Generating Supercharged Protein Ions for Breath Analysis by Extractive Electrospray Ionization Mass Spectrometry

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S *Supporting Information*

ABSTRACT: Supercharged protein ions produced by electrospray ionization are extremely efficient proton donors for secondary ionization. Here, by electrospraying the protein solutions containing 5% 1,2-butylene carbonate, the supercharged protein ions with unusually high proton density were produced as the primary ions for the ionization of exhaled breath samples in the extractive electrospray ionization mass spectrometry (EESI-MS), which resulted in the enhanced ionization efficiency for the breath analytes even with relatively low gas phase basicity. Moreover, the total number of metabolites detected in breath increased by about 260% in the mass range of 200−500 Da, owing to the substantial signal enhancement for breath metabolites, providing complementary and additional information to conventional SESI.
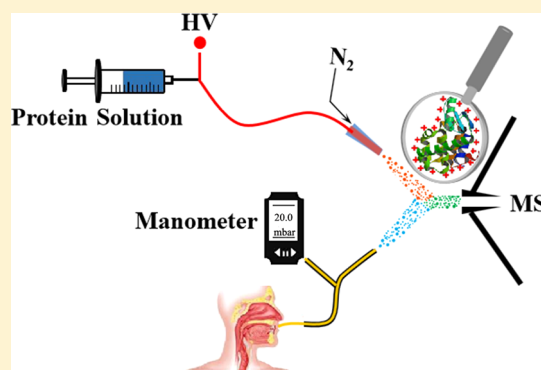
The exhaled breath contains molecular information on personal metabolic states, which makes breath analysis attractive for noninvasive diagnoses.[1,2] Traditionally, breath samples are analyzed by solid-phase microextraction in combination with gas chromatography−mass spectrometry (SPME-GC-MS),[3] which is the gold standard for the off-line breath analysis. However, sample collection and pretreatment can be complicated. Selected ion flow tube mass spectrometry (SIFT-MS)[4] and proton transfer reaction-mass spectrometry (PTR-MS)[5] have been increasingly successful in real-time breath analysis without sample pretreatment. Alternatively, extractive electrospray ionization mass spectrometry (EESI-MS, Figure 1), a technique validated for reliable analysis of perfume,[6] skin,[7] and viscous samples,[8,9] requires no sample pretreatment for real-time breath analysis.[10,11] Typically, a mixture of water with organic solvent is used as an ionizing solution in EESI. As featured by the unique design of EESI, reactive chemical reagents are easily implemented in the ESI spray to produce suitable ionic reagents for ionization of specific analytes. For example, reactive silver cations (Ag$^+$) addictive was used for the detection of acetonitrile[11] and sulfur-containing compounds,[12] as well as tetrabromobisphenol A derivatives,[13] and acetic acid (HAc) agent was employed for the selective detection of uranyl species.[14] This promotes EESI as a platform for selective detection of given analytes in a complex matrix with tunable ionization efficiency and improved selectivity.
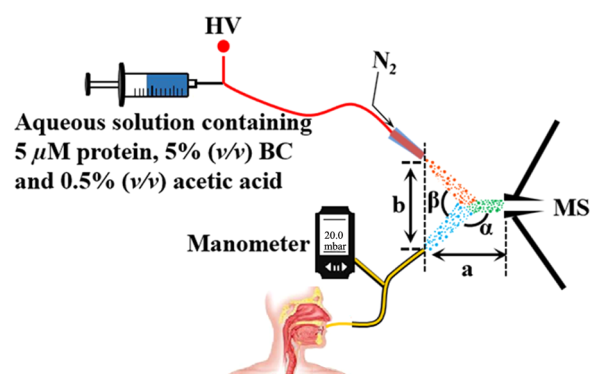

Figure 1. Schematic illustration of the protocol of real-time breath analysis by EESI-MS with supercharged proteins as the extractive ionic reagent.

The charge states of protein ions in native MS can be increased using *m*-nitrobenzyl alcohol,[15,16] sulfolane,[17] and 1,2-butylene carbonate (BC).[18] This phenomenon is collectively termed as protein "supercharging". Supercharging additives can be used to increase the ion abundances of peptides in LC-MS.[19,20] In addition, such additives can be used to lower the

mass-to-charge ratios (m/z) of macromolecules to maximize the mass resolving power and mass accuracy.[21−24] Protein supercharging has also been demonstrated useful for the improvement in efficiency of protein fragmentation and disulfide bond cleavage.[25−27] Owing to the extremely high density of protons, supercharged protein ions display very distinct properties. Recently, proteins with unusually high charge states were found to be the strongest acids, as pioneered by Donald and co-workers,[28] which are able to protonate N_2 and Ar in the gas phase. This finding opens novel possibilities for EESI to analyze breath metabolites with supercharged proteins.

In this work, supercharged proteins prepared by doping 1,2-butylene carbonate were electrosprayed in the EESI-MS to generate primary supercharged protein ions for interaction with the exhaled breath samples (Figure 1). During the collisions occurring at ambient conditions, the protons carried by the supercharged protein ions were apt to transfer from the supercharged protein ions to the analytes in the breath aerosols, resulting in the protonated breath analytes for mass analysis. Our experimental data showed that more analytes were detected with increased abundances using the supercharged protein ions instead of methanol/water/acids solution, with improved sensitivity and complementary information for breath analysis.

EXPERIMENTAL SECTION

The materials, chemical reagents, and samples were directly used without sample pretreatment (see SI for details). The supercharged protein ions were formed by spiking 5% 1,2-butylene carbonate into the protein solution,[28] which was then directly sprayed for production of the supercharged protein ions (see SI for details).

The real-time EESI-MS breath experiments were carried out using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA, U.S.A.) coupled with a homemade EESI source for ion generation and Xcalibur software for instrument control and data processing. The EESI source design and principle were detailed elsewhere.[29,30] Briefly, the EESI source was composed of two major parts: channels 1 and 2 (Figure 1). Channel 1 was used to generate the supercharged protein ions as the primary ions. Channel 2 was made of a piece of Teflon tube (ID 2 mm, OD 4 mm, length 30 cm, $T = 110\ °C$) that was used to introduce the breath sample. The distances (a) between the tips of the EESI source and the MS inlet and the distance (b) between the two spray channels of the EESI source were 5 mm and 1−2 mm, respectively. The angle (β) between the two spray channels and the angle (α) between individual spray and the MS inlet were around 90° and 150°, respectively. For the setting parameters of the Orbitrap Fusion Tribrid, see SI for details. The volunteer then watched the manometer dial and adjusted exhale strength to maintain the pressure of 20 mbar. The analytes in exhaled breath were directly ionized by supercharged protein ions plume and the breath fingerprints were automatically recorded. Breath fingerprint data acquired using supercharged protein ions and the conventional methanol/water/acetic acid (49.75%/49.75%/0.5%, $v/v/v$) were classified by t-distributed stochastic neighbor embedding (t-SNE), providing a visual representation of the heterogeneity of the data (see SI for details).

RESULTS AND DISCUSSION

Formation of Supercharged Protein Ions. Supercharged proteins were formed by electrospraying the solution with added supercharging reagent 1,2-butylene carbonate rather than m-nitrobenzyl alcohol or sulfolane. The representative ESI mass spectrum of 99.5%/0.5% water/acetic acid solutions containing 5 μM insulin with no supercharging additive showed a low charge state (Figure 2a), in which the

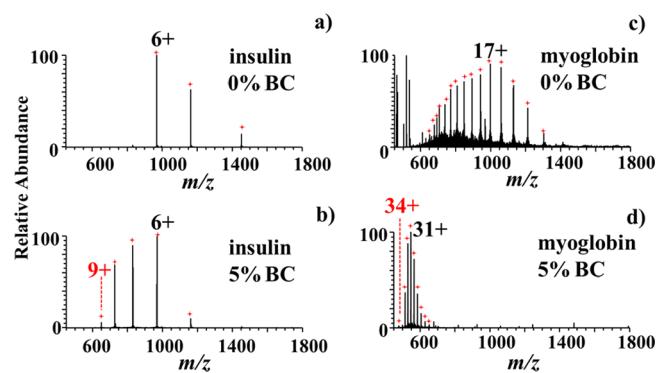**Figure 2.** ESI mass spectra of 0.5% acetic acid in water solution containing: (a) 5 μM insulin, 0% BC; (b) 5 μM insulin, 5% BC; (c) 5 μM myoglobin, 0% BC; (d) 5 μM myoglobin, 5% BC (BC designates the supercharging additive, 1,2-butylene carbonate).

basic peak was a z_{base} of 6$^+$, the highest charge state was a z_{max} of 7$^+$ and the averaged charge state was z_{avg} of 5.5$^+$. With the addition of 5% 1,2-butylene carbonate, the charge state was shifted to the lower mass range (Figure 2b) with z_{base} of 6$^+$, z_{max} of 9$^+$, and z_{avg} of 6.7$^+$. Similarly, myoglobin was shifted from z_{max} of 27$^+$ to 34$^+$, z_{base} of 17$^+$ to 31$^+$, and z_{avg} of 19.0$^+$ to 31.5$^+$ (Figure 2c and d); lysozyme was shifted from z_{max} of 12$^+$ to 20$^+$, z_{base} of 10$^+$ to 17$^+$, and z_{avg} of 10.0$^+$ to 16.8$^+$ (Figure S1a,b); cytochrome c was shifted from z_{max} of 20$^+$ to 25$^+$, z_{base} of 9$^+$ to 19$^+$, and z_{avg} of 9.1$^+$ to 20.0$^+$ (Figure S1c,d). These data confirmed that the proteins were successfully charged with unusually high charge states by adding the 5% BC as the supercharging additive, which were similar to those previously observed.[18,31] A possible reason for protein supercharging by butylene carbonate is that the cyclic nonvolatile butylene carbonate, which is an ultraweak base/poor proton acceptor, decreases the degree of charging for solvent clusters and increases the degree of protein charging. This mechanism has been proposed earlier by Lakshmanan et al.[32] The increased surface tension of ESI droplets due to the presence of 1,2-butylene carbonate is another factor that could be partially responsible for the enhanced protein charging. The surface tension of 1,2-butylene carbonate (>35 mN m$^{−1}$) is higher than that of mixtures of acetic acid and water. Acetic acid is less volatile than water and should be enriched in the ESI droplets prior to ion formation.

Breath Analysis by EESI-MS with Supercharged Protein Ions. In order to evaluate the contribution of supercharged proteins to the increased ionization efficiency of breath metabolites, four ionizing solutions in channel 1 (Figure 1) were compared: (1) methanol/water/HAc (49.75%/49.75%/0.5% $v/v/v$), (2) 5% butylene carbonate/94.5% water/0.5% HAc (without protein), (3) 5 μM protein in 99.5% water/0.5% HAc (without supercharging additive), (4) 5 μM protein in 5% butylene carbonate/0.5% HAc/94.5% water (supercharged protein ions). The obtained breath data

were then processed to identify characteristic metabolites (see SI and Figure S2 for details). As can be seen in Figure 3, higher
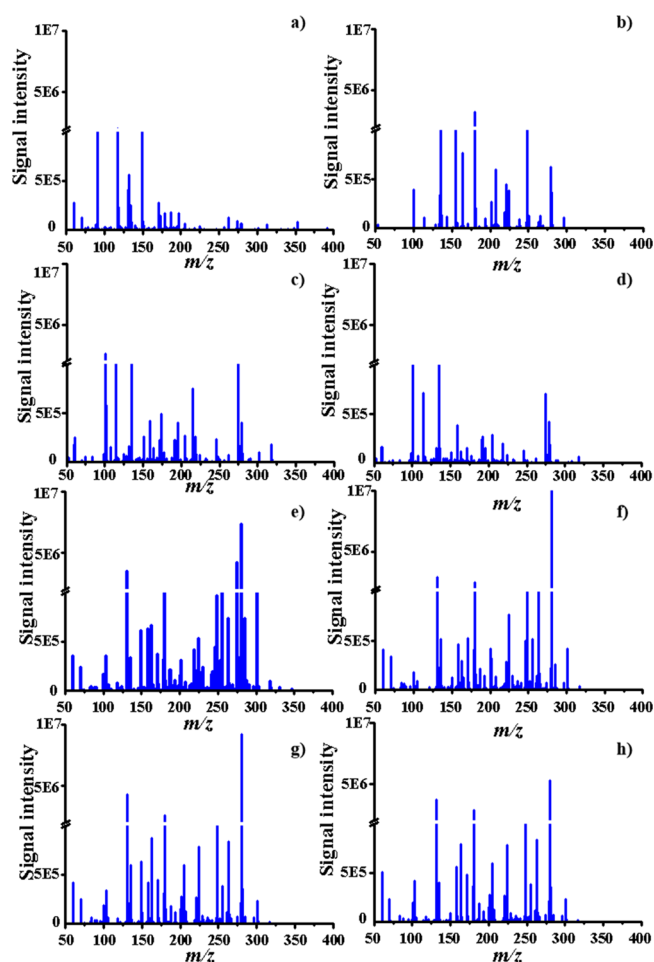

Figure 3. Characteristic metabolite signals acquired by EESI-MS with different composition of ionizing solution: (a) 49.75%/49.75%/0.5% methanol/water/HAc; (b) 5% butylene carbonate/94.5% water/0.5% HAc; (c) 5 μM lysozyme in 99.5% water/0.5% HAc; (d) 5 μM cytochrome c in 99.5% water/0.5% HAc; (e) 5 μM insulin in 5% butylene carbonate/94.5% water/0.5% HAc (supercharged insulin); (f) 5 μM lysozyme in 5% butylene carbonate/94.5% water/0.5% HAc (supercharged lysozyme); (g) 5 μM cytochrome c in 5% butylene carbonate/94.5% water/0.5% HAc (supercharged cytochrome c); (h) 5 μM myoglobin in 5% butylene carbonate/94.5% water/0.5% HAc (supercharged myoglobin).

signal intensity of metabolites was achieved using ionizing solution containing both protein and butylene carbonate (Figure 3e−h) in comparison with using ionizing solutions in which either protein or supercharging reagent (or both) were absent (Figure 3a−d). Therefore, the results indicate that the increased ionization efficiency of metabolite is most probably caused by the proton transfer from supercharged proteins. However, further research is needed to definitively clarify whether the proton transfer from supercharged protein ions to metabolites occurs mainly in the gas phase or in mature ESI droplets.

Moreover, *t*-SNE was further used to provide a visual representation of the heterogeneity of the breath metabolites. Figure S3 visualizes distinction between the corresponding data sets of breath fingerprints acquired using supercharged insulin (purple mark), lysozyme (blue mark), cytochrome c

(red mark), myoglobin (yellow mark), and classic solution (green mark) by *t*-SNE. Then, the characteristic metabolite ions detected using supercharged protein and standard ionizing solution were compared as shown in Figure 4. The red peaks
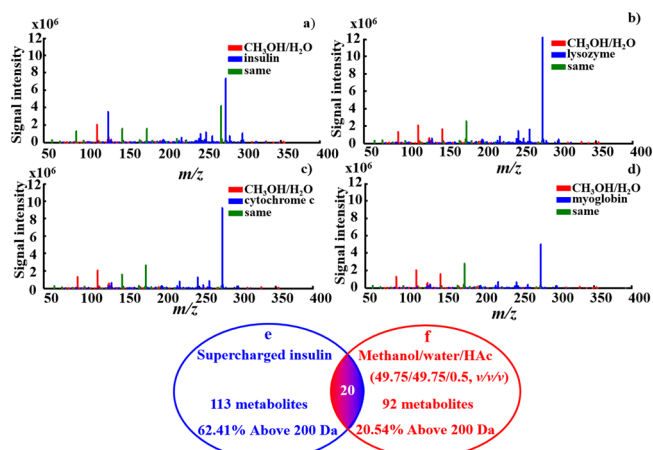

Figure 4. Comparison of the characteristic metabolites detected by different supercharged protein and methanol/water/acetic acid (49.75%/49.75%/0.5%, *v/v/v*): (a) supercharged insulin; (b) supercharged lysozyme; (c) supercharged cytochrome c; (d) supercharged myoglobin; (e) distribution of characteristic metabolites obtained by supercharged insulin; (f) distribution of characteristic metabolites obtained by conventional methanol/water/acetic acid (49.75%/49.75%/0.5%, *v/v/v*).

represent metabolites solely detected using standard ionizing solution. The blue peaks represent metabolites solely detected using supercharged protein. The green peaks represent metabolites detected using both supercharged protein and standard solution. As can be seen in Figure 4, the blue peaks are relatively tightly centered within the range of 200−500 Da, whereas the red peaks are concentrated in the range of 50−150 Da. The spectral patterns illustrate that the supercharged proteins as primary ions in EESI expand the coverage of metabolites in the exhaled breath to species well above 200 Da. In detail, the total metabolites of 133 and 112 were detected from the exhaled breath sample by EESI-MS using either supercharged insulin or the conventional acidic solvent with no supercharged proteins, respectively. Only 20 metabolites were commonly detected by EESI-MS using the two types of electrospray solvents, showing that the mass spectral signals were well complementary to each other (Figure 4e,f). Furthermore, with supercharged proteins as the ionic reagent, 62.41% metabolites (i.e., 83 among 133) were detected in the mass range above 200 Da, while only 20.54% metabolites (i.e., 23 among 112) were detected close to the low end of the mass range above 200 Da using the solvent without supercharged proteins. Thus, the number of metabolites detected among the mass range of 200−500 Da in the breath were increased by about 260% (i.e., 100(83−23)/23%). The specific metabolite with corresponding extraction reagent, abundance, molecular structure, and so on are detailed in Table S1. There is some slight variation in observed analytes using different proteins (Figure 4a−d, Table S1). This variation may be caused by the difference in intrinsic physical properties of these proteins, specifically those related to charge affinity. For example, estimated isoelectric point (pI) for insulin is about 1.0; pI of lysozyme is around 7.0; pI of cytochrome c is around 4.5; pI of

myoglobin is about 7.1. In principle, the efficiency of proton transfer should be maximal for protein ions with the lowest proton affinities in microdroplets and/or in the gas phase. However, due to the lack of a notable experimental trend (Figure 3e−h), at this point it is too preliminary to draw any conclusions regarding the role of protein structure in the observed differences.

Signal Enhancement Obtained by Supercharged Protein Ions. By using the present EESI-MS combined with supercharged protein as the primary ions, some special compounds with relatively low gas phase basicity have been observed. The unsaturated aliphatic hydrocarbons such as tridecene formed an adduct with ammonium $[C_{13}H_{26} + NH_4]^+$ (m/z 200.23752) in the breathand were detected with high abundance of about 3.73E5 (Figure 5a), while no signal was
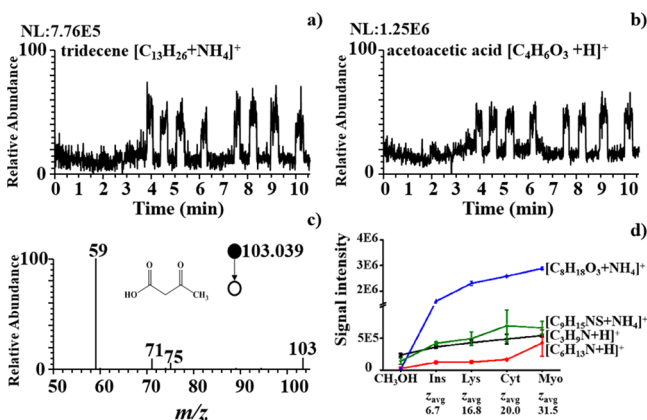
Figure 5. Typical metabolites detected using supercharged protein solutions: (a) the real-time extracted ion chromatogram of tridecene adduct $[C_{13}H_{26} + NH_4]^+$; (b) the real-time extracted ion chromatogram of acetoacetic acid $[C_4H_6O_3 + H]^+$; (c) EESI-MS/MS mass spectra of acetoacetic acid; (d) signal enhancement along the averaged charge states of the ionic reagents observed for $[C_8H_{18}O_3 + NH_4]^+$, $[C_3H_9N + H]^+$, $[C_6H_{13}N + H]^+$, and $[C_9H_{15}NS + NH_4]^+$.

detectable using the methanol/water/HAc solution. The NH_4^+ adducts of breath metabolitesare commonly observed in EESI-MS of human breath since trace amount of ammonia vapor is present both in the breath and in the surrounding environment. Especially, due to its high proton affinity, NH_4^+ ions are readily formed in the ionizing area and can cluster with certain metabolites.[33,34] There are several mechanisms whereby supercharging agent could possibly facilitate ionization by ammonium ion, for example, via the enhanced ionization of ammonia to form NH_4^+ or via the adduction of neutral ammonia to the protonated metabolite. The clarification of exact mechanism is left for future studies. The acetoacetic acid, a species unlikely to be protonated in normal cases, was detected about 6.1E5 as the protonated molecule at m/z 103.03930 (Figure 5b). It was tentatively identified based on the characteristic fragment peak at m/z 59, m/z 75, and m/z 71 (Figure 5c), probably due to the cleavage of neutral CO_2, CO, and O_2 from the precursor ions to generate $[C_3H_7O]^+$, $[C_3H_7O_2]^+$, and $[C_4H_7O]^+$, respectively. However, the acetoacetic acid showed no signal using the methanol/water/HAc solution. Acetoacetic acid is usually an indispensable source of energy for newborns to develop extrahepatic tissues and is a metabolite present in the metabolism of those undergoing starvation or prolonged physical exertion as part of ketogenesis.[35] In this work, the presence of acetoacetic acid rightly

reflected the metabolic state of a healthy female adult after fasting for 12 h. Therefore, the supercharged protein ions for breath analysis would provide more metabolic information rather than the classical EESI experiments.

The signal intensity level of breath metabolites were also influenced by the composition of EESI extraction solution. The majority of metabolite signals were enhanced upon the addition of supercharged proteins by about 2−5 times. The different enhancement magnitude of signal intensity for different breath metabolites may possibly be explained by the differences in their proton affinity. As an example, the signal at m/z 60.08110 was assigned to protonated adduct [trimethylamine + H]$^+$ based on the high-resolution data. When supercharged proteins were respectively sprayed, the signal intensity was enhanced. The magnitude of signal enhancement was in correlation with the magnitude of z_{avg}: the higher the z_{avg} of supercharged protein, the stronger the signal. Thus, the intensity of signal at m/z 60.08110 was about 2.35E5 using methanol/water/acetic acid ionizing solution, 3.60E5 upon the addition of supercharged insulin ($z_{avg} = 6.7$), 4.28E5 upon the addition of supercharged lysozyme ($z_{avg} = 16.8$), 4.85E5 upon the addition of supercharged cytochrome c ($z_{avg} = 20.0$), and 5.39E5 using supercharged myoglobin ($z_{avg} = 30.5$). Thus, the intensity of [trimethylamine + H]$^+$ signal at m/z 60.08110 in the presence of supercharged myoglobin was 2.3× higher than that obtained by electrospraying the methanol/water/acetic acid solution. Similarly, the signal intensity of cyclohexylamine $[C_6H_{13}N + H]^+$ was 2.72E4 without supercharged protein, 1.20E5 with supercharged insulin, 1.26E5 with supercharged lysozyme, 1.6E5 with supercharged cytochrome c, and 4.23E5 with supercharged myoglobin. The signal intensity of $[C_6H_{13}N + H]^+$ in the presence of supercharged myoglobin was 15.6× higher than that obtained by electrospraying the methanol/ water/acetic acid solution. For 5-butyl-4-ethylthiazole $[C_9H_{15}NS + NH_4]^+$, the signal intensity without supercharged protein was 1.38E5, the signal intensity with supercharged insulin was 4.17E5, the signal intensity with supercharged lysozyme was 4.9E5, the signal intensity with supercharged cytochrome c was 6.60E5, and the signal intensity with supercharged myoglobin was 6.90E5 (Figure 5d). The signal intensity of $[C_9H_{15}NS + NH_4]^+$ in the presence of supercharged myoglobin was 5 times higher than that obtained by electrospraying the methanol/water/acetic acid solution. Note that the formation of ammonium metabolite clusters is probably preceded by the protonation of ammonia in the ionizing area by supercharged proteins. Interestingly, while the majority of metabolite signals in our experiments showed the intensity enhancement of about 2−5 fold, the signal at m/z 180.15975 $[C_8H_{18}O_3 + NH_4]^+$ showed the enhancement of about 200-fold (Figure 5d). The particularly high degree of enhancement for $[C_8H_{18}O_3 + NH_4]^+$ is rather curious and presents an interesting topic for further research. These data suggest that protein with high charge state would be helpful for detection of trace analytes or analytes with low gas phase bacisity.

Mechanism of Signal Enhancement. Proton transfer was thereby proposed to account for the signal enhancement using supercharged proteins for EESI breath analysis. The latest work[28] by Donald estimated that the gas-phase basicity (GB) of the highest charge state of supercharged protein ions are extremely low, even much lower than O_2 and N_2. The lower the GB value, the more readily protein ions can protonate other molecules. Thus, supercharged proteins are

particularly prone to lose protons. This mechanistic interpretation was supported by several experiments. First under the condition of the mass range of 150−2000 Da for protein detection, there was only channel 1 used to generate the supercharged protein ions. ESI mass spectra of supercharged proteins before collisions could be obtained. Subsequently, channel 2 was applied to introduce breath sample to record the EESI mass spectra. Clearly, the EESI-MS spectral profiles of supercharged protein ions collected with and without breath samples were dramatically differed from each other (Figure 6). Note that the strongest contribution to
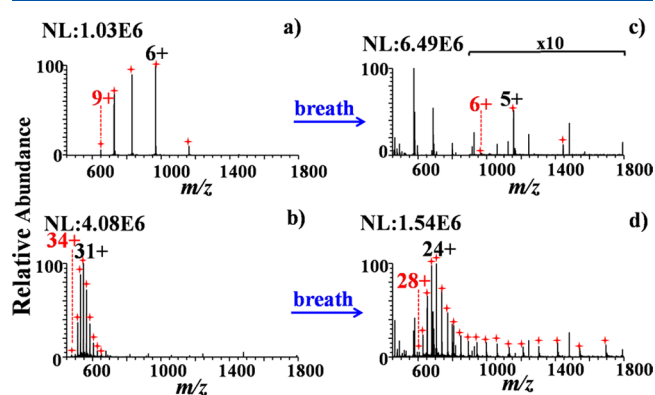

Figure 6. Mass spectra confirmed the proton transfer occurring between the supercharged proteins and the breath analytes in EESI: (a) supercharged insulin without breath sample; (b) supercharged myoglobin without breath sample; (c) supercharged insulin with breath sample; (d) supercharged myoglobin with breath sample.

the ionization process of breath metabolites can be expected either from the protein charge state with the highest signal intensity in the spectrum (z_{base}) or from the protein charge state with the highest number of charges (z_{max}), which should have the highest ionizing affinity. It was observed that, when reacted with a breath sample, the z_{max} of supercharged insulin ions reduced from 9 to 6 accompanied by dropping the z_{avg} from 6.7^+ to 4.9^+. Also, the signal intensity for the peak at z_{base} of 6^+ was further decreased from 1.03E6 to 4.21E4. Figure 6b−d illustrate that the supercharged myoglobin was also decreased from z_{max} of 34^+ to 28^+, z_{base} of 31^+ to 24^+, and z_{avg} of 31.5^+ to 21.79^+, which indicates that higher charge states transfer charge to metabolites more readily than the lower charge states. Similar phenomena were observed in all the proteins tested (Figure S4) and the shifts in z_{avg}, z_{max}, and z_{base} for different proteins are summarized in Table S2. To exclude the possibility that supercharged proteins would transfer their protons to water-saturated air, in a reference experiment water-saturated air was used instead of exhaled breath to react with ionizing spray containing supercharged protein. The results show that water-saturated air has almost no effect both on the charging states and signal intensity of supercharged protein ions (Figure S5). This result presents additional evidence that ionization enhancement of organic metabolites in breath is mainly due to the transfer of protons from supercharged protein ions. All the data confirmed that the charge state of proteins moved from high states to low states by donating more protons to the breath metabolites.However, because proteins are believed to be supercharged in late-stage ESI droplets, there are actually two principal processes possible whereby the proton transfer can occur. The first possible

process is that protein ions at first become fully desolvated and then ionize metabolites via gas-phase ion−molecule reaction. The second possible process is that the metabolite molecules diffuse inside a mature ESI droplet containing supercharged protein prior to complete protein desolvation, and the proton transfer from the protein ion to metabolite occurs in the solution phase.

The second mechanism was also put forward that the increase of surface tension might be another factor contributed to the signal enhancement. The alcohols with different surface tension values were used to prepare the electrospray solution of alcohols: water: acetic acid with a fixed ratio at (49.75%/ 49.75%/0.5% $v/v/v$). Compared with the characteristic metabolites acquired by supercharged insulin (black peaks), the mass spectral patterns (Figure S6) do not show significant difference no matter which solution mixture was used, although the surface tension value of 24 dyn cm^{-1} for methanol (red peaks), 27.18 dyn cm^{-1} for n-butanol (blue peaks), 27.81 dyn cm^{-1} for 1-hexanol (green peaks), 29.09 dyn cm^{-1} for 1-octanol (purple peaks), and 50.21 dyn cm^{-1} for ethylene glycol (orange peaks) were experimentally investigated, respectively. Therefore, no strong correlation was found between the breath fingerprints and the surface tension of the reagents. Also, it should be noted that by changing the solvent, the composition of the droplets are changing, the viscosity is changing, the rates of solvent evaporation are changing, and the gas-phase basicity values are changing, in addition to the surface tension. A batch of control experiments that trying to reduce a multivariate phenomenon to a single variable are left for future studies.

■ CONCLUSION

The primary ions with unusually high charge states were easily obtained by electrospraying proteins solutions with supercharging additive of 1,2-butylene carbonate, allowing more protons to be readily transferred from the ionic reagents to analytes in the exhaled breath, which resulted in the increase of detected metabolites about 260% and the signal intensity enhancement for the majority of metabolite in the range from 2 to 5 times. This method enabled more analytes to be detected form the breath samples in the relatively high mass range such as 200−500 Da, providing complementary information to the mass spectral data obtained by electrospraying methanol/water without supercharged protein ions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Additional information as noted in text (PDF).

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Notes

The authors declare no competing financial interest.

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