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Ionic strength of electrospray droplets affects charging of DNA oligonucleotides

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The fundamental aspects of charging in electrospray ionization (ESI) are hotly debated. In the present study, ESI charging of DNA oligonucleotides was explored in both positive (ESI+) and negative (ESI−) polarity using mass spectrometry detection. Single-stranded 12-mer CCCCAATTCCCC in buffer solution (aqueous NH_4Ac, 100 mM) produced similar charge state distribution (CSD) in either ESI+ or ESI−. Similarity of CSD in ESI+ and ESI− was also observed for the double-stranded 12-mer CGCGAATTCGCG. By adding typical low-vapor reagents (e.g. m-nitro benzyl alcohol, m-NBA; sulfolane) into the same buffer solution (<0.5% w/v), both CCCCAATTCCCC and CGCGAATTCGCG revealed strong supercharging (SC) effect in ESI−, while very little or no SC effect was observed in ESI+. With either sulfolane or m-NBA, the CGCGAATTCGCG duplex dissociated into single strands in ESI−. No SC was observed in both ESI+ and ESI− for thermally denatured CGCGAATTCGCG duplex in NH_4Ac buffer without the reagents. These findings are difficult to reconcile with the earlier model, which attributes SC in aqueous buffer solution to the conformational changes of analytes. Our observations suggest that the ionic strength of ESI droplets strongly affects the CSD of biopolymers such as DNA oligonucleotides and that SC effect is related to the depletion of ionic strength during the ESI process. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: ESI mechanisms; DNA oligonucleotides; charge state distribution; supercharging; gas-phase ions

Introduction

Electrospray ionization (ESI) is widely applied in modern mass spectrometry (MS) owing to its ability of generating intact, multiply charged macromolecular ions, but the mechanistic aspects of this process are not yet fully understood.[1–4] It is generally accepted that multiply charged biopolymer ions are formed in accordance with the charged residue model (CRM) from Rayleigh-charge limited ESI droplets.[5–7] However, experimentally observed charge states and other ESI behaviors of proteins, e.g. supercharging (SC),[8,9] can deviate from those predicted by CRM.[10–16] The interpretation of protein charging is greatly complicated by the presence of solution conformation and its alteration over ESI process. Even the short polypeptides that had been considered random coils in solution were recently found to possess structural motifs sensitive to microenvironment.[17–19] Under present circumstances, the choice of simpler model systems can be beneficial to improve our understanding of ESI process and pinpoint unaccounted factors.

Recently, attention of the community has been brought to DNA oligonucleotides. Unlike polypeptides, no predefined conformation was established for short oligonucleotide sequences.[20–22] This allows elimination of the conformational factor out of consideration for more comprehensive and precise mechanistic interpretation of charging behavior.[23] Gas-phase DNA ions can be generated directly from aqueous buffer without the addition of auxiliary chemicals in both ESI+ [24–27] and ESI−,[23,24,28–32] which allows direct comparison of experimental data. Furthermore, double-helix DNA can be transferred into gas phase with the preserved structure.[33–35] Double-stranded DNA ions represent a simple model to probe noncovalent interactions in the gas phase.[31] In this contribution, charging of DNA oligonucleotides in ESI MS is explored further. Based on our observations for single-stranded and double-stranded 12-mer oligonucleotides in both ESI+ and ESI− as well as on the results of earlier studies,[23,30,31] we propose that the ionic strength of electrospray droplets mediates the key role in the charging process of DNA oligonucleotides and that the SC effect is related to the depletion of ionic strength during the ESI process.

Experimental

DNA oligonucleotides used in this study were purchased from Sangon Biotech Co., Ltd (Shanghai, China) at ≥99% purity. SC reagents m-nitro benzyl alcohol (m-NBA) and sulfolane were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) and Aladdin Industrial Corporation (Shanghai, China) at >97% and ≥99.5% purity, accordingly. Ammonium acetate (NH_4Ac) was obtained from Aladdin Industrial Corporation (Shanghai, China). DNA oligonucleotides were dissolved in pure deionized water (18 MΩ•cm) or in NH_4Ac aqueous buffer to 10 μM concentration. Before MS analysis, the DNA solution in a closed 1.5 ml eppendorf tube was heated in a water bath up to 95 °C and was cooled down to room temperature at ambient conditions. This step prevented nonspecific binding of DNA strands. For consistency, heating was applied during the preparation of both double-stranded and single-stranded sequences. DNA samples were stored as dry-powder aliquots at −20 °C. Each working day, new DNA samples in a fresh buffer were prepared.

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MS experiments were conducted on an LTQ linear ion trap mass spectrometer (ThermoFischer, San Jose, USA). Mass spectra were collected in *m/z* range 300–2000. 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 charge state distribution (CSD) for DNA 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 DNA 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 pressure was 1 MPa. LTQ capillary temperature was 220 °C. No DNA signal was detected at the zero spraying voltage, indicating that there was no component of the signal due to the sonic spray ionization mechanism in our experiments.

Results and discussion

Figure 1 illustrates ESI MS analysis of single-stranded DNA 12-mer CCCCAATTCCCC. The sequence was chosen such as to avoid the formation of a hairpin, double helix or quadruplex structure.[36] When ionized from 100 mM NH_4Ac aqueous buffer, the oligonucleotide was observed within a very narrow CSD, triplet charge state being the most abundant in both ESI+ and ESI− (Fig. 1(a, d)). Similar charging behavior for DNA oligonucleotides was observed in earlier studies.[23,30] The great similarity of CSD in ESI+ and in ESI− is consistent with the CRM mechanism of DNA ionization proposed earlier.[37] In CRM, the average charge state of gas-phase ions is limited by the Rayleigh charge of analyte-sized droplets and depends on their size and surface tension but not on the ion polarity.[5]

The majority of salts are nonvolatile and therefore incompatible with ESI MS of biological molecules. When CCCCAATTCCCC was analyzed from NaCl buffer solution, the analyte signals were totally suppressed by the abundant NaCl clusters in both ESI+ and ESI−. NH_4Ac is the most popular salt in biological ESI MS studies, because it consists of two volatile components, i.e. ammonia (NH_3) and acetic acid (CH_3COOH).[2] NH_4Ac has a poor buffering capacity at pH 7 but effectively maintains the pH value of aqueous solution between 6 and 8. The net charge of ESI droplets results from the excess of NH_4^+ cations in ESI+ and the excess of CH_3COO^- anions in ESI−, respectively. In ESI+, DNA phosphate groups associate with NH_4^+ as the droplet dries out. The resulting adduct then loses NH_3 during collisional activation in the ion sampling region of the mass spectrometer to yield multiply protonated DNA ions.[1] In ESI−, the excess negative charge in ESI droplets results in the formation of DNA poly-anions via partial deprotonation of phosphate groups.

The alterations in CSD of CCCCAATTCCCC ions were studied in response to the addition of a small amount (≤1% w/v) of m-NBA and sulfolane into the spraying solution (100 mM NH_4Ac) in both ESI+ and ESI−. These low-vapor additives commonly promote enhanced charging of gas-phase ions in ESI. The approach is commonly referred to as SC and is of increasing interest in mechanistic studies.[7–11,38–44] In our experiments, we observed much more pronounced SC effect for CCCCAATTCCCC in ESI− (Fig. 1(e, f)) than in ESI+ (Fig. 1(b, c)). The extent of SC in ESI− with sulfolane was lower than with equal amount of m-NBA, but comparable charging was induced at higher concentration of sulfolane (1%). The observed SC behavior is in excellent agreement with the recent study by Tabet and coworkers in which they explored SC of several single-strand DNA 12-mers and 6-mers with m-NBA.[23] All the studied sequences revealed pronounced SC in ESI− but negligible effect in ESI+. This strongly suggests that the preference to SC in ESI− but not in ESI+ is intrinsic to DNA. Moreover, we conclude that conformational factors, if any, have no notable effect during ESI charging of short single-strand oligonucleotides.

Based on their experiments with proteins, Williams and coworkers proposed that, in line with CRM, low-vapor additives affect the observed charge state of gas-phase analyte ions in two major ways: (i) by modifying the surface tension[7] and (ii) by mediating conformational changes of analyte molecules in ESI droplets.[39,41,42,44] For biopolymers electrosprayed from an aqueous buffer, the first factor is actually associated with decreased average charge of gas-phase ions, because the surface

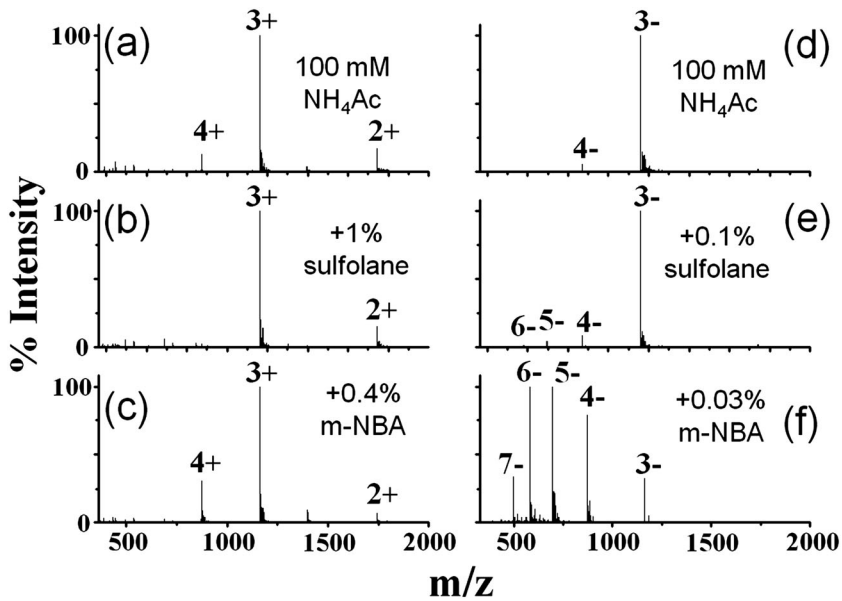

Figure 1. ESI MS of 10 μM single-stranded DNA 12-mer CCCCAATTCCCC in 100 mM aqueous NH4Ac buffer in ESI+ (a–c) and ESI− (d–e): without the addition of SC reagents (a, d); with, sulfolane (b, e); with m-NBA (c, f).

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tension of both m-NBA and sulfolane are lower than that of water.[38] Therefore, increase in the average charge of biopolymer ions analyzed from an aqueous buffer can only occur as a result of conformational changes within ESI droplets, overweighing the opposite effect of decreased surface tension.[44] However, the strong SC of short DNA sequences as well as the occurrence of SC effect only in ESI− is difficult to explain by conformational factors.

In complement to single-stranded 12-mer CCCCAATTCCCC, we also analyzed ESI charging of double-stranded 12-mer CGCGAATTCGCG, which is a popular DNA model in ESI MS studies.[45] When ionized from 100 mM NH_4Ac at a 10 μM concentration, CGCGAATTCGCG duplex was clearly observed in both ESI + and ESI− (Fig. 2(a, g)). While the even charge states of the duplex (e.g. 4+ or 6+) could be superimposed with the CSD of the dissociated strands (2+ and 3+, accordingly), the presence of duplex was unambiguously indicated by the odd charge states (5+ in our case).

SC behavior of CGCGAATTCGCG was very similar to that of CCCCAATTCCCC. In ESI+, only modest CSD shift was induced with m-NBA (Fig. 2(d, e)) and no notable shift was observed with sulfolane, even though relatively higher concentration of sulfolane was applied (Fig. 2(b, c)). The addition of SC reagents in ESI+ did not notably affect duplex stability, as reflected by the mass spectra obtained at different concentrations of SC reagents. In ESI−, both m-NBA (Fig. 2(j, k)) and sulfolane (Fig. 2(h, i)) promoted pronounced SC of CGCGAATTCGCG; m-NBA being more efficient. The observed charging in ESI− was clearly accompanied by duplex denaturation (Fig. 2(i, k)). Very similar SC behavior of double-stranded DNA 14-mer anions was observed by Madsen and Brodbelt.[31] Of note, the high resolution Fourier transform ion cyclotron resonance MS in their experiment distinguished between double-stranded and single-stranded DNA signals. The difference clearly indicated that at low concentrations of m-NBA (<0.5%) notable SC effect occurred without significant onset of duplex dissociation.[31]

Pronounced SC and disintegration of CGCGAATTCGCG duplex due to the addition of m-NBA or sulfolane to DNA solution only in ESI− is another observation that we find difficult to explain with the SC model by Williams and coworkers. Within the framework of this model, supported by the observation of proteins, analyte SC in aqueous buffer occurs as a result of denaturation in ESI droplets aided by the enhanced concentration of low-volatility components.[39,41,42,44] Because the latter process does not depend on the net charge of the droplet, duplex denaturation should occur at the same rate in both ion detection modes, which is however not supported by our data. In a reference experiment, the spraying capillary of the ESI source was heated to induce thermal dissociation of CGCGAATTCGCG double helix in solution before ionization. As a result, only the single-stranded ions were detected in both ESI+ and ESI− (Fig. 2(f, l)). Clearly, thermal dissociation of DNA duplex was not accompanied by notable SC effect in either polarity. Dissociated single strands of CGCGAATTCGCG were dominantly observed as triply charged ions, the same as the single-stranded CCCCAATTCCCC (Fig. 1(a, d)).

Overall, our data suggest that the SC of DNA oligonucleotides is quite unlikely to be caused by conformational changes, as proposed for proteins.[41–43] The following observations are particularly difficult to explain by conformational changes: (i) pronounced SC of very short single-stranded DNA sequences;

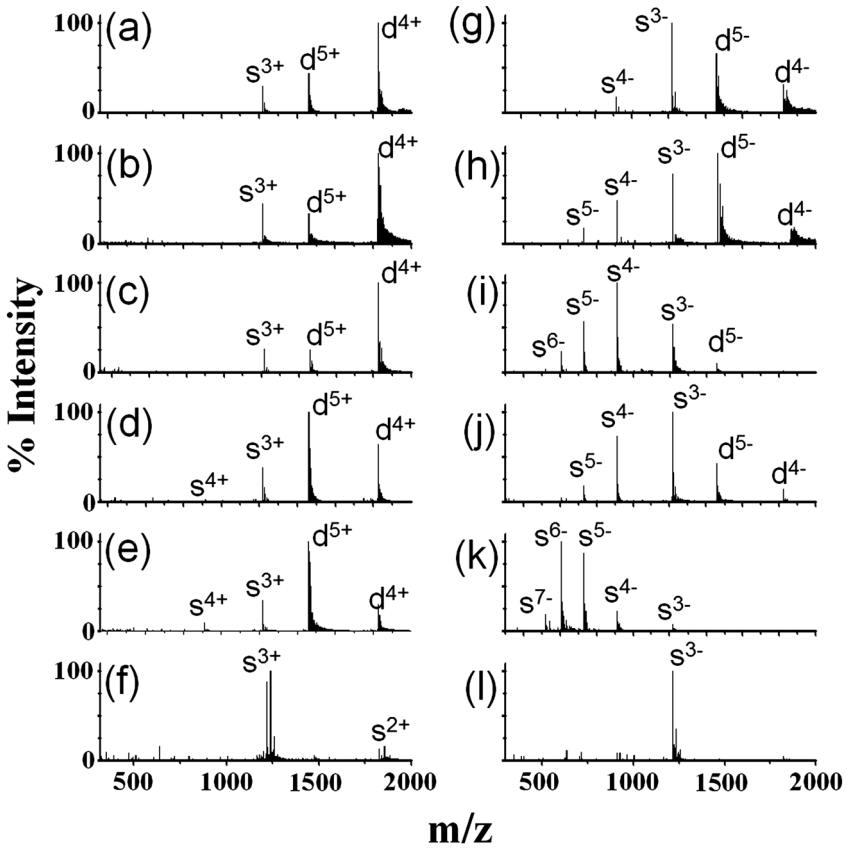

Figure 2. ESI MS of 10 μM self-complementary DNA 12-mer CGCGAATTCGCG in 100 mM aqueous NH4Ac buffer in ESI+ (a–f) and ESI− (g–l): without the addition of SC reagents (a, g); with 0.04% sulfolane (b, h); with 0.4% sulfolane (c, i); with 0.01% m-NBA (d, j); with 0.1% m-NBA (e, k); without SC reagents from heated ESI solution (f, l). Double-stranded and single-stranded ions are denoted as 'd' and 's', accordingly.

(ii) the occurrence of SC effect only in ESI−; (iii) denaturation of DNA duplex following the addition of SC reagents only in ESI−; (iv) the absence of extra-charging for the thermally denatured DNA duplex. To provide a consistent interpretation for the observed SC behaviors of DNA oligonucleotides, the following model is proposed. At a neutral pH (~7), the highly acidic phosphate groups in oligonucleotide are negatively charged. The net negative charge of DNA poly-anions is partially or fully compensated by NH_4^+ cations. During the ESI process, volatile NH_3, CH_3COOH and water evaporate at a much faster rate than the low-vapor SC reagents. As a result, the concentration of SC reagents in ESI droplets is gradually increasing while the concentrations of NH_3, CH_3COOH and water are decreasing. Because neutral NH_3 and CH_3COOH are in equilibrium with NH_4^+ and CH_3COO^- ions, the ionic strength of mature ESI droplets and, therefore, the amount of NH_4^+ per phosphate group decrease as well. As a result, the charge compensation of a DNA poly-anion becomes weaker, and higher charge states are observed in ESI−. Thereby, pronounced SC of DNA in ESI− can simply reflect the strong dependence of analyte charging on the ionic strength of ESI solution. The enhanced ESI− charging of DNA oligonucleotides upon the decrease in ionic strength of spraying solution was observed by Creig and coworkers[30] and confirmed in our lab (data not shown). Furthermore, Tabet and coworkers recently found that the degree of SC for single-stranded DNA anions correlates with the density of thymine groups.[23] Phospho-thymidine units are the most acidic sites in DNA and should have the highest affinity toward the anionic form upon the depletion of electrolytes in ESI droplets. The onset of duplex dissociation in ESI− at a high concentration of sulfolane or m-NBA[31] can be easily explained by the increased coulomb repulsion between supercharged complementary strands. Compared to phosphate groups, nucleobase amines are less ionic and therefore have much weaker interaction with buffer counter-ions. For example, solution studies show that duplex grooves are usually filled with water or with sodium cations but not with anions. This can explain why the depletion of electrolyte concentration in ESI droplets promoted by SC reagents does not notably affect CSD of DNA oligonucleotides in ESI+. In a reference set of experiments, we analyzed CCCCAATTCCCC in ESI+ from aqueous solution with different concentrations of NH_4Ac (100 mM, 10 mM, 1 mM, 100 μM, 10 μM). As the concentration of NH_4Ac was decreased from 100 mM down to 10 μM, we observed gradual decrease of signal intensity but no notable change in the CSD of DNA cations, which is consistent with our model. No DNA signal could be detected at a concentration of NH_4Ac below 100 μM likely due to the lack of solvent cations to compensate deprotonated phosphate groups. Interestingly, the proposed model implies the reverse cause-and-effect relationship between SC and biopolymer denaturation as compared to the model by Williams and coworkers for proteins, *viz.* duplex dissociation occurring in response to SC rather than SC occurring in response to duplex dissociation.

Key role of the ionic strength of ESI droplets in charging and SC processes is further indicated by ESI MS of DNA oligonucleotides dissolved in pure deionized water (Fig. 3). Pure water can be regarded as the extreme case of NH_4Ac aqueous buffer with zero concentration. At the 10 μM concentration, deprotonated DNA poly-anions and protons dissociated from the phosphate groups represent the most abundant ionic species. Therefore, the compensation of DNA anions in water droplets is much weaker than in the droplets generated from buffer solution. Accordingly, both

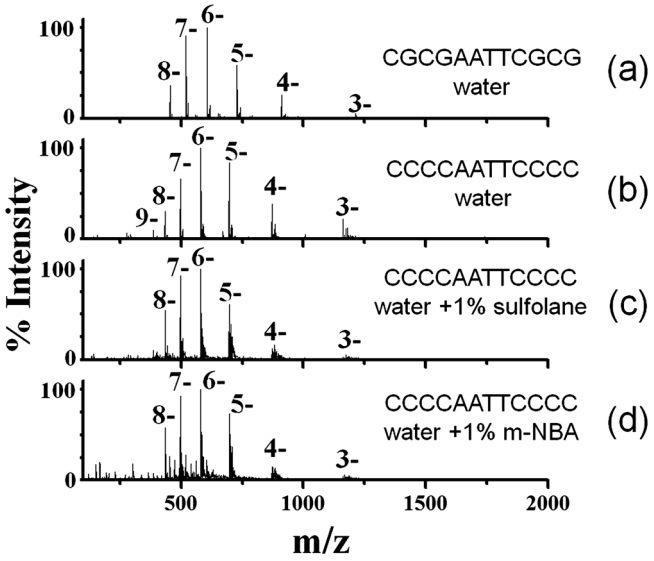

Figure 3. (a): ESI− MS of 10 μM CGCGAATTCGCG in pure deionized water; (b): ESI− MS of 10 μM CCCCAATTCCCC in pure deionized water; (c) ESI− MS of 10 μM CCCCAATTCCCC in pure deionized water with 1% sulfolane; (d) ESI− MS of 10 μM CCCCAATTCCCC in pure deionized water with 1% m-NBA.

CCCCAATTCCCC and CGCGAATTCGCG in ESI− from pure water were observed at even higher charge states than from 100 mM NH_4Ac buffer with high amount of sulfolane or m-NBA (Fig. 3(a, b)). The highest charge state of DNA 12-mer anions in our experiments was 9−, indicating that nearly all the 11 phosphate groups carry negative charge. Indeed, no duplex ions were observed for CGCGAATTCGCG due to the instability of double-helix conformation in buffer-free solution. No notable SC effect could be revealed with either sulfolane or m-NBA (Fig. 3(c, d)), because the ionic strength of pure water is already too low to control DNA charging. Neither of the oligonucleotides could be detected in ESI+, which reflects the lack of solvent electrolytes in water to compensate the negative charge on phosphate groups. These data provide support for the charging mechanism described above.

Unlike DNAs, protein affinity to SC is usually much stronger in ESI+ than in ESI−.[41] According to our model, the depletion of ionic strength due to the addition of SC reagents will result in weaker compensation of cationic protein residues (e.g. arginine, lysine and tyrosine) by CH_3COO^- anions and, thereby, increased charge states in ESI+. This situation is the reverse of that for DNAs, in which SC in ESI− reflects a decreased compensation of anionic phosphate groups by NH_4^+ cations. However, protein charging is more complex and case specific due to the onset of conformational factors. The depleted ionic strength of ESI droplets can promote protein denaturation. Denaturation alters the number of polar groups exposed to solvent, which greatly affects the observed CSD. Furthermore, protein basicity and conformation strongly depend on the primary amino acid sequence and post-translational modifications.

Several studies revealed dependence of protein charging on the ionic strength of ESI buffer. Thus, Klassen and coworkers have documented the increased average charge state of several acidic proteins with the decrease in ionic strength of NH_4Ac buffer in ESI− but not in ESI+.[46] The effect was attributed to electrostatically driven protein unfolding occurring in charged droplets. However, our model suggests that, just like for DNA anions,

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higher charge states of acidic proteins in ESI− with a low ionic strength of solution can be primarily caused by the weaker charge equilibration of deprotonated groups. Protein denaturation is a possible but not inevitable aftermath of increased charge state. In fact, Loo and coworkers have recently demonstrated increased charging of noncovalent protein complexes by m-NBA with little-to-no dissociation of noncovalently bound ligands/subunits from aqueous, near neutral-pH solutions.[11,16] This interesting observation was discussed in the literature, but no consistent interpretation has been offered.[10,11,39] In another study, Williams and coworkers observed much weaker SC for hen egg white lysozyme when this protein was electrosprayed from 200 mM NH_4Ac compared to pure water solution using dimethyl sulfoxide (DMSO) as the SC reagent.[43] To account for this observation, it was proposed that buffer ions fully or partially counteract the destabilizing effect of DMSO during the formation and evaporation of ESI droplet.[43]

Conclusion

In summary, charging behaviors of DNA oligonucleotides in ESI revealed in this study are difficult to interpret within the framework of existing models. Based on our findings and earlier published data, we suggest that the ionic strength of ESI droplets strongly affects CSD of biopolymers such as DNA oligonucleotides and that the charge increase of gas-phase ions upon the addition of low-vapor reagents into buffer solution is related to the depletion of ionic strength during ESI process. Interestingly, the proposed mechanism accounts for the possibility of analyte SC with preserved native-like conformation, for example if the analyte conformation is sufficiently stable. Seemingly, the refined model can rationalize some poorly understood protein behaviors,[11,15,16,46] but its general relevance to proteins is yet to be explored.

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