SUPPORTING INFORMATION

Sample Flow Rate Scan in Electrospray Ionization Mass Spectrometry Reveals Alterations in Protein Charge State Distribution

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ADDITIONAL EXPERIMENTAL DETAILS

Materials

Ammonium acetate (≥99%, for HPLC) and ammonium formate (≥99.99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (for LC-MS) and water (for chromatography, LiChrosolv) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (99%, for analysis), cytochrome *c* (90%, from horse heart muscle), and piperidine were purchased from Acros Organics (Pittsburgh, PA, USA). Ubiquitin (human recombinant) was purchased from Boston Biochem (Cambridge, MA, USA). Both cytochrome *c* and ubiquitin were used without purification.

Protein samples (5 μM) were prepared in 25% (v/v) methanol in water solution, with or without ESI additives. Considering the influence of methanol on the structure of proteins,[1-5] the methanol concentration of 25% (v/v) in water was chosen. The presence of methanol helped to maintain a stable electrospray at all flow rates. Moreover, it prevented clogging of the ESI capillary.

Four additives, which are commonly used in ESI-MS studies of biological macromolecules, were selected: AA,[6-12] AB,[9,11-14] AF,[9,11] and PP.[15-18] All the chemicals were handled in accordance with the relevant MSDS and institutional safety guidelines.

Data processing

The raw data of cumulative ion currents of charge states (a range of *m/z*: 3 to 4) were processed in the LabSolutions software by Savitzky-Golay smoothing function (25 point window), and were exported to ASCII files. The ASCII files of three replicates were imported to Excel (version 2016; Microsoft, Redmond, WA, USA), and averaged. The averaged datasets were smoothed by exponential smoothing with a smoothing factor (α) of 0.05. Further, the $\boldsymbol{z_{av}}$ data were transferred to OriginPro (version 8; OriginLab, Northampton, MA, USA) for plotting against flow rate.

Fluorescence spectroscopy

The analyses were performed on a fluorescence spectrometer (LS-55, Perkin Elmer, Waltham, MA, USA). Fluorescence of tryptophan and tyrosine residues in proteins were recorded at an excitation wavelength of 280 nm. The widths of excitation and emission slits were 5 nm each.

High-resolution mass spectrometry

In addition to the triple quadrupole mass spectrometer (LCMS-8030, Shimadzu, Kyoto, Japan) used in most experiments, a quadrupole (Q) time-of-flight (ToF) mass spectrometer (Impact HD, Bruker, Billerica, MA, USA), equipped with a conventional ESI source, was used to obtain some comparative results. Instrumental parameters in the Q-ToF mass spectrometer: ESI emitter voltage, +4.5 kV; endplate offset voltage, 0.5 kV; nebulizer gas pressure, 0.4 bar; drying gas flow rate, 4.0 L min^{-1}; the temperature of the ion chamber and ion-transfer tube, 200 °C. The ToF mass analyzer was operated in scan mode, in the *m/z* range 50-2000.

ADDITIONAL RESULTS

Possible confounding effects: shear stress before ESI capillary

To verify the influence of hydrodynamic shear stress before ESI capillary, we passed the protein samples (with or without 10 mM AA) through fused silica inert capillary tubings (i.d. 150 μm, o.d. 375 μm; GL Sciences, Tokyo, Japan) of different lengths (10 cm to 80 cm) at two different sample flow rates (200 and 400 μL min^{-1}). If the protein molecules unfolded as they passed through the capillary tubing, then the currents of higher charge states would increase with increasing capillary length. Notably, no significant changes to protein CSD were observed (**Figure S9**). However, a slight reduction in ion currents of some charge states was observed (10+ for cytochrome *c*, **Figure S9D**, and 9+ for ubiquitin, **Figure S9H**). That could be because of the slight reduction in flow rate due to increased back pressure in longer capillaries. Our results are in agreement with an earlier report, where cytochrome *c* solution was forced into the capillary tubing (i.d. 150-180 μm), subjecting it to shear stress, and no change in protein structure was observed.[19] The increased stability of ubiquitin CSD at different capillary lengths can be attributed to its compact structure maintained by a hydrophobic core.[20] Therefore, we have ruled out the possibility of significant structural changes to protein molecules in the flow line.

Possible confounding effects: pH changes in electrospray plume

It is well-known that decreasing the pH of ESI electrolyte can lead to protein denaturation (unfolding) and formation of gas-phase ions with higher charge states.[21] The pH of the infused liquid sample can change in different steps of the ESI-MS process: (a) in the bulk solution; (b) in the ESI capillary; (c) in the sprayed micro/nano-droplets.[10] In the present study, only the processes (b) and (c) could contribute to a pH change because no chemical reaction was performed on the sample. Considering the influence of electrochemical processes occurring in the ESI capillary,[22-24] and the process of droplet shrinkage[10,25] on the pH of the ESI droplets, we designed experiments to directly measure the pH of the solution coming out of the high voltage probe (**Figure S10**). In one experiment, we collected the sample solution after passing it through the ESI capillary but not subjecting to electrospray and drying. In that experiment, we connected a Tygon tubing (i.d. 0.19 mm, o.d. 2.00 mm, length 4 cm) to ESI capillary to collect the solution in a 0.5 mL microcentrifuge tube (**Figures S10A** and **S10B**). Sample solutions containing additives were collected at different flow rates. To collect enough volume of the sample for the immersion of the sensing portion of a micro pH electrode, aliquots were collected at different duration. Then, the pH of the collected solutions were measured with a pH electrode (Ag/AgCl reference electrode, KCl filling solution, InLab Micro; Mettler Toledo, Columbus, OH, USA). Notably, there was no change in the pH of sample solution after passing through the ESI capillary (**Figure S10C**). In another experiment, we set up a small droplet collector beneath the electrospray to collect the droplets at high flow rates. At low flow rates, it was not possible to collect the droplets as they completely evaporated. The electrospray droplet collector consisted of a polytetrafluoroethylene (PTFE) funnel (funnel dimensions: slant height = 4.5 cm, base diameter = 3.0 cm, vertex diameter = 0.5 cm) placed on a 0.5 mL microcentrifuge tube (**Figures S10D** and **S10E**). The funnel and the microcentrifuge tube were

supported by a stainless steel wire and binder clip to withstand the drying gas flow. The pH values of the solutions collected were slightly lower than that of the original sample solution (**Figure S10F**). Therefore, there could be changes in pH happening at flow rates below 400 μL min^{-1}. This is in agreement with the fluorometric droplet pH measurements reported in an earlier study.[25]

ADDITIONAL FIGURES

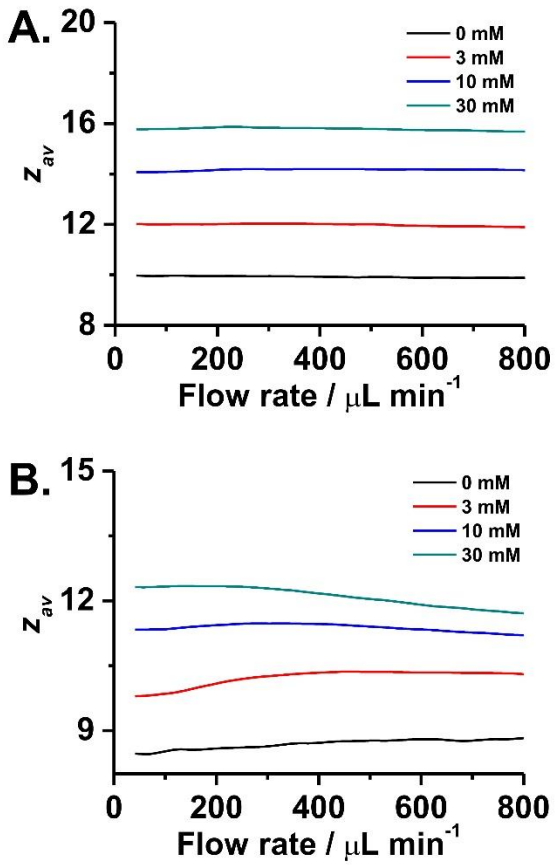

Figure S1. The influence of sample flow rate on z_{av} of proteins in the presence of AB. Sample: 5 μM cytochrome *c* or ubiquitin in 25% (v/v) methanol in water. (A) z_{av} *vs* flow rate at different concentrations of AB for cytochrome *c*. (B) z_{av} *vs* flow rate at different concentrations of AB for ubiquitin. For examples of mass spectra, see **Figure S6**.

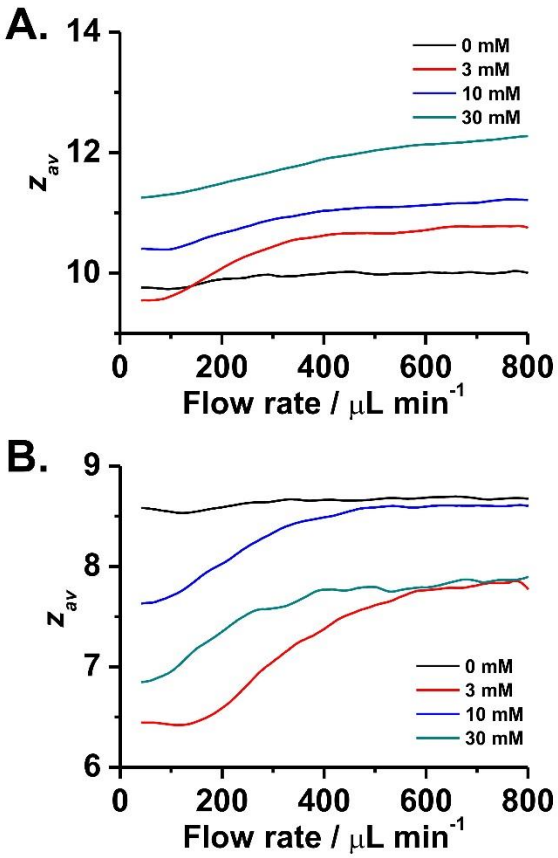

Figure S2. The influence of sample flow rate on z_{av} of proteins in the presence of AF. Sample: 5 μM cytochrome *c* or ubiquitin in 25% (v/v) methanol in water. (A) z_{av} *vs* flow rate at different concentrations of AF for cytochrome *c*. (B) z_{av} *vs* flow rate at different concentrations of AF for ubiquitin. For examples of mass spectra, see **Figure S7**.

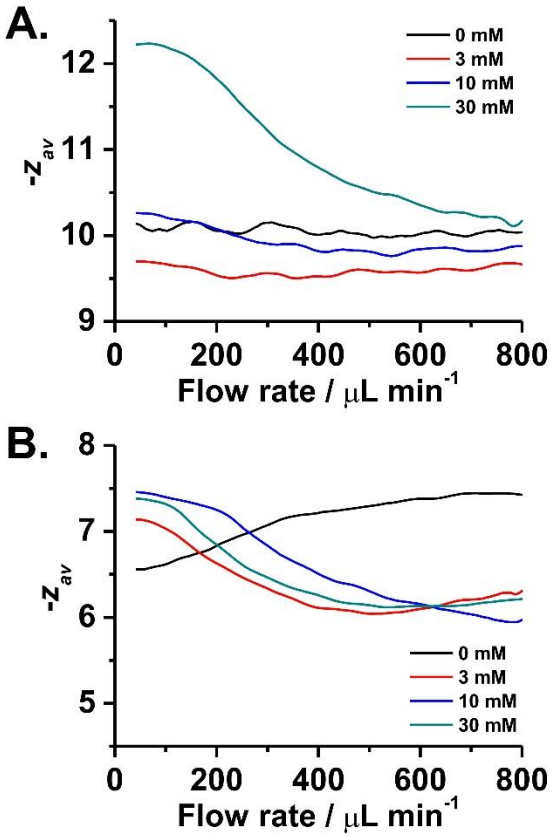

Figure S3. The influence of sample flow rate on z_{av} of proteins in the presence of PP. Sample: 5 μM cytochrome *c* or ubiquitin in 25% (v/v) methanol in water. (A) z_{av} *vs* flow rate at different concentrations of PP for cytochrome *c*. (B) z_{av} *vs* flow rate at different concentrations of PP for ubiquitin. For examples of mass spectra, see **Figure S8**.

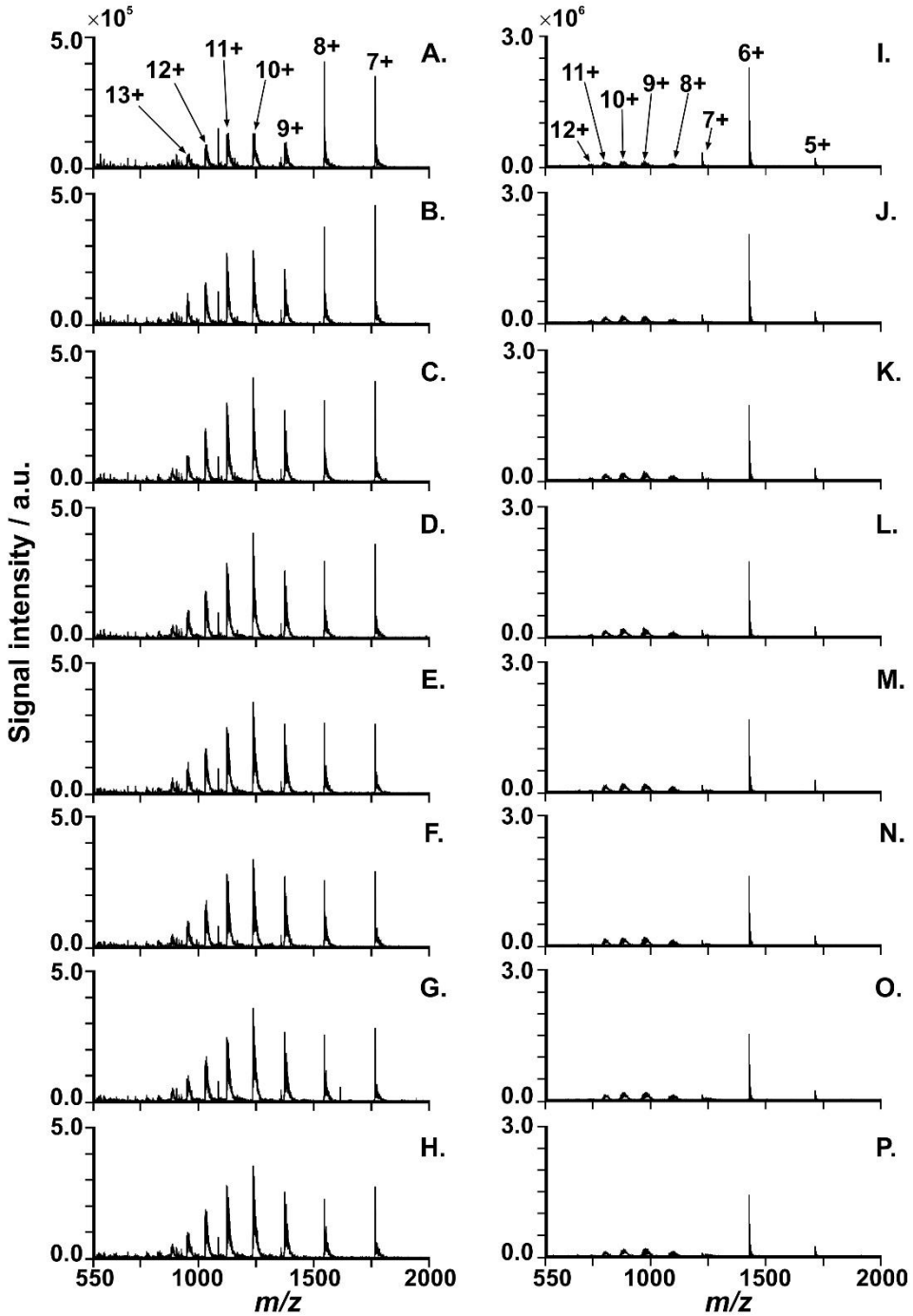

Figure S4. The influence of sample flow rate on intensities of multiply charged ions of proteins in the presence of AA in the positive-ion mode. Sample: (A-H) 5 μM cytochrome *c* and (I-P) 5 μM ubiquitin in 25% (v/v) methanol in water containing 10 mM AA. Spectra averaged for the flow rate ranges, (A,I) 40-100 μL min^{-1}; (B,J) 101-200 μL min^{-1}; (C,K) 201-300 μL min^{-1}; (D,L) 301-400 μL min^{-1}; (E,M) 401-500 μL min^{-1}; (F,N) 501-600 μL min^{-1}; (G,O) 601-700 μL min^{-1}; (H,P) 701-800 μL min^{-1}. Some peaks in the cytochrome *c* spectra could be due to the impurities in the protein sample.

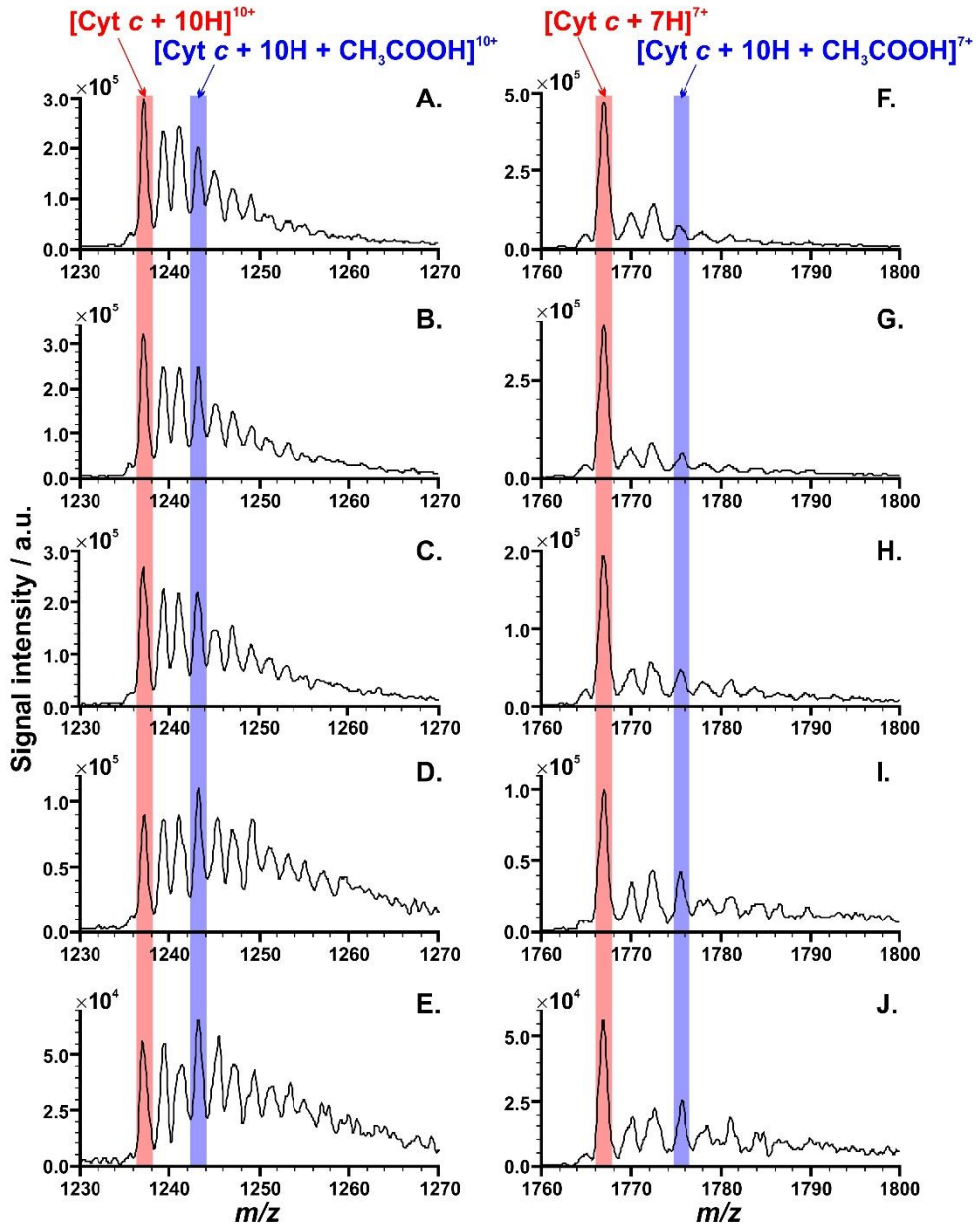

Figure S5. Averaged mass spectra for sample flow rates 40-400 µL min^{-1} showing a possible acetic acid adduct associated with cytochrome *c* in positive-ion mode (in addition to other adducts). Sample: 5 µM cytochrome *c* in 25% (v/v) methanol in water with different concentrations of ammonium acetate. Concentration of ammonium acetate: (A,F) 3 mM; (B,G) 10 mM; (C,H) 30 mM; (D,I) 100 mM; (E,J) 300 mM. Number of charges: (A-E) 10+; (F-J) 7+. Peaks highlighted in red are attributed to cytochrome *c*, and peaks highlighted in blue are attributed to acetic acid adduct of cytochrome *c*. Apart from H^+ ions, the charge carriers can be other cations (*e.g.* NH_4^+, Na^+) resulting in the formation of other adducts observed in the spectra. Notably, with increasing concentration of ammonium acetate, the intensity of cytochrome *c*-acetic acid adduct peak increases relative to decrease in intensity of cytochrome *c* peak.

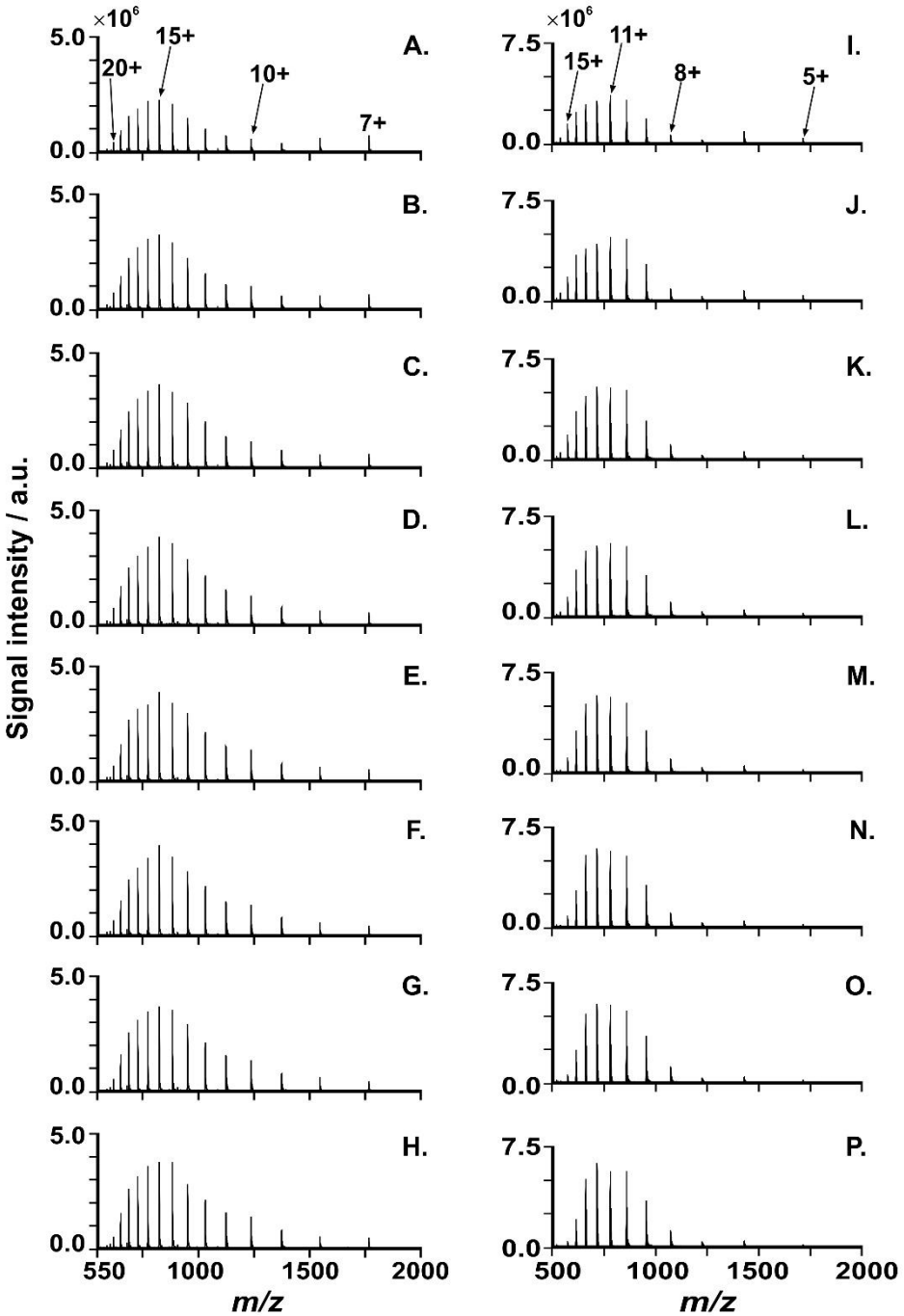

Figure S6. The influence of sample flow rate on intensities of multiply charged ions of proteins in the presence of AB in the positive-ion mode. Sample: (A-H) 5 μM cytochrome *c* and (I-P) 5 μM ubiquitin in 25% (v/v) methanol in water containing 10 mM AB. Spectra averaged for the flow rate ranges, (A,I) 40-100 μL min^{-1}; (B,J) 101-200 μL min^{-1}; (C,K) 201-300 μL min^{-1}; (D,L) 301-400 μL min^{-1}; (E,M) 401-500 μL min^{-1}; (F,N) 501-600 μL min^{-1}; (G,O) 601-700 μL min^{-1}; (H,P) 701-800 μL min^{-1}. Some peaks in the cytochrome *c* spectra could be due to the impurities in the protein sample.

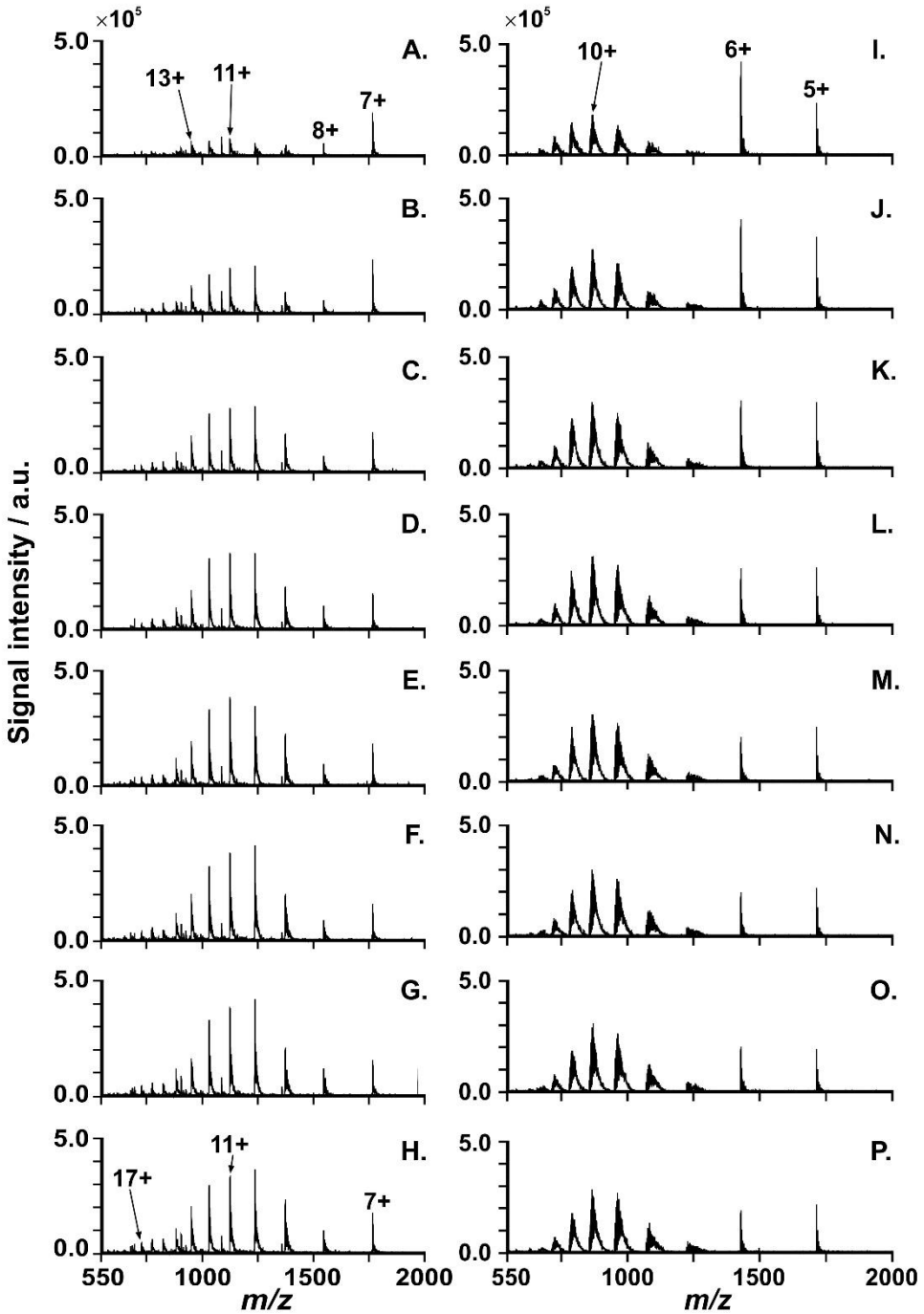

Figure S7. The influence of sample flow rate on intensities of multiply charged ions of proteins in the presence of AF in the positive-ion mode. Sample: (A-H) 5 μM cytochrome *c* and (I-P) 5 μM ubiquitin in 25% (v/v) methanol in water containing 10 mM AF. Spectra averaged for the flow rate ranges, (A,I) 40-100 μL min^{-1}; (B,J) 101-200 μL min^{-1}; (C,K) 201-300 μL min^{-1}; (D,L) 301-400 μL min^{-1}; (E,M) 401-500 μL min^{-1}; (F,N) 501-600 μL min^{-1}; (G,O) 601-700 μL min^{-1}; (H,P) 701-800 μL min^{-1}. Some peaks in the cytochrome *c* spectra could be due to the impurities in the protein sample.

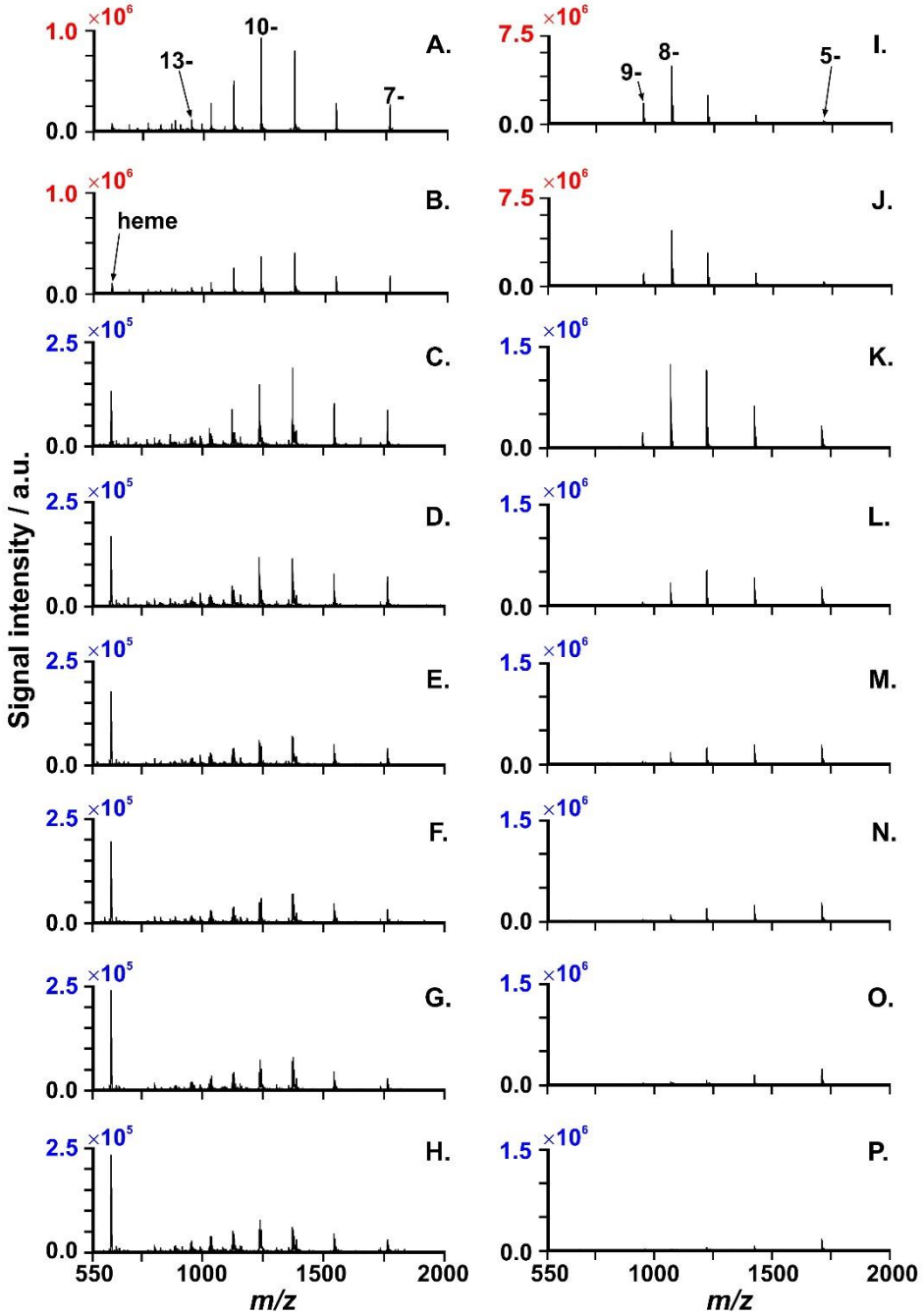

Figure S8. The influence of sample flow rate on intensities of multiply charged ions of proteins in the presence of PP in the negative-ion mode. Sample: (A-H) 5 μM cytochrome *c* and (I-P) 5 μM ubiquitin in 25% (v/v) methanol in water containing 10 mM PP. Spectra averaged for the flow rate ranges, (A,I) 40-100 μL min^{-1}; (B,J) 101-200 μL min^{-1}; (C,K) 201-300 μL min^{-1}; (D,L) 301-400 μL min^{-1}; (E,M) 401-500 μL min^{-1}; (F,N) 501-600 μL min^{-1}; (G,O) 601-700 μL min^{-1}; (H,P) 701-800 μL min^{-1}. Some peaks in the cytochrome *c* spectra could be due to the impurities in the protein sample. Note that the signal intensity scales are adjusted to magnify the spectra.

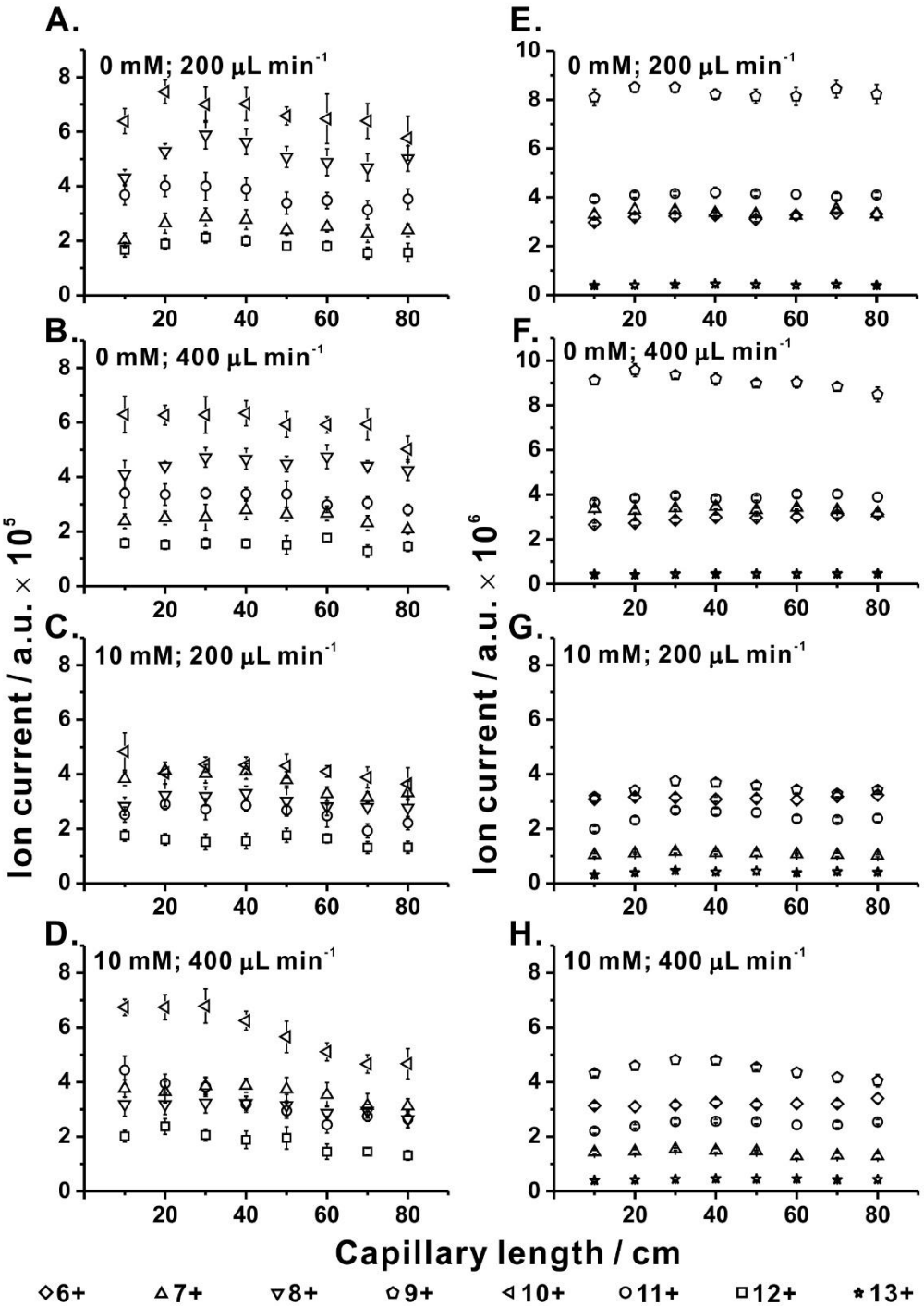

Figure S9. Dependencies of charge state ion currents of two proteins at different flow rates (200 or 400 µL min^{-1}) on capillary lengths: (A-D) cytochrome *c*; (E-H) ubiquitin. Sample: 5 µM cytochrome *c* or ubiquitin in 25% (v/v) methanol in water without (A,B,E,F) or with (C,D,G,H) AA (10 mM). Different symbols indicate charge states. Error bars represent the standard deviation for 31 data points from 1 min ion current measurement.

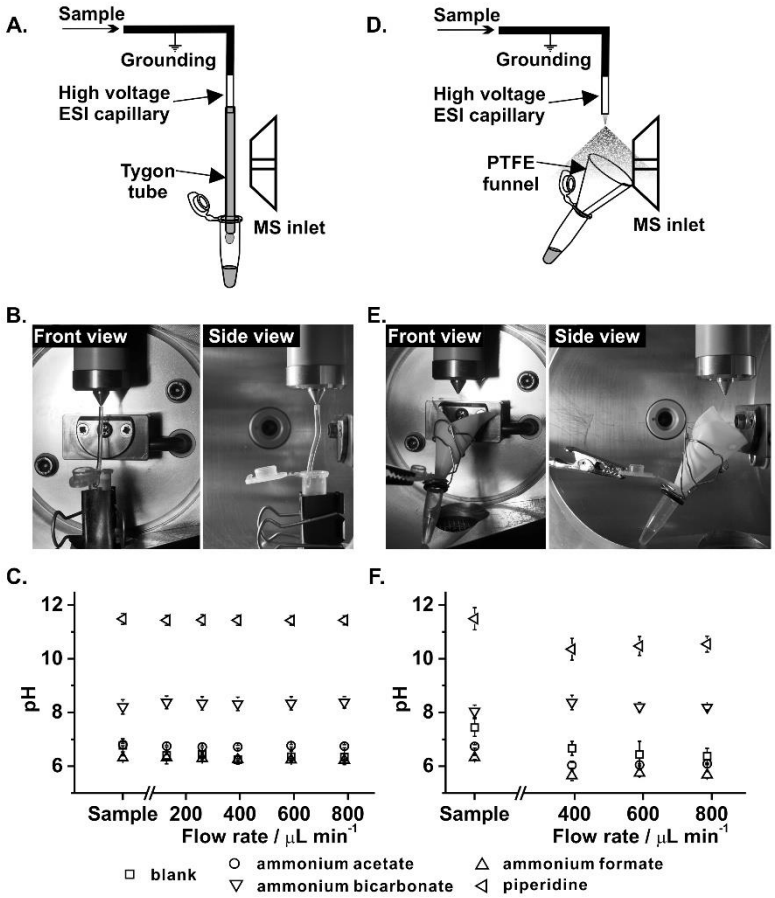

Figure S10. pH values of ESI solution and plume at different flow rates with different electrolytes. (A-C) ESI solution collected before the desolvation process *via* Tygon tubing at different flow rates and the pH values of electrolyte solution before and after passing it through ESI voltage. (D-F) ESI plume droplets collected *via* PTFE funnel in the presence of nebulizing gas (2.0 L min^{-1}), drying gas (15.0 L min^{-1}), and the pH values (n = 6) of electrolyte solution before and after passing it through ESI voltage. Sample: 10 mM additives were dissolved in 25% (v/v) methanol in water. Error bars indicate standard deviation.

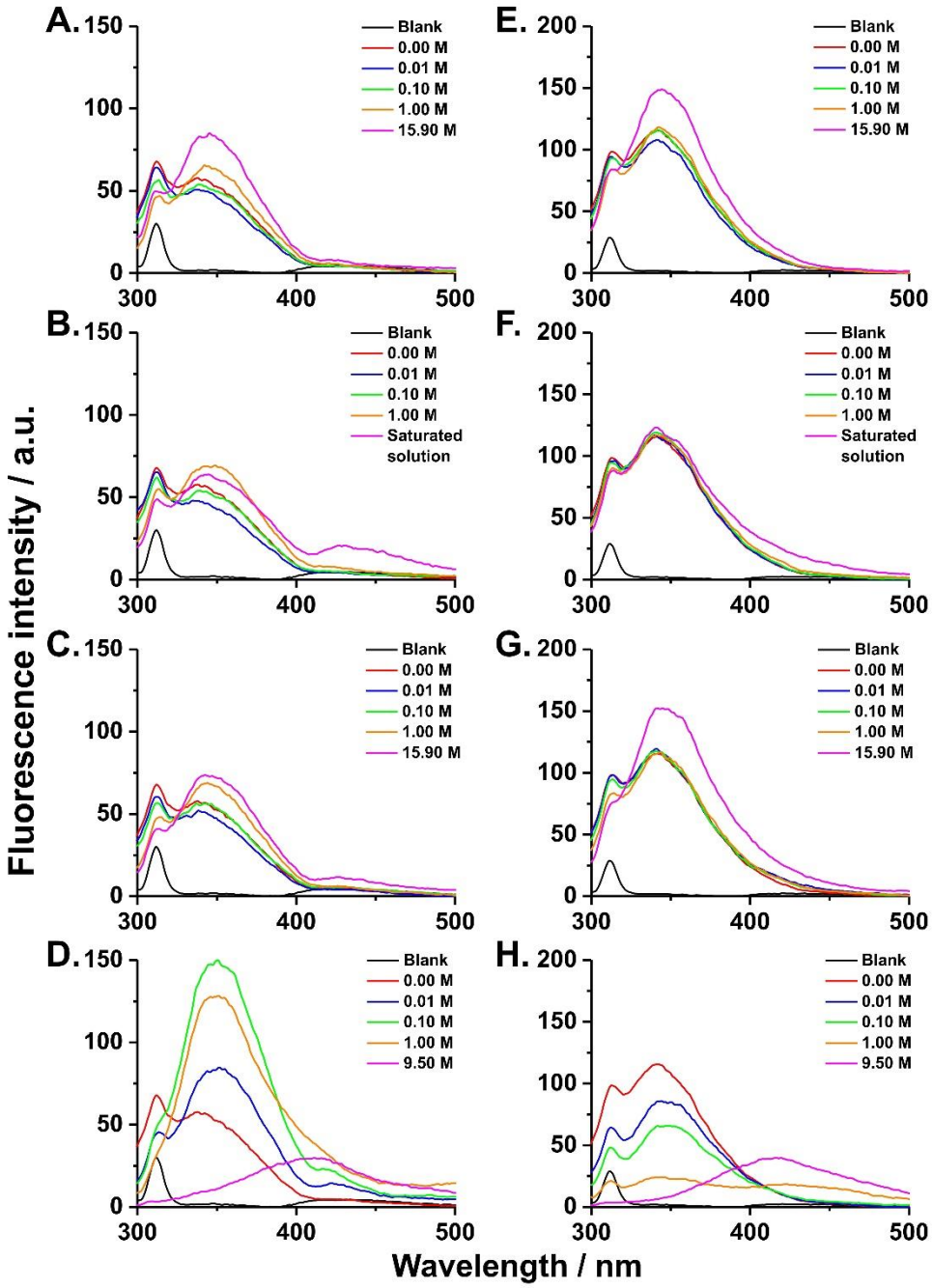

Figure S11. Fluorescence emission spectra of cytochrome *c* and ubiquitin recorded at the excitation wavelength of 280 nm in the presence of different concentrations of ESI additives. Sample: (A-D) 5 μM cytochrome *c* and (E-H) 5 μM ubiquitin in 25% (v/v) methanol in water containing different concentrations of AA (A,E); AB (B,F); AF (C,G); and PP (D,H). The maximum emission wavelength for tyrosine and tryptophan residues are at 303 nm and 350 nm, respectively.[26] Note that the band at 311 nm is attributed to the Raman scattering caused by solvent.[26]

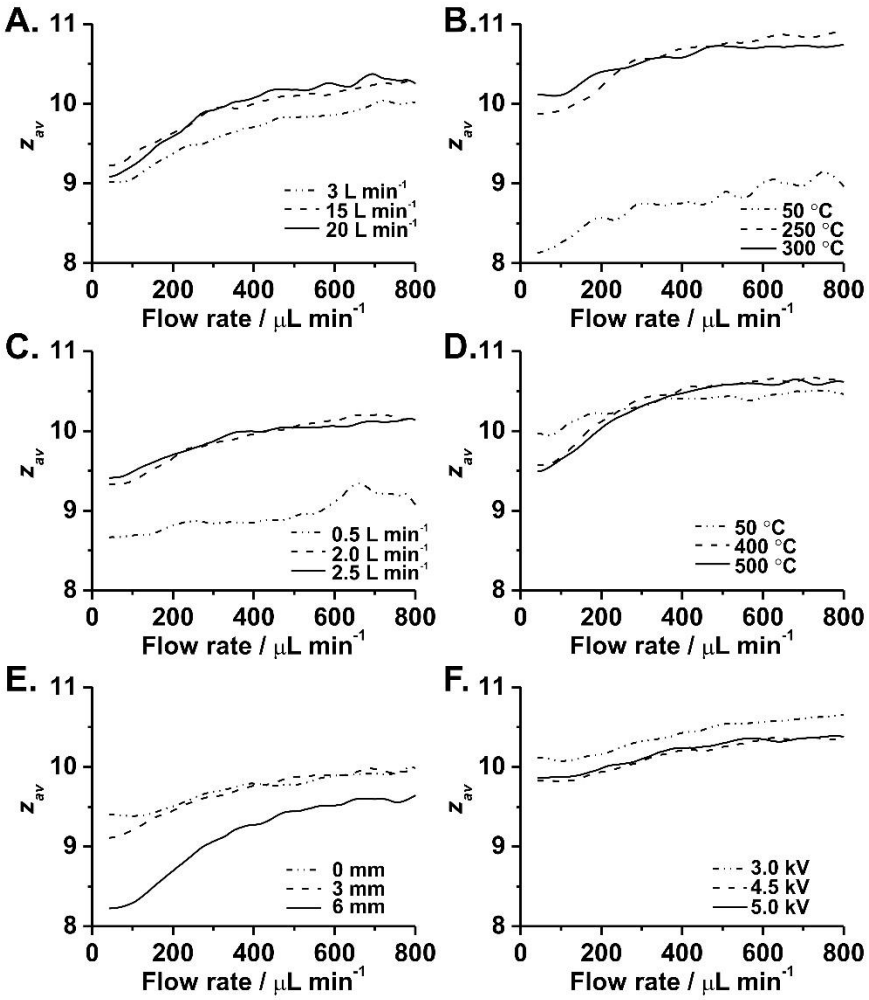

Figure S12. The influence of various ESI parameters on the z_{av} of proteins observed during sample flow rate scan. Sample: 5 μM cytochrome *c* in 25% (v/v) methanol in water containing 10 mM AA. (A) Drying gas flow rate; (B) ion-transfer tube temperature; (C) nebulization gas flow rate; (D) drying gas temperature; (E) distance between ESI capillary axis and MS inlet; (F) ESI voltage. Dashed-double dotted lines represent minimum value, dashed lines represent recommended default value, and solid lines represent the maximum value of different ESI parameters. While performing sample flow rate scan at three different values of a given parameter, all the other parameters were kept constant.

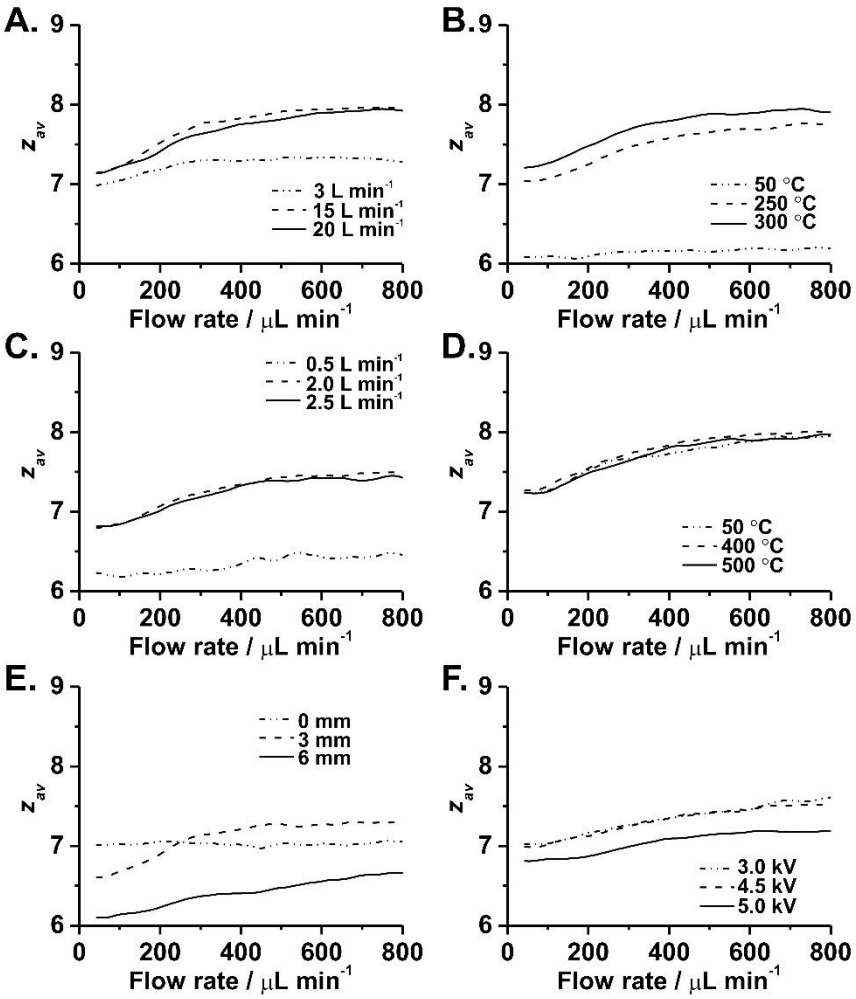

Figure S13. The influence of various ESI parameters on the z_{av} of proteins observed during sample flow rate scan. Sample: 5 μM ubiquitin in 25% (v/v) methanol in water containing 10 mM AA. (A) Drying gas flow rate; (B) ion-transfer tube temperature; (C) nebulization gas flow rate; (D) drying gas temperature; (E) distance between ESI capillary axis and MS inlet; (F) ESI voltage. Dashed-double dotted lines represent minimum value, dashed lines represent recommended default value, and solid lines represent the maximum value of different ESI parameters. While performing sample flow rate scan at three different values of a given parameter, all the other parameters were kept constant.

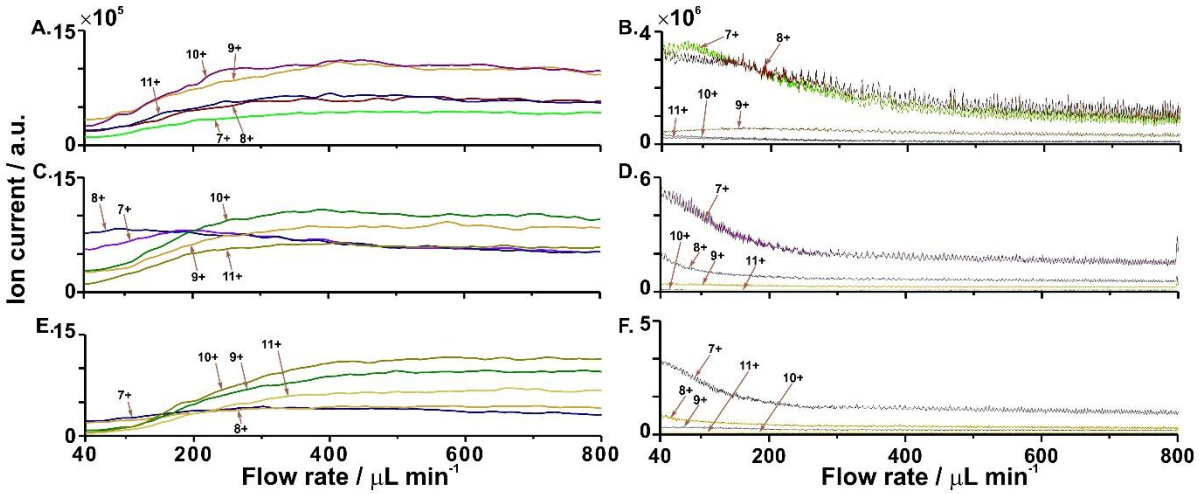

Figure S14. The influence of sample flow rate on ion currents of selected charge states of cytochrome *c* in the presence of AA. (A,C,E) Results obtained using triple quadrupole mass spectrometer (LCMS-8030, Shimadzu, Kyoto, Japan); (B,D,F) results obtained using quadrupole time-of-flight mass spectrometer (Impact HD, Bruker, Billerica, MA, USA). Sample: 5 μM cytochrome *c* in 25% (v/v) methanol in water containing (A,B) 0 mM; (C,D) 10 mM; and (E,F) 30 mM AA.

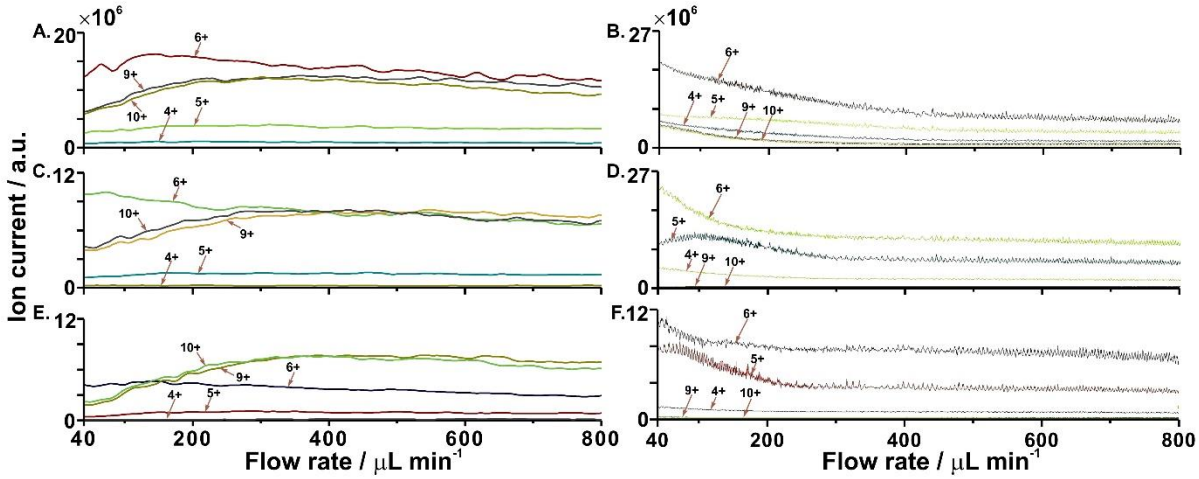

Figure S15. The influence of sample flow rate on ion currents of selected charge states of ubiquitin in the presence of AA. (A,C,E) Results obtained using triple quadrupole mass spectrometer (LCMS-8030, Shimadzu, Kyoto, Japan); (B,D,F) results obtained using quadrupole time-of-flight mass spectrometer (Impact HD, Bruker, Billerica, MA, USA). Sample: 5 μM ubiquitin in 25% (v/v) methanol in water containing (A,B) 0 mM; (C,D) 10 mM; and (E,F) 30 mM AA.

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