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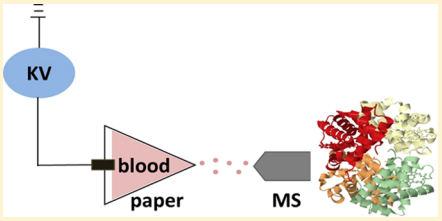
Paper Spray Ionization of Noncovalent Protein Complexes

Yun Zhang, Yue Ju, Chengsi Huang, and Vicki H. Wysocki*

Department of Chemistry and Biochemistry, The Ohio State University, 484 W. 12th Ave., Columbus, Ohio 43210, United States

Supporting Information

ABSTRACT: Paper spray (PS) ionization, an ambient ionization method, has previously been explored as a direct and fast method for mass spectrometric analysis of complex mixtures. It has been applied to the analysis of a wide variety of compounds, mostly small molecules. The work reported here extends the application of PS ionization to noncovalent protein complexes on an ion mobility tandem mass spectrometer. Similar mass spectra for protein complexes were obtained by PS ionization and nanoflow electrospray ionization (nESI), indicating that intact protein complexes can be preserved in PS ionization. In addition, collisional cross sections measured by ion mobility provide evidence that the protein assemblies may remain compact by PS ionization. With PS, it is possible to detect hemoglobin tetramer from a blood sample with minimal sample preparation. This is the first report to show that PS ionization is a promising ionization method for nonconvalent protein complexes.



Native mass spectrometry (NMS) has become an increasingly important method in structural biology.[1−3] In NMS, the noncovalent interactions stabilizing protein complexes can be maintained under gentle interface conditions. Nanoflow electrospray ionization (nESI) coupled to an ion mobility tandem mass spectrometer provides information on stoichiometry, size, and structure of noncovalent protein complexes.[4−7]

The Nobel Prize-winning discovery of electrospray ionization (ESI) by John Fenn[8] is a soft ionization method with little or no source fragmentation, making it particularly useful for the analysis of fragile biological macromolecules. The lower flow version of ESI, nESI, introduced by Matthias Wilm and Matthias Mann[9,10] proves to be superior for studies of native protein complexes. With smaller spray orifices, nESI allows better dispersion of the liquid to nanodroplets, avoiding harsh desolvation conditions. It also allows lower flow rates than those in conventional ESI, improving the ionization efficiency. With smaller required sample volumes and concentrations, less sample is consumed during a typical nESI experiment than a conventional ESI experiment. This also leads to reducing the amount of volatile buffer (e.g., ammonium acetate) needed to make a more native environment. In addition, nESI is more tolerant to nonvolatile salts and buffers.[11]

Ambient ionization[12] methods such as desorption electrospray ionization (DESI)[13] and direct analysis in real time (DART)[14] have opened a new area in MS, allowing MS of samples on a variety of surfaces/substrates. In addition, some new ionization methods such as matrix-assisted ionization vacuum (MAIV) combined with ion mobility mass spectrometer (IM-MS) show potential for clinical study.[15] Paper spray (PS) ionization, a relatively recent ambient ionization method, was first introduced by Graham Cooks and Zheng Ouyang's groups from Purdue University in 2010.[16] This method has the characteristics of both ESI and ambient ionization methods. It involves loading a sample in solvent onto a paper with a macroscopically sharp point and generating ions by applying a high voltage to the wetted paper.

Studies have shown that a wide variety of molecules including small molecules, peptides, and proteins can be ionized by PS.[16] This method is also important for clinical applications,[16−19] with numerous drugs in whole blood,[16] dried blood spots (DBS),[18] and urine[17] directly analyzed by PS. Very recently, a high throughput PS ionization device for fast quantitative analysis of drugs in blood was reported.[20] However, the PS ionization method has not been applied to ionize protein complexes. In the research described in this paper, PS ionization was used for the study of noncovalent protein complexes both in purified form and in human blood detritus with plasma removed. PS ionization in conjunction with an IM-MS was also used to show that protein assemblies can remain intact and compact/native-like by PS ionization. The results show that PS ionization is a promising method for study of protein complexes.

EXPERIMENTAL SECTION

Transthyretin (TTR) from human plasma, concanavalin A (ConA) from *Canavalia ensiformis*, human hemoglobin (Hb) standard and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Human serum amyloid P (SAP) was purchased from EMD Millipore Corporation (Chicago, IL). The protein powders including TTR, ConA, and SAP were dissolved in deionized water to desired concentrations and buffer exchanged into 100 mM ammonia acetate with Micro Bio-Spin 6 chromatography columns (Bio-Rad Laboratories, Inc., Hercules, CA) before analysis. Deformyl antitryptophan binding RNA attenuation protein (dfAT) was provided by Prof. Mark Foster and Dr. Elihu Ihms from The Ohio State

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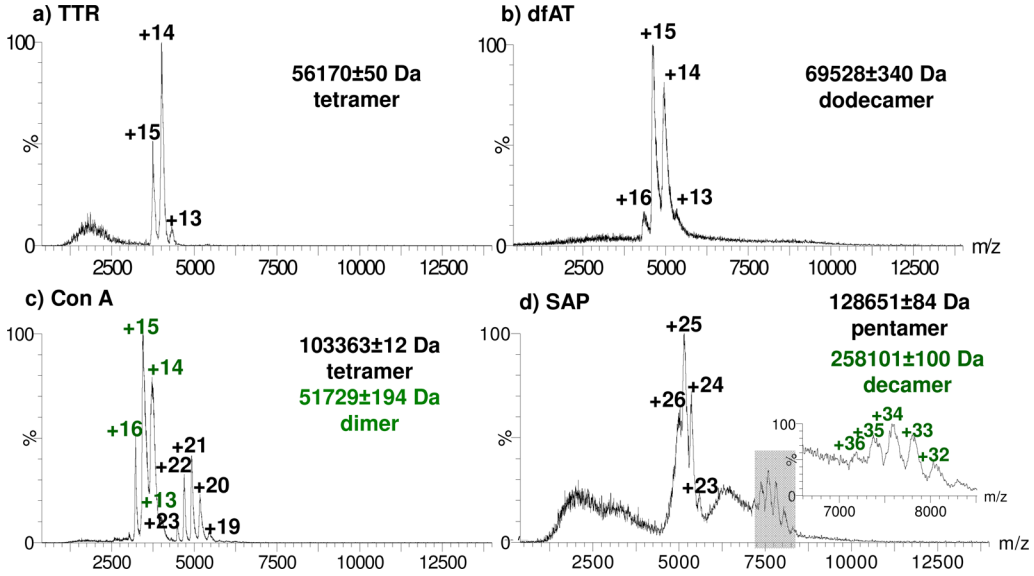

Figure 1. PS mass spectra of (a) TTR, (b) dfAT, (c) ConA, and (d) SAP. The region between m/z 6500 and m/z 8500 is expanded and labeled and shown in the inset of Figure 1d.

University and dialysized into 300 mM ammonium acetate. We usually use 100 mM ammonium acetate for protein complexes, but for dfAT, we used an ammonium acetate concentration of 300 mM because dfAT is most stable around this ionic strength. Human Hb standard was dissolved in 100 mM ammonium acetate for nESI and PS without buffer exchange. Human blood detritus/discard with plasma removed was generated from blood provided for an unrelated IRB approved study. The blood detritus sample (1 μL) was mixed with 400 μL of 100 mM ammonium acetate for nESI and PS. The concentrations of proteins used for nESI and PS are 25 μM for TTR tetramer, 20 μM for dfAT dodecamer, 25 μM for ConA tetramer, 20 μM for SAP pentamer, and 20 μM for human Hb tetramer.

All experiments were performed using a Waters Synapt G2 HDMS (Waters MS Technologies, Manchester, U.K.), an instrument whose source pressures are appropriate for stabilization and transmission of noncovalent protein complexes. The cone voltage was set at 50−100 V, and the source temperature was 50−70 °C to minimize denaturation of protein complexes. The instrument conditions are 5.2 mbar for the backing pressure, 2 mbar for nitrogen gas pressure in the IM cell, 120 mLmin^{-1} gas flow to the helium cell, and 7 × 10^{-7} mbar in the time-of-flight (TOF) analyzer. The ion mobility spectrometry (IMS) wave height and velocity were set at 26 V and 260 ms^{-1}, respectively, and were optimized based on the literature and previous experience with large complexes.[7,21−23] Collision-induced dissociation (CID) was performed in the trap cell, a collision cell located in front of the IM cell. Calibration of the collisional cross section (CCS) was performed following a published protocol[24,25] with TTR tetramer, SAP pentamer, SAP decamer, and ConA tetramer, sprayed by nESI, as the standard calibrants. All mass spectra were processed with MassLynx v4.1 and DriftScope v2.4 (Waters Corporation, Manchester, U.K.).

For paper spray ionization, the paper was cut into a triangular shape, approximately 6 mm base width and 6 mm height. A voltage-supply line with an alligator clip was used to hold the paper triangle and to apply the high voltage needed for the spray. A small plastic pin was used to bypass the source interlock. A moveable platform was used to adjust the paper position. The tip of the paper is in-line with the cone of the mass spectrometer at a distance around 3 mm (Figure S1 of the Supporting Information). Whatman filter paper grades 1 and 42 with a pore size of 11 and 2.5 μm (Whatman International Ltd. Maidstone, England), respectively, were used for PS. The cellulose filter paper grade 1 contains some trace elements such as 185 μg/g calcium, 160 μg/g sodium, 130 μg/g chlorine, etc. Whatman filter paper grade 42 has a lower amount of typical trace elements, including 13 μg/g calcium, 33 μg/g sodium, 80 μg/g chlorine, etc.[26] Figure S2 of the Supporting Information shows the mass spectra resulted when 10 μL of 100 mM ammonium acetate was added to Whatman filter paper grades 1 and 42. The two experiments were performed back-to-back on the same day with the same instrumental conditions. Only the ammonium acetate solution and components from the paper are present for this experiment. A broad peak around m/z 2000 was observed for both filter papers. Only the ammonium acetate solution and components from the paper are present for this experiment. In addition, the intensity of the broad peak for Whatman filter paper grade 42 was lower than that of Whatman filter paper grade 1. This is consistent with the lower amount of trace elements of Whatman filter paper grade 42. If present as anions and cations, elements from the paper could stabilize proteins in the gas phase, while they can also cause peak broadening.[27] Because narrower analyte peaks were obtained by using Whatman filter paper grade 42, Whatman filter paper grade 42 was used for most of protein complexes in the PS experiments. The protein dfAT was run only on Whatman filter paper grade 1 because good quality spectra were acquired before Whatman filter paper grade 42 was obtained, and we did not have enough dfAT to use with Whatman filter paper grade 42. For PS experiments, 10 μL of sample was dropped onto the mounted filter paper. This was sufficient to wet the entire triangular paper. For PS ionization in the positive mode, 2.5−3 kV was then applied. The sample signal lasted for 1 to 15 min. In nESI, each sample was loaded into a tapered glass capillary pulled in-house using a Sutter Instruments P-97 micropipette puller (Novato, CA). A platinum wire was inserted into the nontapered end of the capillary,[7] and a voltage of 1.1−1.5 kV was applied.

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RESULTS AND DISCUSSION

We first examined four protein complexes in ammonium acetate by PS ionization: TTR, dfAT, ConA, and SAP. TTR is a 56.0 kDa homotetramer that circulates in both blood and cerebrospinal fluid (CSF) and has a dimer of dimers quaternary structure under physiological conditions.[28] TTR is the major protein component in CSF.[29] There is a potential correlation between the concentration of TTR in CSF and Alzheimer's disease, as it has been reported that TTR concentration is significantly lower in CSF of patients with Alzheimer's disease.[30] dfAT exists as a 67.8 kDa dodecamer.[31] ConA is a homotetramer with a mass of 104 kDa.[32] SAP is a 125 kDa pentameric protein complex.[33]

Figure 1a shows PS ionization of TTR tetramer, resulting in charge states +13∼+15, values similar to those produced by nESI (Figure S3a of the Supporting Information). The calculated mass based on these charge states is 56170 ± 50 Da and 55746 ± 36 Da for TTR tetramer by PS and nanospray, respectively. Similarly, dfAT dodecamer with charge states +13∼+16, ConA dimer and tetramer with charge states +13∼+16 and +19∼+23, and SAP pentamer and decamer with charge states +23∼+26 and +32∼+36 were observed by PS (Figures 1, panels b, c, d). Although ConA favored the tetramer in early nESI experiments and PS experiments, more recently both nESI and PS have favored the dimer; it is not yet clear whether this is a sample issue or other factors. The calculated masses based on the observed charge states are slightly larger than the theoretical mass for all the complexes as is common in NMS. PS mass spectra of the four protein complexes are very similar to the corresponding nanospray mass spectra (Figure S3 of the Supporting Information). However, the peaks in nanospray mass spectra give results closer to the theoretical masses, and those peaks are narrower than those in PS mass spectra. This was probably caused by insufficient desolvation in PS ionization when compared with nESI. In addition, the anions and cations from paper are nonvolatile and may tend to cluster to the protein complexes, causing peak broadening.[27] Different source temperatures were used to improve the peak shapes in PS, and the optimized temperature of 50−70 °C was chosen to get sharper peaks while keeping a relatively gentle source condition to preserve the native state of protein complexes. Furthermore, the broad peak in the low m/z region shown in Figure 1 (panels a and d) could be from the solvent and components from paper since the paper was not pretreated. It also matched with the broad peak observed in the PS mass spectrum of 100 mM ammonium acetate (no analyte added, Figure S2 of the Supporting Information).

Figure 2 shows CCSs of TTR tetramer, dfAT dodecamer, ConA tetramer, SAP pentamer, and SAP decamer of different charge states, ionized by PS and nanospray. In this Figure, PS and nanospray are labeled in blue and red, respectively. IM-MS spectra show similar drift times for the corresponding charge state for each protein complex ionized by nanospray versus PS. CCSs calculations thus show concordance between the two ionization methods for each of the four protein complexes. On the basis of comparison with the reported CCSs of native-like TTR tetramer, ConA tetramer, SAP pentamer, and SAP decamer,[24] complexes ionized by PS and nanospray are native-like, but ConA tetramer is partially denatured according to the increasing CCS values and the broader peak with a shoulder at longer drift time for the arrival time distribution of charge states

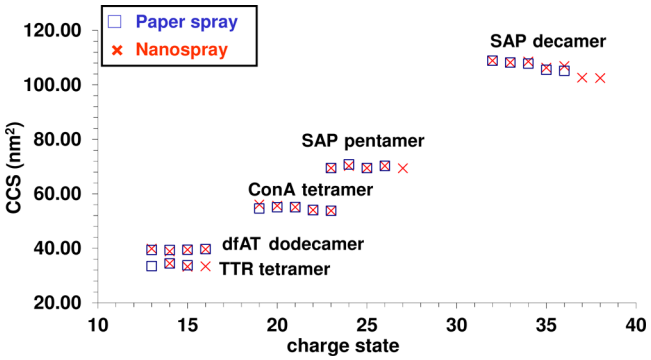

Figure 2. CCSs of TTR, dfAT, ConA, and SAP of different charge states, ionized by PS and nanospray. PS and nanospray are labeled in blue and red, respectively.

+22 and +23 (data was not shown). This could be caused by the source condition. In addition, the 100 mM ammonium acetate concentration used to spray the protein complexes might not be the optimal condition to preserve the compact/native-like structures in PS for those complexes.

PS ionization has been used previously for direct analysis of dried blood spots[18] and whole blood.[16] The previous focus, however, has been mostly on small-molecule drug analysis, although Hb variants were also analyzed by direct surface sampling of DBSs with high-resolution mass spectrometry.[37] Measurement of Hb is one of the most commonly performed blood tests for many clinical diagnoses and therapeutic interventions such as anemia detection.[27] In blood, the quaternary structure of Hb consists of four subunits in a tetrahedral arrangement, and each subunit is associated with one heme group.[34,35] In adult humans, more than 95% of the Hb is in the form of $\alpha_2\beta_2$.[35] However, some Hb variant forms are associated with diseases. For instance, Hb S ($\alpha_2\beta^S_2$) is a variant form of Hb resulting from a single-point mutation and is found in people with sickle cell disease.[36] PS ionization could be a rapid and convenient method for direct analysis of different Hb variants in the blood. In the present work, we examined the Hb protein complex in the blood using PS ionization in comparison to nESI. Figure 3a shows the nanospray mass spectrum of 1 μL of human blood detritus (without plasma) in 400 μL of 100 mM ammonium acetate. Hb tetramer with charge states +14∼+18 was observed. The measured mass for tetramer was 64848 ± 37 Da. In addition, Hb dimer was seen and the measured mass was 32410 ± 11 Da. The same sample was also analyzed by PS ionization. The mass spectrum is exhibited in Figure 3b. Similarly, Hb tetramer and dimer were observed, and the measured masses for tetramer and dimer were 65358 ± 66 Da and 32450 ± 46 Da, respectively. Both of the masses are slightly larger than those from the nanospray mass spectrum (Figure 3a). As proposed for the earlier example in Figure 1, this is likely caused by the insufficient desolvation in PS ionization at lower source temperature. A control experiment that used human Hb standard was performed. The nanospray and PS mass spectra for human blood detritus (Figure 3) and human Hb standard (Figure S4 of the Supporting Information) are comparable, and the measured masses for Hb tetramer and dimer are similar. To further confirm the structure of Hb tetramer, the +16 precursor of Hb tetramer was selected and activated. Figure 3c shows the PS CID mass spectrum of m/z 4158 at a collision energy of 100 V. The iron-containing heme group was observed at m/z 616.

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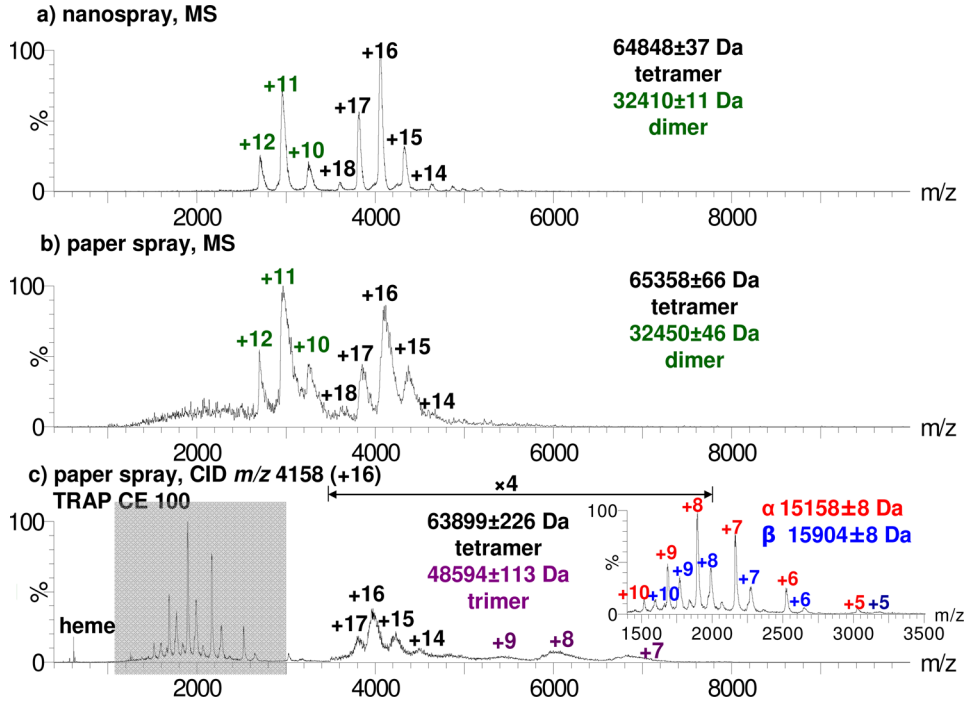

Figure 3. (a) Nanospray mass spectrum and (b) PS mass spectrum of human blood detritus without plasma diluted by 400 times in 100 mM ammonium acetate, and (c) PS CID mass spectrum of m/z 4158 (+16) at 100 V. The region between m/z 1400 and m/z 3500 is expanded and labeled and shown in the inset of Figure 3c.

In addition, Hb α and β monomers with charge states +5∼+10 were observed, as shown in the inset of Figure 3c. The measured mass for Hb α monomer was 15158 ± 8 Da, and β monomer was 15904 ± 8 Da. Furthermore, the complementary trimers were observed with charge states +7∼+9, and the measured mass based on the charge states was 48594 ± 113 Da. However, due to the broadness of the trimer peaks, it is hard to characterize the exact composition of the trimer peaks. Using the monomer information, we can speculate that the trimer peaks observed would be a mixture of $\alpha_2\beta$ and $\alpha\beta_2$. PS ionization is a potential tool for analyzing noncovalent protein complexes in crude biological samples such as the different Hb variants in blood.

CONCLUSIONS

This is the first reported evidence of successful PS ionization of noncovalent protein complexes. Similar mass spectra were obtained for nESI and PS ionization but with poorer desolvation in PS. On the basis of the measured drift time and corresponding calculated CCSs, the native structure of the protein complexes may be preserved in the gas-phase by PS ionization when appropriate source and buffer conditions are used. The advantage of simple sample preparation and ease of multiplexing could potentially make this a viable and attractive method for lower-cost and high-throughput clinical diagnostic measurements of large biomolecules in biofluids, as shown by the successful detection of Hb tetramers from blood. Another potential application is a multiplexing platform based on PS for the determination of appropriate solution conditions for optimized spray of a previously uncharacterized complex. This includes monitoring effects of changing ionic strength and changes in the composition of additives in the solvent. Future studies will focus on the improvement of experimental conditions for better desolvation of the ions generated by PS ionization and preservation of native-like protein complexes.

ASSOCIATED CONTENT

Supporting Information

A schematic of the PS ionization apparatus on a Waters Synapt G2 mass spectrometer, PS mass spectra of background using Whatman filter paper grades 1 and 42, nanospray mass spectra of four protein complexes, and nanospray and PS mass spectra of human Hb standard are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: wysocki.11@osu.edu.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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