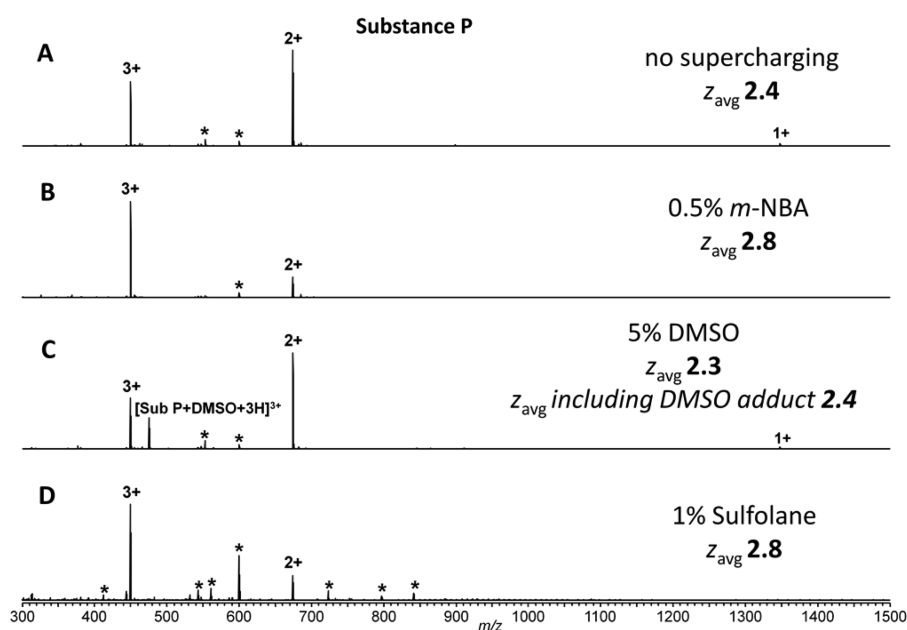


Figure 3. LTQ FT-ICR MS of substance P when its solution was electrosprayed via one channel of the dual-sprayer ESI microchip whereas (A) water/methanol, (B) 0.5% *m*-NBA, (C) 5% DMSO, and (D) 1% sulfolane were sprayed through the second microchannel. Asterisks denote spontaneous chemical decomposition products, not originated due to the in-spray reaction with the supercharging reagents.

z_{avg} of 21.7, a z_{base} of 23+, and a z_{max} of 31+, Figure 1B. The effect of DMSO supercharging was comparable to the results of *m*-NBA supercharging: the addition of DMSO increased z_{avg} to 21.8, z_{base} to 20+, and z_{max} to 29+, Figure 1C. The supercharging with sulfolane produced myoglobin mass spectra abundant with sulfate adducts, Figure 1D. Protein sulfate adducts in sulfolane in-solution supercharging were reported previously.²²

The influence of sulfolane quantity mixed with myoglobin within the Taylor cone on the ion abundance is shown in Figure S1, Supporting Information. The formation of sulfate adducts influenced the signal abundance of myoglobin ions in

an inverse manner to the sulfolane concentration. At the same time, the intensity of the heme signal was constant because sulfate adducts were not observed with the heme ion. Besides adduct formation, the presence of sulfolane increased z_{avg} to 23.6, z_{max} to 30+, and z_{base} to 26+.

The efficiency of ion supercharging is protein dependent; therefore, even proteins of a comparable size (e.g., myoglobin and cytochrome C) may demonstrate very different supercharging tendencies. Naturally, efficient supercharging of larger proteins, e.g., transferrins and immunoglobulins, is the desired outcome of ion supercharging applications. However, the current development of even a solution-based ion super-

charging method and technique, does not allow for efficient supercharging of large intact proteins. Presumably, this decrease in supercharging efficiency results from the significantly higher extent of structure folding in larger proteins. Furthermore, separation efficiency of proteins by liquid chromatography is underdeveloped for large proteins, whereas for peptides and small, 10–20 kDa, proteins, considered in the current work, LC separation is well established. Therefore, the in-spray supercharging developed here primarily targets bottom-up and middle-down proteomics, where enzymatically produced peptides of up to 20 kDa might have different ionization and fragmentation capabilities compared to myoglobin and cytochrome C. An extension of in-spray ion supercharging to larger proteins would require further developmental work.

In-spray supercharging of cytochrome C demonstrated similar behavior to both myoglobin in-spray supercharging and cytochrome C in-solution supercharging, Figure 2. Specifically, cytochrome C electro sprayed from a nonsupercharging solution showed a z_{avg} of 14.6 with a z_{base} of 14+ and a z_{max} of 20+, Figure 2A. In-spray supercharged cytochrome C with *m*-NBA showed a z_{avg} of 18.4 with a z_{base} of 20+ and a z_{max} of 23+; with DMSO a z_{avg} of 17.5 with a z_{base} of 20+ and a z_{max} of 21+; and with sulfolane a z_{avg} of 18.3, a z_{base} of 19+, and a z_{max} of 23+, see Figure 2B,C,D, respectively. In-spray supercharged cytochrome C with DMSO showed mass spectrum with a bimodal charge distribution, Figure 2C, whereas sulfolane caused sulfate adduct formation, Figure 2D. The origin of the bimodal charge distribution in Figure 2C is presumably due to the variation of DMSO concentration during ESI droplet evolution. It is known that supercharging efficiency of DMSO depends on its concentration in the analyte solution and ESI droplet.¹⁹

Substance P is a peptide consisting of 11 amino acids, a length comparable with proteolytic peptides generated in bottom-up proteomics experiments.²⁷ In a regular ESI MS experiment, substance P showed a z_{avg} of 2.4 with the z_{base} of 2+, whereas in-spray supercharged substance P with *m*-NBA had a z_{avg} of 2.8 with a z_{base} of 3+, and supercharged DMSO had a z_{avg} of 2.3 with a z_{base} of 2+; see Figure 3A,B,C, respectively. The z_{max} was 3+ in all experiments with substance P. When DMSO was used as a supercharging reagent, a DMSO adduct was observed exclusively on the triply charged ion, [Substance P + DMSO + 3H]³⁺, Figure 3C. The adduct signal was observed only when the capillary temperature of the MS was colder than 200 °C and was easily lost upon very mild vibrational activation, indicating the noncovalent nature of adduct formation. Nevertheless, [Substance P + DMSO + 3H]³⁺ species were sufficiently stable to be isolated in the LTQ for CID-based MS/MS and transferred intact to the ICR ion trap for IRMPD and ECD-based MS/MS. However, even application of soft ion activation and dissociation conditions has not resulted in product ions containing the adduct (data not shown), further confirming the noncovalent nature of the complex. When substance P was supercharged with sulfolane, the z_{avg} was 2.8 and the z_{base} was 3+, Figure 3D. However, the signal intensities of 2+ and 3+ ions decreased significantly, whereas sulfate adducts were not observed. Note, that the observed spontaneous decomposition products shown in Figure 3D are not the result of in-spray supercharging reaction but arise from the sample decomposition in solution, which is known to be substantial for substance P, from a separate experiment without supercharging (data not shown).

The results reported in Figures 1–3 nicely demonstrate the shift in the charge states observed upon in-spray addition of supercharging reagents but do not directly address the impact of supercharging on the overall signal magnitude. Indeed, the benefit of in-spray or in-solution supercharging for ECD/ETD-based middle-down and top-down proteomics could be counterbalanced by a possible signal intensity drop due to a supercharging reaction. First, appearance or substantial intensity increase of ions with charge states higher than the present without supercharging reaction are beneficial even if intensity of lower charge state ions drops. Indeed, fragmentation efficiency of peptide and protein ions, especially by ETD and ECD, drastically depends on the precursor ion charge state. As such, even a moderate increase in charge state may significantly improve the obtained sequence coverage. Second, signal abundance variation can be compared for lower charge state ions, including ions corresponding to the average charge state and most abundant ions. Here, we observed that the signal intensity of ions detected in FTMS is not dramatically affected by the in-spray addition of supercharging reagents; the variation is always within an order of magnitude. *m*-NBA and DMSO can even cause a moderate signal increase, whereas the impact of sulfolane on the signal magnitude was always negative under the applied experimental conditions, including the employed concentration levels of supercharging reagents (see Supporting Information Tables S1 and S2 for more details).

CONCLUSIONS

To improve the performance of LC-MS/MS-based peptide and protein structure analysis, we developed an in-spray ESI supercharging method. To accomplish the in-spray supercharging, we employed a dual-sprayer ESI microchip containing two microchannels ending near each other at the tip of the emitter, which allows mixing of the two liquids within the Taylor cone. Our results include successful demonstrations of in-spray supercharging using the direct infusion of proteins, cytochrome C and myoglobin, and a peptide, substance P, with three commonly employed supercharging reagents, *m*-NBA, DMSO, and sulfolane. The effect of the in-spray supercharging method using dual-sprayer ESI microchip is comparable to previously reported results where supercharging reagents were added directly into electrospray solutions. It was found that *m*-NBA-based in-spray supercharging abilities were superior to both DMSO and sulfolane, producing not only slightly higher average and most abundant charge states but also fewer adducts.

Overall, the reported results demonstrate that peptide and protein supercharging processes require a short interaction time between analytes and supercharging reagents. On the basis of the previously suggested dynamics of ESI droplet evolution in time and considering that supercharging reaction takes place in the droplet, the interaction time can be estimated as less than 1–2 ms when low flow rate ESI conditions are realized, as performed in the current work.^{31,32} Importantly, the analytes in the online LC-MS/MS experiments, presumably, could be supercharged once eluted from the chromatographic column without influencing chromatographic performance. This opens an attractive prospect for supercharging applications in routine LC-MS/MS, including bottom-up, middle-down, and top-down protein analysis.

■ ASSOCIATED CONTENT**■ Supporting Information**

Influence of sulfolane concentration on myoglobin peak intensities. Myoglobin and substance P signal intensity variation during in-spray supercharging with *m*-NBA, DMSO, and sulfolane. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION**Corresponding Author**

*Address: EPFL ISIC LSMB, BCH 4307, 1015 Lausanne, Switzerland. E-mail: yury.tsybin@epfl.ch.

Notes

The authors declare no competing financial interest.

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