Mobility of Proteins in Porous Substrates under Electrospray Ionization Conditions

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ABSTRACT: Proteins are important substances in living organisms and characterization of proteins is an indispensible part for protein study. Analysis of proteins using electrospray ionization mass spectrometry (ESI-MS) with porous substrates was investigated in this study. The results revealed that the ionization process had two stages. At the first stage, mobility and resulting spectra of proteins were similar to those obtained with conventional capillary-based ESI-MS. At the second stage, hydrophobic-hydrophobic interactions between proteins and the tip surfaces play an important role in mobility and detectability of proteins, which were size and shape dependent, and a linear relationship could be found between the relative integration area of selected ion chromatogram and the cross section of protein ions. Preparative separation of proteins could be achieved by collecting the proteins remained on the porous substrates. These results propose a new approach for analysis of proteins and investigation of protein structures and conformations.

Proteins play critical roles in life activities and are hot targets of fundamental and applied research in chemistry and biology. Characterization of proteins provides information crucial to understanding the structures and functions of proteins. Techniques such as liquid chromatography[1,2] and gel electrophoresis[3,4] are commonly used for separation of proteins, and mass spectrometry (MS),[5-9] nuclear magnetic resonance spectroscopy[10-12] and X-ray crystallograpgy[13,14] are widely used for investigation of protein structures, conformations and interactions. Among these techniques, MS is superior to others in speed, sensitivity and specificity for characterization of proteins. Combination of MS with hydrogen/deuterium exchange[15,16] and ion mobility (IM)[17-21] have allowed mapping and measurements of different conformations of proteins. Electrospray ionization (ESI) is one of the most powerful ionization techniques for MS characterization of proteins.[7,22] Under ESI conditions, a protein is typically detected as a series of multiply charged ions that can be correlated to the protein conformations, and selective deposits of these multiply charged ions of proteins on a surface *in vacuo* using the soft-landing method has been reported.[23]

Conventional ESI uses capillary for sample loading and ionization. Upon application of a high voltage, sample solution sprays out of the capillary to form fine droplets. Solvent evaporation and repeated droplet fission ultimately lead to production of gas-phase ions of analytes. ESI is a very gentle ionization technique and allows maintenance and investigation of protein conformations and interactions. Non-capillary ESI has also been developed and solid substrates such as metal needles, paper and wood tips have been successfully applied for ESI analysis of various samples.[24-27] Use of solid substrates for sample loading and ionization much facilitates sample introduction, avoids sample clogging and allows analysis of samples of various forms.[28,29] Moreover, new ionization features were found with ESI on solid substrates. For example, it was reported that proteins could be ionized separately from salts or detergents in the solutions with ESI using stainless steel needles,[30,31] presumably because the small amount of solution loaded on the hard and smooth tip end could be considered as a large droplet, and proteins, the more hydrophobic components of the solutions, were easier to accumulate on the surface of the droplet to be ionized.

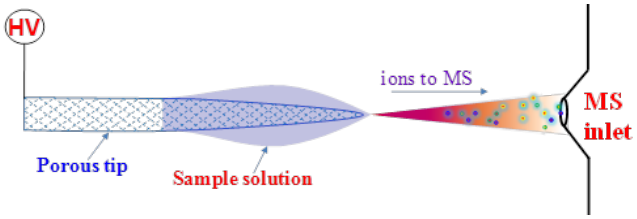

Figure 1. Schematic diagram of porous-tip ESI-MS

Herein we report that ESI with porous substrates could allow separation of proteins based on their sizes and shapes. The separated proteins can be monitored by the ESI spectra or by analysis of the proteins remained on the substrates, and the latter can also allow preparative separation of proteins. This new observation implies a new approach for investigation of proteins.

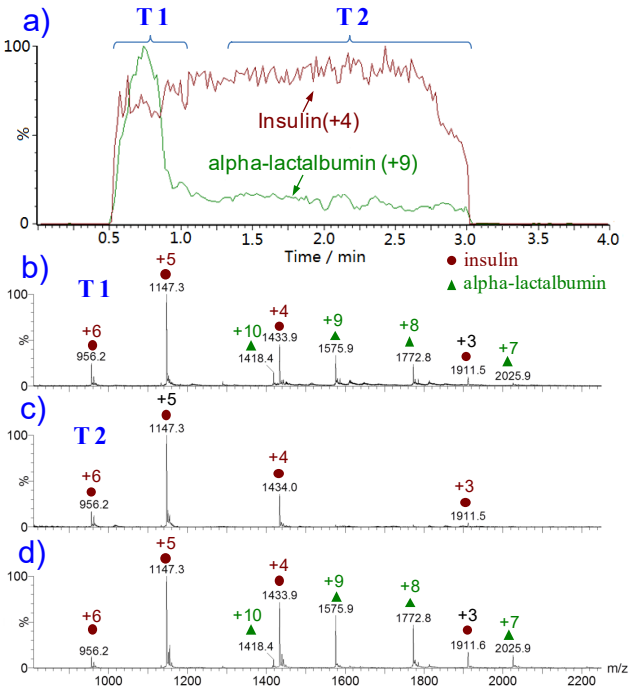

Figure 2. Analysis of the mixture of insulin and alpha-lactalbumin. a) SICs of the 4+ ion of insulin and the 9+ ion of alpha-lactalbumin obtained by polyester-tip ESI-MS; b) mass spectrum obtained for duration T1 in a); c) mass spectrum obtained for duration T2 in b); d) mass spectrum obtained by conventional ESI-MS.

The settings of solid-substrate ESI-MS is shown in Figure 1. When a sample solution is loaded onto the solid-substrate tip, with the application of a high voltage, the solution can be sprayed out of the tip and spectra of the sample can be obtained. In this study, an aliquot of 10 μL solution containing two proteins, alpha-lactalbumin (14.2 kDa) and insulin (5.7 kDa), was loaded onto a porous polyester tip and analyzed with the technique. The spraying lasted for about 2.5 min and the obtained selected ion chromatograms (SICs) of the two proteins are shown in Figure 2a. Two obviously different stages were observed during the ionization period. At duration 1 (T1), both proteins were abundantly observed in the mass spectrum (Figure 2b), which was very similar to the spectrum obtained by conventional ESI (Figure 2d). At duration 2 (T2), however, insulin, the smaller protein, was almost exclusively detected in the spectrum (Figure 2c). Such two-stage ionization and elution order could be reproducibly observed with the same polyester tip (after cleaning and reuse) or with differernt polyester tips (see Figure S1 in the Supplementary Information), and was assumed to be related to the use of the porous substrate for ionization.. As illustrated in Figure S2, when a solution was loaded onto a porous tip for electrospray ionization, initially, the pores on the tip were overwhelmed by the solution and the movement and ionization of the sample solution was mainly a bulk solution behavior, in which all the protein molecules sprayed out together, leading to a spectrum similar to that obtained by capillary-based ESI-MS. The second stage occurred when the solution was consumed to a low level. At this stage, driven by the electrospray, protein molecules migrated along the microchannels at the surface of porous substrate, and the results suggested that smaller molecules were easier to be sprayed out to be detected (Figure 2c).

To verify the separation effect of the porous tip and detect the proteins remained on the tip, after the ESI process, the front 1.0 mm part of the tip was cut out and extracted with solvent. The spectrum obtained by analysis of the extract using conventional ESI-MS showed that only insulin, the smaller protein, was in this most front part (see Figure S3a). Analysis of the other part of the tip revealed that it contained both alpha-lactalbumin and insulin, with alpha-lactalbumin predominated (see Figure S3b), indicating that alpha-lactalbumin, the larger protein, was more retained in the porous substrate. These results were consistent with spectral results obtained by polyester-tip ESI-MS, and suggested that under the ESI conditions, porous substrates could allow separation of proteins with smaller proteins moving faster to be separately detected.

Such a separation effect was further confirmed by using polyester-tip ESI-MS for analyzing a mixture containing alpha-lactalbumin (14.2 kDa) and lysozyme (14.3 kDa), two proteins having similar amino acid sequences and molecular weights but different sizes.[32,33] Such a mixture allowed comparison of the behaviors of ions from two proteins with the same charge states and close *m/z* values. The SICs of the 9+ ions of these two proteins were showed in Figure S4a. Again, at stage 1 (T1), both proteins were observed, and the spectrum obtained (Figure S4b) was very similar to that obtained by conventional ESI-MS (Figure S4d). At stage 2 (T2), lysozyme was almost exclusively detected in the spectrum (Figure S4c). Although the mass of lysozyme (14.3 kDa) is slightly larger than that of alpha-lactalbumin (14.2 kDa), X-ray crystallography measurements showed that lysozyme had a smaller size than alpha-lactalbumin.[34] These results again demonstrated the two-stage ionization in porous-tip ESI-MS and the size-dependent separation in the second stage.

Scanning electron microscope (SEM) was used to examine the properties of the polyester tips in this study. As shown in Figure S5, the SEM photos of the surfaces and cross sections of polyster tips revealed the existence of microchannels in the polyster tips. However, the pore size of the microchannels were in the range of tens of micrometers, much larger than the cross sections of proteins, which are typically in the range of nanometers.[35] Therefore, the polyster tips should not be able to physcially serve as filters for separation of proteins.

To better understand the mechanism for the separation, effect of tip materials on the protein separation was invetsigated in this study. In addition to polyster tips, wooden tips as well as polyethylene tips of different densities were used for analysis of the alpha-lactalbumin/insulin and alpha-lactalbumin/lysozyme samples. Among these tips,

polyethylene tips are most hydrophobic while wooden tips are most hydrophilic, yet wooden tips contain lignins[36] that also allow for some hydrophobic-hydrophobic interactions. As shown in Figures S6 and S7, similar two-stage ionization and the same elution and ionization orders were observed for these porous tips, as for the polyester tips (Figures 2 and S4). However, it could be found that the proteins were more retained by the more hydrophobic tips, and compared to wooden tips and polyester tips, polyethylene tips allowed better separation of both protein mixtures (see Figures S6b and Figure S7b). This suggested that hydrophobic-hydrophoobic interactions between proteins and the substrate surfaces played an important role in the protein mobility and separation. Alpha-lactalbumin, which has a larger size than insulin and lysozyme, had stronger hydrophobic-hydrophobic interactions with the tip surfaces and was thus more retained in the tips. Such behavior was more significant with the more hydrophobic tips, leading to the better separation effect with the polyethylene tips. Much longer signal duration was observed with the denser polyethylene tips for analysis of the same volume of the samples (Figures S6c and S7c), due to the larger surface areas for interactions between analytes and surface and the reduced diffusion of the solution.

To gain more insight into the effect of protein conformation on its mobility in the porous substrate, a solution of bovine cytochrome *c* was investigated with polyester-tip ESI-MS. Cytochrome *c* has been well studied with conventional ESI-MS, and the results revealed that the larger charge state of the protein observed in the ESI-MS had larger collision cross section (CCS).[35] The total ion chromatogram (TIC) obtained with polyester-tip ESI-MS analysis of the cytochrome *c* sample is shown in Figure 3a, together with the SICs of the 7+ and 17+ ions of the protein. It can be seen that in the first minute (T1), the 7+ and 17+ ions had very similar intensities and the spectrum obtained (Figure 3b) was very similar to that obtained by conventional ESI-MS (Figure 3d), indicating that these two charge state ions had similar tendency to be eluted and ionized. These spectra (Figures 3b and 3d) indicated that under the present conditions, the protein existed in two conformations with charges centered at 8+ and 14+, respectively, in the spectra. For a later spraying time (T2), however, the signal of the 17+ ion was significantly reduced, while the signal of the 7+ ion maintained relatively abundant (Figure 3c), leading to the protein ions with lower charge states much predominated in the spectrum (Figure 3c) and indicating that the protein species with higher charge states, i.e., with larger CCSs,[35] were more retained in the porous substrate. These results were consistent with those obtained for the protein mixtures, since higher charge states and larger CCSs meant more unfolded states of the protein and thus more hydrophobic-hydrophobic interactions of the protein with the tip surface, leading to more retainment of the protein by the tip. The observation that proteins of higher charge states did not have higher mobility also indicated that electrophoretic migration played a less important role in porous-tip ESI, consistent with the previous results obatined with paper spray.[37] Area of SIC of each protein ion was proportional to the eluted and ionized amount of the species and can be used as an indicator for the mobility or elution tendency of the species in the substrate. The relative integral area (RIA) of SIC of the protein ion at each charge state was calculated (see Figure S8 for the calculation details) and plotted against the CCS value of the protein ion.[35] Although these CCS values were previously obtained for the protein ions in the gas phase, they were for the same protein at different charge states and were thus assumed to be proportional to the values of the species in the solution phase in this study. A linear correlation ($R^2 = 0.82$), as shown in Fig. 3e, was obtained, indicating that the mobility of the protein in the porous substrate was size and shape dependent and the present technique could be used to measure the cross sections of proteins in solution and to study protein size, shape and conformations. Note that according to the literature,[35] more than one CCS values were used for some charge states of cytochrome *c*. This might, to some extent, reduce the linearity of the plot.

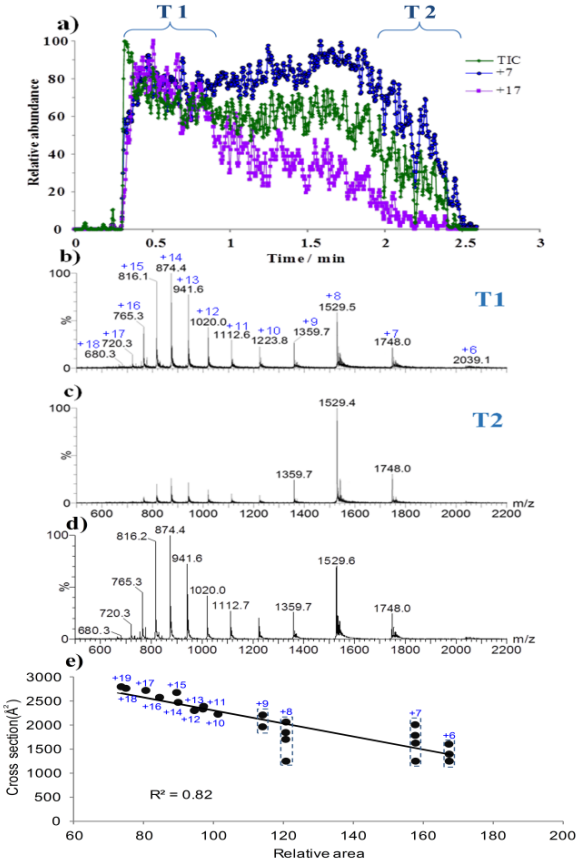

Figure 3. Analysis of the cytochrome *c* sample. a) TIC of and SICs of the 7+ and 17+ ions; b) mass spectrum obtained for duration T1 in a); c) mass spectrum obtained for duration T2 in b); d) mass spectrum obtained by conventional ESI-MS. e) Plot of CCSs of the protein ions with different charge states against their relative integral areas.

The solution of bovine cytochrome *c* was also analyzed using porous tips of different properties, including wooden tips and polyethylene tips of different densities. Similar two-stage ionization and the same elution order of different charge states of cytochrome *c* were observed with these tips (Figure S9). It can be seen that more hydrophobic tips or porous tips with larger surface areas allowed more retainment of the proteins and better separation of the different charge states,

again demonstrating that hydrophobic-hydrophobic interactions of proteins and the tip surfaces were the key for the protein elution and separation in porous-tip ESI-MS.

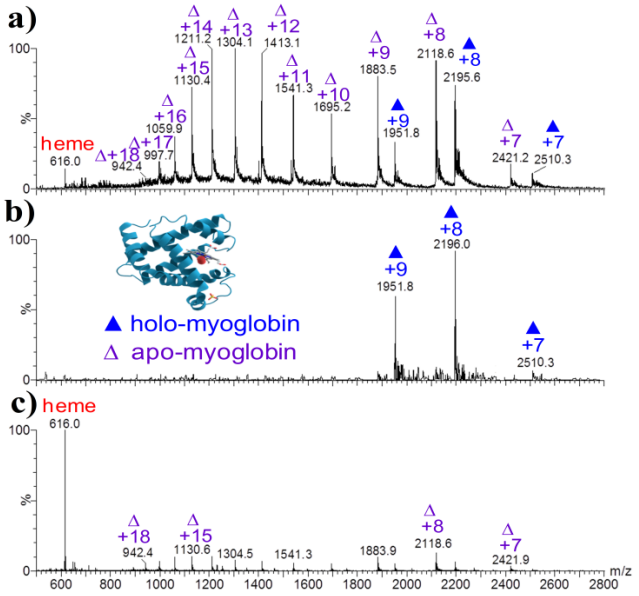

Figure 4. Analysis of the myoglobin sample. a) Spectrum obtained by conventional ESI-MS. Ions of apo-myoglobin are labelled with hollow triangle (Δ) and ions of holo-myoglobin are labelled with filled triangle (▲). b) Spectrum obtained for the front 1.0 mm part of the polyester tip after the polyester-tip ESI-MS analysis. c) Spectrum obtained for the other part of the polyester tip after the polyester-tip ESI-MS analysis.

In addition to preparative separation of smaller proteins from larger proteins as demonstrated with polyester tips (see Fig. S3), separation of different conformations of the same protein could also be achieved by porous-tip ESI. In this study, a myoglobin solution (20 μM in 12.5% acetonitrile), which contained both intact holo-myoglobin and denatured apo-myoglobin as well as heme (as indicated in Figure 4a), was analyzed using polyester-tip ESI-MS. Similar to the study with insulin/ alpha-lactalbumin, after the electrospray, the polyester tip was cut into two parts to determine the residues on the tip. Extraction and analysis with aqueous ammonium acetate allowed maintenance of the protein conformations. As shown in Figure 4b, only native holo-myoglobin with compact conformation, indicated by a narrow charge state distribution in higher mass range in the spectrum, was found in the front 1 mm part of the tip; while the spectrum obtained for the remainder part of the tip (Figure 4c) was predominated with heme and a broad charge state distribution in lower mass range, which corresponded to denatured apo-myoglobin with loosen conformations. These results demonstrated that the technique could be used for preparative separation of proteins and isolation of native forms from denatured forms of proteins.

In conclusion, we demonstrated that porous-tip ESI-MS could allow separation of proteins on porous substrates, followed by ESI-MS detection. The separation was size and shape dependent, and allows separation of different conformations and charge states of the same proteins and measurements of their cross sections. These features are not available with LC-MS approaches, including those involving use of nanospray emitters, which are commonly used for protein separation and detection. Compared to IM-MS that can also separate and detect proteins based on their sizes and shapes, the protein separation in the present technique is caused by the mobility differences of proteins in microchannels of substrates in solution phase, while the separation in IM-MS is caused by the differences in collision with drift gas in the gas phase. Therefore, the information obtained by the present technique is directly about the proteins in the solution phase, and the present technique could be better for maintaining and studying intact proteins and protein complexes. Our results showed that the technique could also be used for preparative separation of proteins. These together indicated the high potential of the technique for separating proteins and studying protein structures and conformations. Further investigation on effects of various factors and developing the technique for more efficient protein analysis and studies is on-going.

ASSOCIATED CONTENT

Supporting Information

Materials and experimental methods, experimental procedures, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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