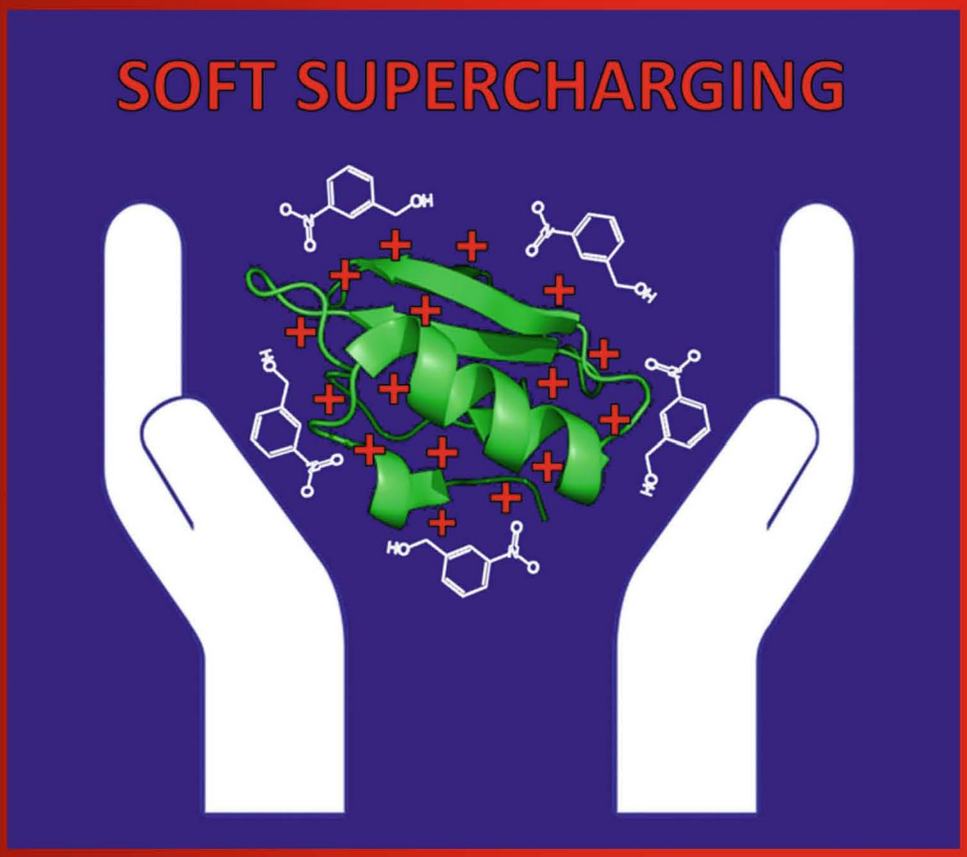
Volume 25 Number 6 June 2014

Journal of The American Society for MASS SPECTROMETRY

Journal of The American Society for Mass Spectrometry

VOLUME 25 | NUMBER 6 | JUNE 2014 | PAGES 903 | 1088



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In this Issue:

ISSN 1044-0305 • 25 (6) 903–1088 (2014) • 13361

Springer

J. Am. Soc. Mass Spectrom. (2014) 25:928–934
DOI: 10.1007/s13361-014-0887-y

RESEARCH ARTICLE

Soft Supercharging of Biomolecular Ions in Electrospray Ionization Mass Spectrometry

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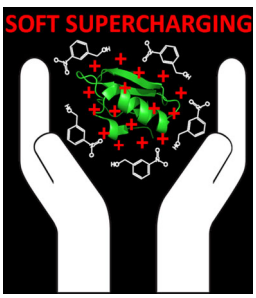

Abstract. The charge states of biomolecular ions in ESI-MS can be significantly increased by the addition of low-vapor supercharging (SC) reagents into the spraying solution. Despite the considerable interest from the community, the mechanistic aspects of SC are not well understood and are hotly debated. Arguments that denaturation accounts for the increased charging observed in proteins sprayed from aqueous solutions containing SC reagent have been published widely, but often with incomplete or ambiguous supporting data. In this work, we explored ESI MS charging and SC behavior of several biopolymers including proteins and DNA oligonucleotides. Analytes were ionized from 100 mM ammonium acetate (NH^4Ac) aqueous buffer in both positive (ESI+) and negative (ESI–) ion modes. SC was induced either with m-NBA or by the elevated temperature of ESI capillary. For all the analytes studied we, found striking differences in the ESI MS response to these two modes of activation. The data suggest that activation with m-NBA results in more extensive analyte charging with lower degree of denaturation. When working solution with m-NBA was analyzed at elevated temperatures, the SC effect from m-NBA was neutralized. Instead, the net SC effect was similar to the SC effect achieved by thermal activation only. Overall, our observations indicate that SC reagents enhance ESI charging of biomolecules via distinctly different mechanism compared with the traditional approaches based on analyte denaturation. Instead, the data support the hypothesis that the SC phenomenon involves a direct interaction between a biopolymer and SC reagent occurring in evaporating ESI droplets.

Key words: Native mass spectrometry, ESI mechanisms, Supercharging, Gas-phase ions, 3-nitrobenzyl alcohol, Charge state distribution, Biopolymers, Protein complexes, DNA, Oligonucleotides

Received: 10 February 2014/Revised: 8 March 2014/Accepted: 17 March 2014/Published online: 15 April 2014

Introduction

"Native" electrospray ionization mass spectrometry (ESI MS) is of growing popularity in structural biology [1, 2]. By ionizing biomolecules from aqueous solution adjusted to physiological pH with volatile buffer and by applying gentle desolvation conditions, weak noncovalent interactions can be transferred into the gas phase. This enables the observation of intact proteins, DNA, macromolecular complexes such as ribosomes [3] and ATPase [4], and even viruses [5].

Compared with the traditional ESI MS, native ESI MS produces ions with much lower charge states (and, hence, much higher *m*/*z* values), which greatly limits the sensitivity and mass resolution of detection as well as the efficiency of structural analysis by tandem MS. Moreover, the observation of native ions can be totally prevented if their *m*/*z* values are beyond the detection range of mass analyzer.

The common approaches to increase the charge states of biomolecular ions in ESI-MS require denaturation of their native structure. Proteins can be unfolded in solution before the analysis using the high concentration of organic solvents [6] and/or extreme pH [7]. Denaturation can also be induced during ionization process using thermal activation and/or elevated spray voltage [8–11].

Williams and coworkers introduced an alternative approach to form high charge-state ions. In this approach, a small amount of supercharging (SC) reagents (e.g., m-nitro benzyl alcohol, m-NBA; sulfolane) is added to the ESI solution before the analysis [12–14]. The low concentration of SC reagents (<1%) does not affect the solution conformation of biomolecules [15–20]. However, because the SC

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reagents have much higher boiling points compared with water, their concentration is gradually increased in evaporating ESI droplets [18, 21]. The saturation of SC reagents in mature ESI droplets is believed to be directly related to the formation of supercharged gas-phase ions. The key role of droplet environment in mediating SC process is evidenced by the observations of SC phenomenon when SC reagents are introduced into ESI plume externally (e.g., using dual-spray configuration [22]). Analyte SC in the presence of m-NBA was originally attributed to the increased surface tension of mature ESI droplets saturated with SC reagent [14]. However, further experiments by the groups of Grandori and Loo questioned the role of surface tension in SC of proteins [20, 23]. Later, Williams and coworkers attributed the charge enhancement of protein ions to unfolding and/or conformational changes to the increased concentration of SC reagents in the ESI droplets [15–19, 24], but this interpretation is not universally accepted [25, 26]. Thus, a number of studies demonstrated the preservation of noncovalent complexes in ESI MS upon SC [20, 23, 25–28].

To improve our understanding of SC process, in this study we compared the ESI MS response of several model biopolymers to the addition of SC reagent m-NBA with the ESI MS response of the same biopolymers to thermal activation during ESI process. In both cases, analyte activation is achieved in evaporating ESI droplets rather than in bulk solution, which allows the direct comparison of experimental data. Model polypeptides and DNA oligonucleotides were ionized from 100 mM ammonium acetate (NH_4Ac) aqueous buffer in both positive (ESI+) and negative (ESI–) ion modes. m-NBA was added to the ESI solution at 1% concentration (w/v). Thermal activation of analytes was achieved by heating the MS inlet capillary. Apart from the addition of SC reagents and heating, the same conditions were used in all the experiments. Overall, our observations indicate that SC reagents affect ESI MS charging of biomolecules via distinctly different mechanism compared with traditional approaches. Based on our data and recent results from other groups, the action mechanism of SC reagents is discussed.

Experimental

Proteins used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) at ≥99% purity. DNA oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) at ≥99% purity. m-NBA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) at >97% purity. Ammonium acetate (NH_4Ac) was obtained from Aladdin Industrial Corporation (Shanghai, China). To prepare buffer solution, NH_4Ac was dissolved in pure deionized water (18 MΩ•cm) to the concentration of 100 mM. Analytes were dissolved in the buffer solution to 10 μM concentration.

MS experiments were conducted on an LTQ linear ion trap mass spectrometer (ThermoFischer, San Jose, CA, USA). Mass spectra were collected in *m/z* range 300–3500. To avoid the effects caused by different ion transmission, the same instrument parameters were used in ESI+and ESI–, with only the inversions of voltage polarities whenever necessary. We did not observe any notable dependence of CSD for analyte ions on the ESI voltage (0.5–4.5 kV) and solution flow rate (1–10 μL min^{-1}). Both parameters were optimized based on the signal-to-noise ratio of ion signals. Spraying voltage was usually +1 kV in ESI+and –1 kV in ESI–. Analyte solution was introduced at a flow rate of 4 μL min^{-1}. Nebulizer nitrogen gas pressure was 1 MPa. LTQ capillary temperature was varied from 220 to 450°C. No analyte signal was detected at the zero spraying voltage, indicating that there was no component of the signal attributable to the sonic spray ionization mechanism in our experiments.

Results and Discussion

Figure 1 illustrates ESI MS analysis of cytochrome *c* in 100 mM NH_4Ac aqueous solution. Despite its low buffering capacity, NH_4Ac is the most widely used additive to stabilize biomolecules in native ESI MS [29]. It consists of two volatile components [i.e., ammonia (NH_3) and acetic acid (CH_3COOH)], and provides gentler droplet environment during ESI process compared with ammonium bicarbonate [8, 30]. When ionized without activation, cytochrome *c* was observed within a very narrow charge state distribution (CSD). The most abundant charge states in ESI+and ESI– were +6, +7, +8, and –4, –5, –6 accordingly (Figure 1a and e). The observed CSDs are very similar to those reported in the earlier ESI MS study of native cytochrome *c* by Konermann and Douglas [31].

When the MS inlet capillary was heated from 220 to 450°C, the CSD of cytochrome *c* ions in ESI+became broader and notably shifted toward higher charge states (Figure 1b). In contrast, the CSD of cytochrome *c* in ESI– remained nearly unchanged, some in-source fragmentation being evident (Figure 1f). Konermann and Douglas observed very similar ESI MS response of cytochrome *c* to unfolding in solution: pronounced broadening of CSD and shift to higher charge states in ESI+, but only marginal changes in ESI– [31]. This similarity strongly suggests that the change in the CSD of cytochrome *c* occurring at the elevated temperature of MS inlet corresponds to protein denaturation in ESI droplets.

In contrast to thermal activation, remarkably different ESI MS response was observed for cytochrome *c* upon activation with SC reagent. The addition of 1% m-NBA into ESI solution of cytochrome *c* induced the shift of CSD toward higher charge states in both ESI+(Figure 1c) and ESI– (Figure 1g). The absolute magnitude of the shift was nearly equal between ESI+and ESI– and higher than the magnitude of the shift in ESI+caused by heating (Figure 1b). In a reference experiment, the two modes of activation were applied together (i.e., 1% m-NBA was added into ESI solution and the MS inlet was heated to 450°C). Interest-

ingly, no further enhancement of SC effect was revealed, as could be expected if the SC mechanisms of these activation modes were similar. Instead, in both ESI+and ESI– the net effect on the CSD of cytochrome *c* was very close to that achieved by droplet heating only (Figure 1d and h). These observations suggest that the SC induced by m-NBA is mechanistically distinct from the SC induced by protein unfolding in ESI droplets. Furthermore, the effect of m-NBA appears to be neutralized at elevated temperatures.

In the same way, we explored the ESI charging behavior of myoglobin. Myoglobin contains a noncovalently bound heme group in its hydrophobic pocket. Upon the unfolding of globular myoglobin structure, the heme moiety becomes much weaker bound and can be readily extracted into solution. Myoglobin with the preserved heme moiety is referred to as holomyoglobin. Myoglobin without heme is commonly referred to as apomyoglobin. In our experiments, myoglobin ionized without activation was observed as holomyoglobin in a narrow CSD in both ion modes. The most abundant charge states in ESI+and ESI– were +7, +8, +9 and –6, –7 accordingly (Figure 2a and e). The CSDs of native myoglobin in ESI+and ESI– are very similar to those reported in the earlier study by Sterling et al. [15].

When the MS inlet capillary was heated from 220 to 450°C, myoglobin ions were observed in two distinct CSDs in both ESI+and ESI– (Figure 1b and f). In ESI+, the first distribution had the intensity maximum at 8+ and was very similar to that produced by native myoglobin (Figure 1a) but contained both holomyoglobin and apomyoglobin ions. The second distribution was broader, had the intensity maximum at 11+, and only contained apomyoglobin ions (Figure 2f). Concomitant with the formation of apomyoglobin, free heme ions were also detected. In ESI–, the "native" CSD had the intensity maximum at 7–, and "denatured" CSD had the intensity maximum at 10– (Figure 2f). The poor visibility of free heme in ESI– is likely related to its low ionization efficiency in ESI–. Both in ESI+and in ESI–, the ratio of apomyoglobin to holomyoglobin for a given charge state increased with charge, which reflects the higher degree of denaturation for ions with higher charge states. The lower rate of heme dissociation in ESI– than in ESI+can be explained by the stabilization of heme binding by electrostatic forces, (e.g., by binding to deprotonated carboxylic groups. Katta and Chait observed very similar ESI+MS response of myoglobin to unfolding in solution: narrow CSD at low charge states with holomyoglobin partially preserved and broader CSD at higher charge states constituted entirely by apomyoglobin [7]. In another study, Kelly et al. found that the CSD of unfolded myoglobin in solution was very similar in ESI+and in ESI– [32]. Compared with the native myoglobin CSD, denatured CSD was broader, shifted to higher charge states, and mostly contained apomyoglobin ions [32]. Overall, this suggests the charge-increased myoglobin ions that occur in response to heat activation result from partial protein unfolding in evaporating droplets. Myoglobin unfolding in solution occurs within milliseconds [33, 34], which is the estimated time frame of droplet evaporation in ESI.

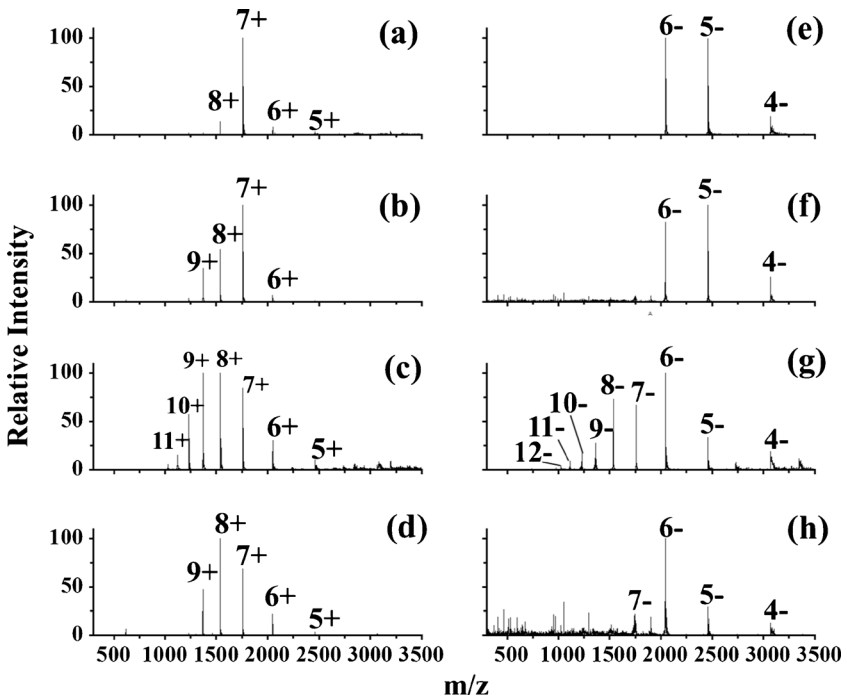

Figure 1. ESI MS of 20 μM cytochrome *c* in 100 mM aqueous NH_4Ac buffer in ESI+(**a**)-(**d**) and ESI– (**e**)–(**h**): without activation (**a**), (**e**); at the increased temperature of MS inlet (**b**), (**f**); with 1% m-NBA added into ESI solution (c, g); at the increased temperature of MS inlet with 1% m-NBA added into ESI solution (**d**), (**h**)

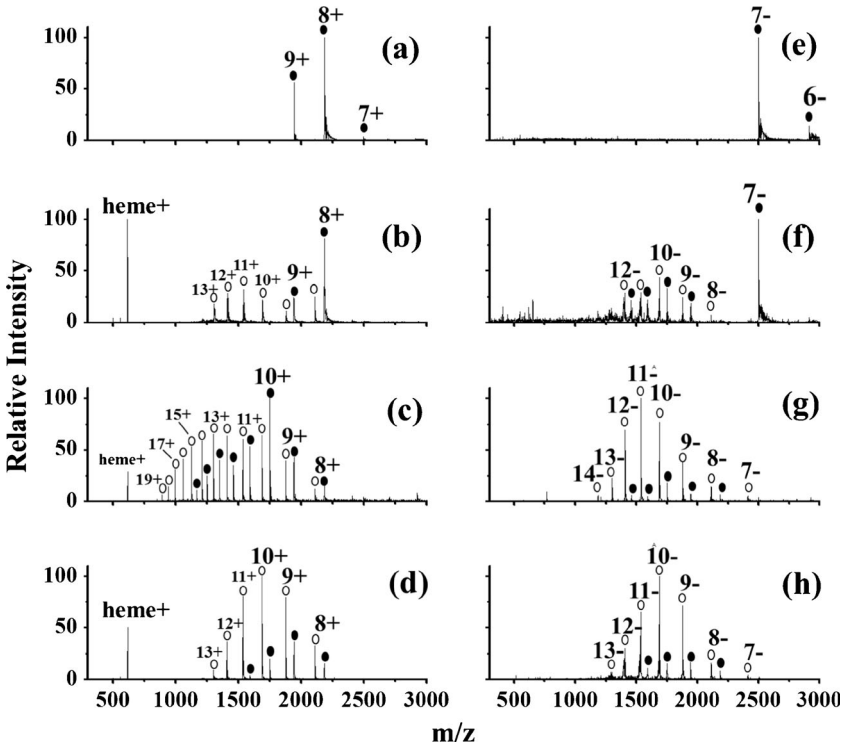

Figure 2. ESI MS of 20 μM myoglobin in 100 mM aqueous NH_4Ac buffer in ESI+(**a**)–(**d**) and ESI– (**e**)–(**h**): without activation (**a**), (**e**); at the increased temperature of MS inlet (**b**), (**f**); with 1% m-NBA added into ESI solution (**c**), (**g**); at the increased temperature of MS inlet with 1% m-NBA added into ESI solution (**d**), (**h**). Holomyoglobin and apomyoglobin ions are marked with filled and open circles accordingly

In contrast to thermal activation, remarkably different ESI MS response was observed for myoglobin upon activation with m-NBA. The addition of 1% m-NBA into native ESI solution of myoglobin induced the shift of CSD toward higher charge states in both ESI+(Figure 2c) and ESI– (Figure 2g). The absolute magnitude of the shift in each ion mode was higher than the magnitude of the shift in the same ion mode caused by heating (Figure 2b and f). In ESI+, the maximal charge state of 19+ was observed (Figure 2c). Remarkably, for each charge state, the intensity ratio of holomyoglobin to apomyoglobin was much higher than upon thermal activation (Figure 2b), which suggests "softer" SC process. In earlier experiments, very similar charge increase was demonstrated for native myoglobin in ESI+by Lomeli et al. using sulfolane [25] and by Sterling et al. using m-NBA [15]. In fact, the authors were able to achieve SC of myoglobin with much lower degree of denaturation than in the current study. Holomyoglobin signal intensity exceeded that of apomyoglobin even for the highest charge state [15]. The more pronounced dissociation of holomyoglobin in our experiments can reflect additional energy uptake during ionization and ion transfer specific to instrument settings. Thus, the extent of in-source heme dissociation could be decreased by applying lower voltages to LTQ capillary and tube lens. However, the total ion current and, in particular, the signal intensity of charge states with m/z >1500 dropped dramatically under those conditions, probably due to the lower transfer efficiency. In contrast to ESI+, rather similar response was observed in ESI– to either heating or the addition of m-NBA; however, activation by m-NBA did induce slightly stronger charging. Interestingly, Sterling et al. did not observe a notable SC effect for the native myoglobin activated with m-NBA in ESI– [15]. Again, this can be related to the difference in experimental conditions. Thus, in the same study, Sterling et al. reported no significant charge increase of myoglobin in ESI– upon unfolding in solution [15], which is in contrast to our observations and observations by Kelly et al. [32]. The different mechanism of myoglobin SC with m-NBA and temperature is further indicated by the results of the experiments in which m-NBA and heating were applied together (Figure 2d and h). Like in the case of cytochrome *c*, the charge increase in the combined approach was very similar to that achieved by droplet heating only (Figure 2b and f) and weaker than that achieved with m-NBA (Figure 2c and g).

Apart from myoglobin, some other noncovalent protein complexes have been reported to preserve integrity in ESI MS upon SC [20, 25, 27], although some complexes were found to be significantly destabilized by SC reagents [24]. Lomeli et al. employed the addition of m-NBA (<1%) to promote the charge increase for 20S proteasome complex, zinc-bound carbonic anhydrase-II protein, enolase dimer, alcohol dehydrogenase tetramer, and phosphorylase *b* dimer

without dissociation of the noncovalently bound ligands/subunits [20]. In another study, Hogan et al. used sulfolane to induce SC of phosphorylase *b* dimer analyzed from charge-reducing triethylammonium formate aqueous buffer [27]. Using complementary ion-mobility analysis, the authors found that sulfolane addition did not substantially alter the structure of phosphorylase ions generated by ESI MS [27]. These findings strengthen the evidence that the charge enhancement of proteins in ESI MS using SC reagents is not directly related to conformational changes in ESI droplets.

Earlier studies also indicate highly characteristic effect of SC reagents to the ESI charging of DNA oligonucleotides [28, 35, 36]. The addition of m-NBA and sulfolane into ESI solution (NH_4Ac) induced substantial charge enhancement of DNA ions in ESI– but had little to no effect in ESI+for all the oligonucleotides studied [28, 35, 36]. Furthermore, at a low concentration of m-NBA (<0.5%), supercharged double-stranded DNA anions could be observed without significant onset of duplex dissociation. Here, we compared ESI MS response for a single-stranded 12-mer CCCCAATTCCCC analyzed from 100 mM NH_4Ac aqueous solution, when activated with either the addition of m-NBA to ESI solution, increased temperature of MS inlet capillary or both (Figure 3). Whereas m-NBA induced significant CSD shift in ESI– (Figure 3g), thermal activation had no effect to CSD but, instead, caused notable in-source fragmentation of DNA ions in either ESI+or ESI– (Figure 3b and f). Similarly, no charge enhancement could be revealed in either detection mode for DNA oligonucleotides ionized from heated solution [36]. When m-NBA and heating were applied together, the net effect in the both ion modes was reduced to that by thermal activation only (i.e., notable in-source dissociation without charge increase (Figure 3d and g).

Overall, the results of our experiments, together with earlier published data, strongly suggest that SC reagents enhance ESI charging of biomolecules via distinctly different mechanism compared with traditional approaches. A number of recent studies reported the observation of gas-phase adducts between charge-enhanced protein ions and SC reagents [25–27]. The degree of adduct formation was found to correlate with the degree of charge enhancement [25, 26]. It was therefore proposed that a more direct interaction between protein and SC reagent, other than denaturing or increased charge availability, could be responsible for the occurrence of SC phenomenon [25, 26]. The decreased degree of SC observed at elevated temperatures of MS inlet further supports this hypothesis because adducts are generally destabilized upon thermal activation. Based on their experiments with cytochrome *c* and sulfolane, Douglas and Venter recently proposed that through large scale solvent reordering, proximal highly polar SC reagents delocalize charge on a biopolymer [26]. As a result, the electrostatic barrier for additional charging is reduced. This mechanism is supported by the clear correlation between the dipole moments of several SC reagents and the extent of charging for cytochrome *c* [26]. This mechanism also rationalizes why the proteins, which have a large number of

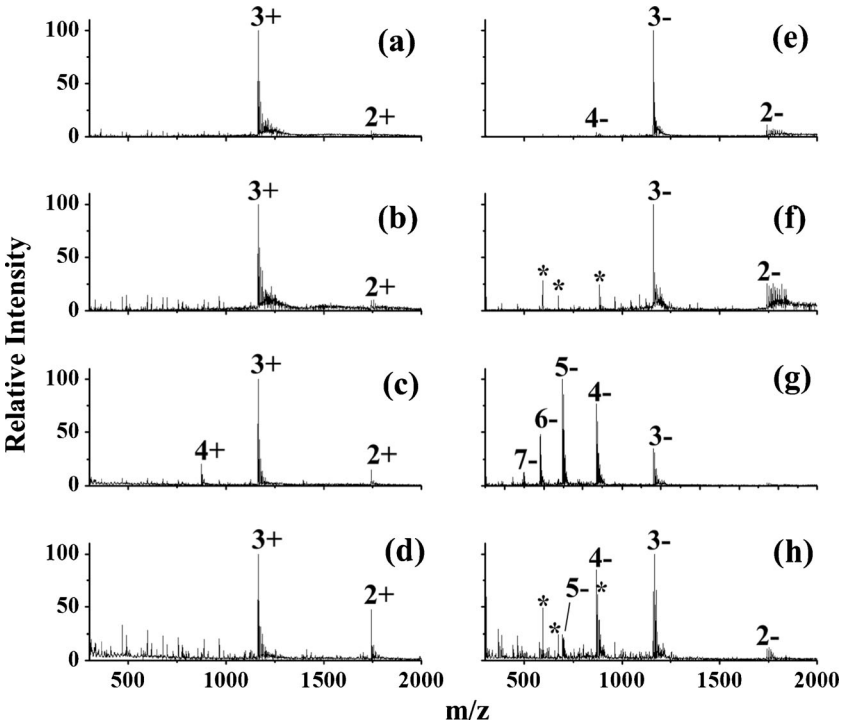

Figure 3. ESI MS of 10 μM single-stranded DNA 12-mer CCCCAATTCCCC in 100 mM aqueous NH_4Ac buffer in ESI+(**a**)–(**d**) and ESI– (**e**)–(**h**): without activation (**a**), (**e**); at the increased temperature of MS inlet (**b**), (**f**); with 1% m-NBA added into ESI solution (**c**), (**g**); at the increased temperature of MS inlet with 1% m-NBA added into ESI solution (**d**), (**h**). Abundant in-source fragment ions are marked with asterisk

highly basic amino groups, are preferentially supercharged in ESI+, whereas the DNA oligonucleotides, which have multiple acidic phosphate groups, are preferentially supercharged in ESI–.

Conclusion

Our observations indicate that SC reagents enhance ESI charging of biopolymers such as proteins and DNA via distinctly different mechanism compared with the traditional approaches based on analyte denaturation. Overall, the data support the hypothesis that the SC phenomenon involves a more direct interaction between a biopolymer and SC reagent, such as charge delocalization on the biopolymer via dipolar interaction with the reagent occurring in evaporating ESI droplets. The growing evidence that SC reagents allow pronounced charge enhancement of biopolymers with little destabilization to the native analyte structure, and contributing to a better understanding of the underlying mechanism, increase the interest in SC reagents as a tool for structural MS and top-down proteomics studies.

Acknowledgments

This work was supported by the National Natural Science Fund of China (no. 21305012), Science and Technology Planning Project at the Department of Science and Technology of Jiangxi Province, China (no. 20124ACB00700), and the Jiangxi Province Program for Training Talents in Science and Technology (no. 2010DD01300).

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