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Direct Analysis of in-Gel Proteins by Carbon Nanotubes-Modified Paper Spray Ambient Mass Spectrometry

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The in-situ and direct extraction, desorption and ionization of in-gel intact proteins after electrophoresis has been achieved by a carbon nanotubes (CNTs)-modified paper spray mass spectrometry at ambient conditions. Characteristics of CNTs (including larger surface area, smaller pore diameter and enhanced conductivity) were endowed to the porous filter paper substrate by uniformly dispersing CNTs on the filter paper. Upon applying electric potential to the CNTs-modified paper, the in-gel proteins were extracted from the gel and subsequently migrated to the tip of the filter paper by electrophoresis-like behavior for paper spray ionization, which was monitored by extracted ion chronograms. The characterizations of modified filter papers and CNTs nanoparticles further confirmed the role of CNTs in the in-gel protein extraction, protein migration as well as spray ionization at the paper tip. Under optimized conditions, a mixture of cytochrome c, lysozyme and myoglobin was successfully separated by native electrophoresis and subsequently analysed by the present method, showing a limit of detection of 10 ng per gel band. The present strategy offers a new pathway for direct detection of in-gel intact proteins at ambient conditions without any pre-treatment (e.g. digestion, chemical extraction and desalting), showing potential application in top-down proteomics.

Introduction

Proteomics, aimed at developing and applying techniques for the global and rapid analysis of proteins, is of great significance in the functionalization of biomolecules as well as in the discovery of disease-state biomarkers and therapeutic targets.[1] In both bottom-up and top-down proteomics, gel electrophoresis is the most widely used separation technology for complex protein mixtures when coupled with mass spectrometry (MS).[2 3 4] In the traditional bottom-up proteomics, in-gel proteins are digested into peptides by proteolytic enzymes that are more amenable to MS analysis.[5] However, the necessary procedures of in-gel digestion, chemical extraction and desalting of the separated proteins are time-consuming and may cause sample loss resulting in the irreplaceable recovery of peptides.[6 7 8 9] In addition, although this strategy is of good sensitivity, the sequence coverage is not satisfied in general and it is often tacitly assumed that the enzymatic efficiency is at or near stoichiometric levels.[5 10 11]

With the development of proteomics, the complete structural analysis of intact proteins after gel electrophoresis becomes crucial. Top-down proteomics is one such strategy that reduces that amount of sample preparation before MS analysis. Without further proteolytic preparation, it has significant advantages in analyzing protein isomers, identifying post-translational modifications and charactering complex proteins samples.[12] The advent of soft ionization strategies to produce intact molecular ions, especially matrix-assisted laser desorption/ionization (MALDI), has greatly extended the application of MS in top-down proteomics.[13] However, most MALDI techniques are performed under vacuum and can hardly achieve adequate desorption/ionization without matrix.[14] Thus, it is still urgent to develop facile techniques for top-down proteomics, which can directly couple gel electrophoresis to MS for direct analysis of in-gel proteins at ambient conditions, without processes of digestion, chemical extraction and desalting.

Ambient ionization methods, including desorption electrospray ionization (DESI),[15] extractive electrospray ionization (EESI),[16, 17] dielectric barrier discharge ionization (DBDI)[18, 19] and low temperature plasma ionization (LTP),[20 21] have been extensively applied to rapid and direct analysis of complex mixtures with little or no sample pre-treatment. However, there is still no report on the use of these techniques for direct desorption/ionization of in-gel proteins, which might be due to the difficulties in extraction, desorption or ionization of proteins from the gel. Through a piece of paper cut into triangular shape,[22] paper spray ionization (PSI) can achieve the rapid analysis of various biological samples such as urine,[23] dried serum[24] and whole blood,[25 22] as well as plant tissue[26] and animal tissue.[27] In addition, silica-coated paper substrate has been reported to improve the sensitivity of PSI for drug analysis.[28] Recently, ambient ionization of small molecules was reported by spraying from a carbon nanotubes (CNTs)-impregnated paper surface at a small voltage of 3 V.[29] Interestingly, electrostatic spray ionization (ESTASI), another ambient ionization strategy, was applied to in situ ionization of samples inside a surfactant-

free polyacrylamide gel. Charged samples in the gel could be directly extracted by an electric field and then electro-sprayed for MS detection.[9] Under similar conditions, with modified paper as the substrate, paper spray could be utilized for in-situ desorption/ionization of in-gel proteins for MS analysis.

In the present work, direct extraction, desorption and ionization of in-gel proteins at ambient conditions was performed by a CNTs-modified paper spray system, which was easily coupled with MS for detection. Via simple dipping procedures, the characteristics of CNTs were endowed to the paper substrate, which enhanced the extraction, migration, desorption and ionization of in-gel proteins. Without any pre-treatment, this offers a new pathway for in-situ detection of in-gel intact proteins at ambient conditions, which is significant in top-down proteomics.

Experimental Section

Chemicals and materials

Cytochrome c from horse heart (12.3 kDa), myoglobin from horse skeletal muscle (17 kDa), and lysozyme from chicken egg white (14 kDa) were from Sigma Aldrich (St. Louis, MO, USA). Carbon nanotubes (CNTs) were purchased from Boyu High-Tech New Material Co. Ltd. (Beijing, China). SiO_2 nanomaterial was supplied by Nanjing Haitai Nano. Co. Ltd. (Nanjing, China). α-Cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Common qualitative filter paper was from Hangzhou Special Paper Industry Co. Ltd. (Hangzhou, China). Methanol, ethanol, isopropanol, acetic acid (HAc) and sodium hydroxide (NaOH) were obtained from Beijing Chemical Co., Ltd. Water was deionized/purified using a Mili-Q water purification system (Millipore, Bedford, MA).

Gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed in a vertical discontinuous polyacrylamide gel system, which was composed of 7.5% (m/v) separating gels and 4.0% (m/v) stacking gels. After loading samples for each channel, electrophoresis was carried out at 90 V for about 2 h. Next, the gel was washed and stained in Coomassie Brilliant Blue (CBB R-250) (0.1 g/100 mL) solution (35% methanol-10% acetic acid). Finally, it was destained in ethanol-acetic acid solution (45%/5% in water) and washed by water before MS analysis.

Apparatus and software

All paper spray experiments were performed using a LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The LTQ operating parameters were as follows: mass spectra displayed from *m/z* 850 to 1800, capillary temperature was 200 ℃, maximum injection and microscans were 10 ms and 1, respectively. All of the mass results were obtained and processed by Xcalibur 2.0 software.

Scanning electron microscope (SEM) images (Hitachi S-4800, Japan) were recorded at 10 kV. Before SEM imaging, a thin layer of Pt was coated on the film surface by an Auto Fine Coater (JEOL Tokyo, Japan) at a current of 20 mA for 100 s. N_2 adsorption-desorption isotherms were collected on an QUADRASORB SI Automated Surface Area & Pore Size Analyzer (Quantachrome Instruments, USA) at 77.3 K.

Modification of the paper

Using 3 mL water/methanol (1:1) solvent, 0.5 mg of the nanomaterials of SiO_2 and CNTs, as well as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were uniformly dispersed on a φ-9 cm filter paper for the modification. Then, after air drying, different filter papers were applied for the paper spray ionization of in-gel proteins.

Configuration of the CNTs-modified paper spray system

As shown in **Figure 1**, after native PAGE, gel strips containing proteins were cut off and placed on the triangular shaped filter paper (10 mm long and 5 mm wide at the base). A copper clip was used to hold the CNTs-modified paper at the base while maintaining a distance of 10 mm from the tip of the paper triangle to the mass spectrometer inlet. Then, after addition of spray solvent onto the paper, a high voltage of 5 kV DC was applied to the wet paper to induce spray ionization at the tip of the paper triangle. Unless otherwise noted, 20 μL of 50% methanol with 49% water and 1% acetic acid was used as the spray solvent.

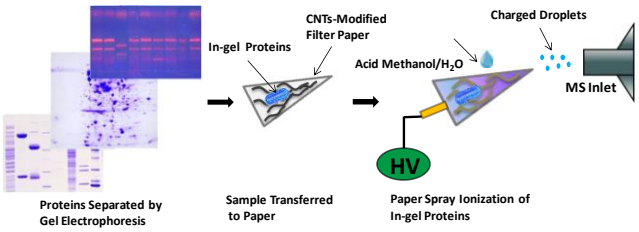

Figure 1. Schematic diagram of CNTs-modified paper spray mass spectrometry for in-gel protein analysis.

Results and discussion

Different substrates for in-gel proteins detection

Initial experiments investigated the effect of different paper substrates on MS analysis. Common qualitative filter paper, printing paper and weighing paper were chosen for the detection of in-gel cytochrome c. As demonstrated, common qualitative filter paper could exhibit spray ionization of in-gel cytochrome c (Figure 2A), obtaining mass spectra peaks from $[M+8H]^{8+}$ to $[M+13H]^{13+}$. The data is in accordance with the detection of cytochrome c in solution phase by traditional ESI-MS.[30] However, no obvious signal was recorded using printing paper and weighing paper, i.e., the in-gel protein detection by paper spray could not be achieved on these two substrates. This is likely due to the different cellulose network of microchannels from different types of papers, which plays an important role in the paper spray ionization [25] as well as the extraction of proteins from the gel. Thus, it is demonstrate that the paper spray-based method is feasible for the direct extraction, desorption and ionization of proteins from the gel without any pre-treatment. However, to further improve the sensitivity of in-gel protein detection by the

paper spray technique, improvements are necessary.

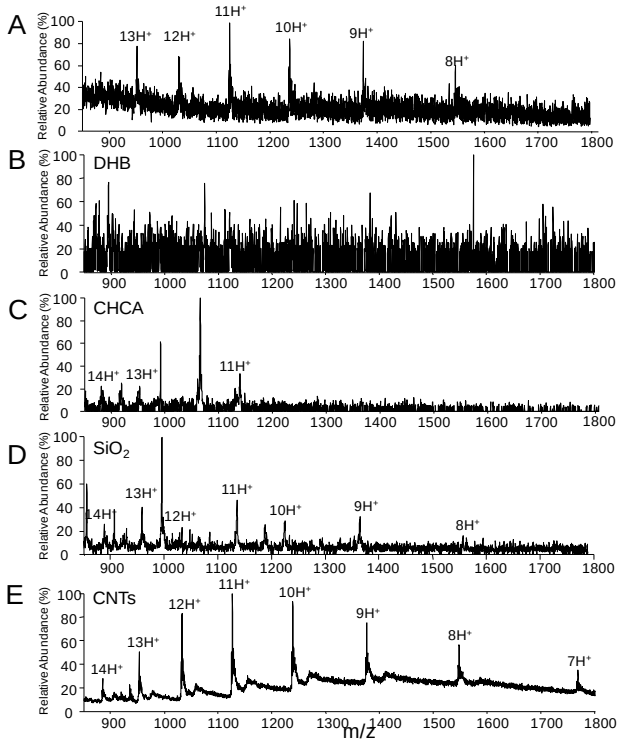

Figure 2. Mass spectra of in-gel cytochrome c on different filter papers: Without modification (A), and modified with 0.5 mg of DHB (B), CHCA (C), nano-SiO_2 (D), and CNTs (E).

Different modifications for in-gel protein detection

Due to the fact that silica coated [28] or CNT-impregnated paper [29] has been reported to show improvement for paper spray analysis, similar modifications of the filter paper may also exhibit good spray ionization of in-gel proteins. In the experiment, SiO_2 and CNTs nanomaterials, as well as the traditional matrix of CHCA and 2,5-DHB for MALDI-MS, were selected for the modification of filter paper and subsequent paper spray ionization of in-gel cytochrome c. As demonstrated, no obvious signal was obtained using DHB-modified paper (Figure 2B), and only weak signals for $[M+11H]^{11+}$, $[M+13H]^{13+}$ and $[M+14H]^{14+}$ were recorded when ionized on CHCA-modified paper (Figure 2C). Relatively recognizable mass spectra peaks from $[M+8H]^{8+}$ to $[M+14H]^{14+}$ could be obtained with nano-SiO_2-modified paper as the substrate (Figure 2D). This might be due to the reduced adsorption between nano-SiO_2 and cytochrom c at pH 2.3, which might depend on the pKa value of silanol group and pI value of cytochrom c. In addition, the nano-SiO_2 particles may play a role to produce electro osmotic flow which drives the solution to form a stable ESI. However, the data was still not satisfactory compared to data obtained on common qualitative filter paper (Figure 2A). Strong signals for $[M+7H]^{7+}$ to $[M+14H]^{14+}$ were obtained with 0.5 mg CNTs-modified paper as the substrate (Figure 2E), which also exhibited better signal to noise ratio and more protein ions compared to common qualitative filter paper (Figure 2A).

It should be noted that the quantity of CNTs used for modification also has an effect on the in-gel protein detection. For example, 0.25 mg and 1 mg of CNTs-modified filter paper resulted in lower signal to noise ratio relative to that on 0.5 mg CNTs-modified paper, especially below m/z 1100 (Figure S1, see Supporting Information for details). As demonstrated, the CNTs-modified paper has the outstanding ability for the direct extraction, desorption and ionization of in-gel proteins, which could show potential in top-down proteomics.

Migration of proteins during detection

For further examination, the extracted ion chronograms were recorded for monitoring the extraction, desorption and ionization of proteins from the gel. Charge states of cytochrome c from +8 to +10 were selected for the investigation. As shown in Figure 3, the curves show abundant peaks as time goes on, which are similar to the migration of proteins during electrophoresis.[31] In detail, no signal was obtained until 2 min for all three charge states. Subsequently, the signals gradually increased and reached the strongest at about 4 min, then began to decrease and reached the lowest at approximately 6.5 min. It was further confirmed by the real-time mass spectra of in-gel cytochrome c during its spray ionization in 0-1 min (Figure 4A), 3.5-4.5 min (Figure 4B) and 6.5-7.5 min (Figure 4C). This indicates that in-gel cytochrome c might be first extracted from the gel onto the CNTs-modified paper substrate, then migrate to the tip of the paper by solvent transport or paper electrophoresis-like movement [32] (resulting from capillary action through the micro-channels in the paper substrate), and finally ionized at the tip of the paper by field-assisted evaporation.[33]

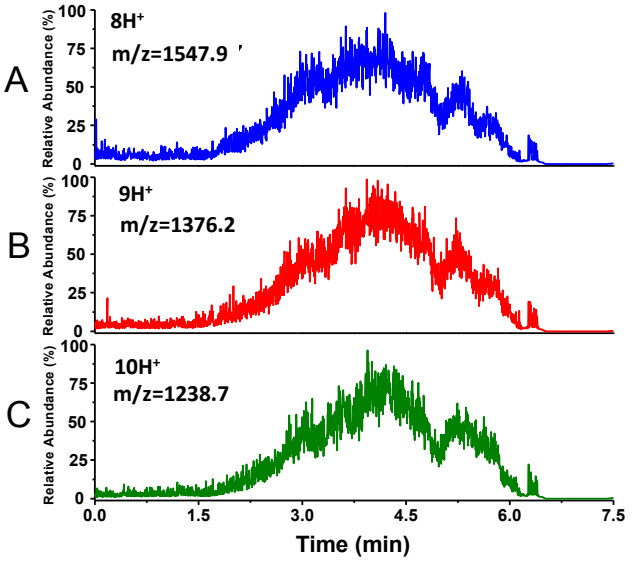

Figure 3. Extracted ion chronograms of cytochrome c for $[M+8H]^{8+}$ of m/z 1547.9 (A), $[M+9H]^{9+}$ of m/z 1376.2 (B) and $[M+10H]^{10+}$ of m/z 1238.7 (C).

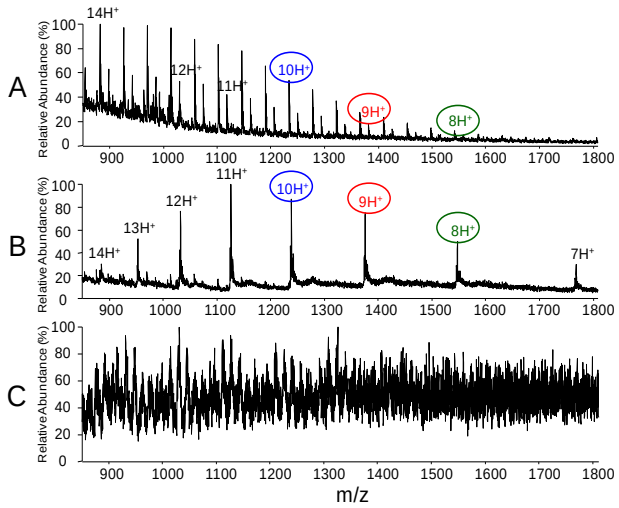

Figure 4. Mass spectra of in-gel cytochrome c with time going on. (A) 0-1 min, (B) 3.5-4.5 min and (C) 6.5-7.5 min.

Role of pH in detection

As in-gel cytochrome c needs to be extracted from the gel, and then migrate to the tip of the paper by the electrophoresis-like behavior for spray ionization, pH value of the spray solvent may influence its extraction, migration, as well as ionization. Five solvent pH values were used for the investigation. Figure 5 shows the signal intensity of $[M+10H]^{10+}$, $[M+11H]^{11+}$ and $[M+12H]^{12+}$ obtained at different pH values. As demonstrated, strong signal intensity was obtained under high acid conditions (pH=2.3), and with increasing pH, the peak intensity of three charge states declined. At strong alkaline solvent conditions, nearly no ion signal was recorded. This phenomenon might be initially explained by the different charge states of cytochrome c in different pH conditions. Cytochrome c (pI=10.7) [34] is positively-charged under strong acid conditions, which not only contributes to the electrophoresis-like migration in an electric field, but also to the paper spray ionization for MS analysis.[31 34 35] However, under strong alkaline conditions, cytochrome c will be negatively-charged, which would be against the desired migration of proteins from gel to the tip of the filter paper and thereby useless to the paper spray. We also didn't obtain significant signals of cytochrome c in the negative ion mode under alkaline conditions. This might be due to the difference between the onset voltage of electrospray and that of corona discharge.[22, 24] Therefore, based on the favourable conditions of strong acid solvent to protein ionization[34 35] as well as the electrophoresis-like migration of proteins during electric field, low pH value of solvent was selected for direct extraction, migration and ionization of in-gel proteins.

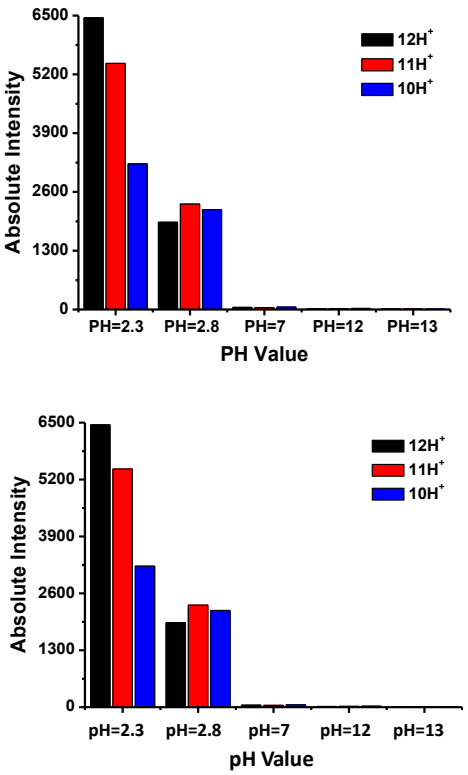

Figure 5. Comparsion of corresponding ion signals of cytochrome c obtained by the CNTs-modified paper spray at different pH values ($[M+10H]^{10+}$, m/z 1238.7; $[M+11H]^{11+}$, m/z 1126.2; $[M+12H]^{12+}$, m/z 1032.5).

Preliminary mechanism discussion

To investigate the CNTs function in the analysis of in-gel proteins, scanning electron microscope (SEM) images of different substrates were recorded. As illustrated in Figure S2, all paper substrates have a framework of cellulosic fibers, whereas filter paper (Figure S2-C) seems more porous than weighting paper (Figure S2-A) and printer paper (Figure S2-B). This difference may contribute to the extraction (permeation and adsorption) of proteins from the gel and the migration of proteins through the microchannels of the cellulose network.[25] In addition, the hydrophilic porous filter paper can hold a certain amount of solvent to increase conductivity, which is important for the fabrication of a high electric field to induce the Coulomb explosion at the apex of the paper triangle for ionization.[25] For this reason, the dispersion of soluble DHB and CHCA on the filter paper may damage the porous structure of the substrate due to crystallization of CHCA or DHB (Figure S2-D and E), leading to the poor detection of in-gel proteins (Figure 2B and C).

For modified paper substrates of SiO_2 (Figure S2-F) and CNTs (Figure S2-G, to I), the pores were filled with nanomaterials particles, which could enable field emission of micro-droplets with the analyte, and is likely responsible for the ionization.[29] These structures could not only maintain the microchannels of the cellulose network, but also endow the filter paper with nanomaterials characteristics, such as larger surface area, smaller pore diameter and enhanced conductivity. Improved detection of in-gel proteins by CNTs-modified filter paper relative to SiO_2

(Figure 2) might be due to the different characteristics of the two nanomaterials. CNTs show larger surface area than nano-SiO_2, resulting in a stronger adsorbing ability for the extraction of proteins from the gel. In addition, the much smaller pore diameter of CNTs compared to nano-SiO_2 may hinder other ions from interfering in the MS analysis (Table S1). In fact, unmodified filter paper shows better ionization than SiO_2-modified filter paper. which might be due to the viscosity change of spray solvent and the interference of other ions in the presence of SiO_2, making proteins difficult to extract and migrate.

Furthermore, the enhanced conductivity of CNTs could also play a role in the extraction and migration of proteins from gel onto the filter paper, as well as to the tip of the filter paper for ionization.[25 36 37] In the CNTs-impregnated paper spray of small molecules at 3 V, it was stated that the small conductive CNT-structures on the surface of the filter paper acted as electrodes.[29] As a result, the voltage could be applied at the "CNT electrode" to induce an electric filed between the paper tip and MS inlet, where the ionization occurred. That is to say, CNTs may play a role of the electrode on which electrochemical reaction occurred so that the electrons can be transmitted to complete the circuit between the paper spray and the MS for ESI formation. Thus, the CNTs density on the filter paper surface could affect the electric properties of the CNTs-modified paper, which could have resulted in the difference in detection of in-gel proteins. This has been confirmed by the different detection efficiencies with the filter paper modified by different amount of CNTs (Figure S1). Thus, the enhanced detection of in-gel proteins by CNTs-modified paper spray could be ascribed to the unique characteristics of CNTs, such as larger surface area, smaller pore diameter, and high conductivity.

Other optimizations

As many factors can affect the paper spray ionization, such as the distance between paper tip and MS-inlet, the paper tip angle and the spray solvent,[28 38] optimization experiments were performed. As demonstrated, a distance of 10 mm away from the MS-inlet (Figure 6A) and the paper tip angle of 30 ° (Figure 6B) were more suitable for the detection of in-gel proteins. Both conditions are in accordance with common paper spray analysis.[38] Furthermore, compared with the ion intensity obtained with isopropanol and ethanol as the solvent, relatively strong signal was achieved with methanol (Figure S3); therefore 50% methanol was selected as the optimal percentage (Figure S4). Using these optimized conditions, the sensitivity was then examined for detection of in-gel cytochrome c, which showed a limit of detection of 10 ng per gel band (Figure S5).

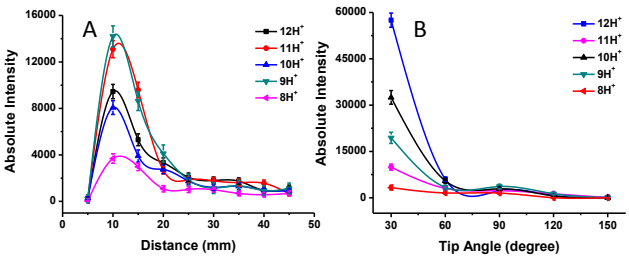

Figure 6. Comparsion of ion signals of cytochrome c obtained by the CNTs-modified paper spray at different distance between paper tip and MS-inlet (A), and the paper tip angle (B).

Real sample detection

For an application, a mixture of cytochrome c, myoglobin and lysozyme was selected as a model for the direct analysis of in-gel proteins by CNTs-modified paper spray mass spectrometry. After PAGE, three protein bands were separated, and then subjected to the in-gel detection by CNTs-modified paper spray MS. As illustrated in Figure 7A, a charge state distribution ranging from +10 to +26 is observed for myoglobin, relatively strong signals of lysozyme from $[M+8H]^{8+}$ to $[M+14H]^{14+}$ was obtained (Figure 7B), and recognizable mass spectra peaks from $[M+7H]^{7+}$ to $[M+14H]^{14+}$ were recorded for cytochrome c (Figure 7C). This demonstrated the feasibility of coupling paper spray-MS with gel electrophoresis for protein separation and subsequent direct MS analysis.

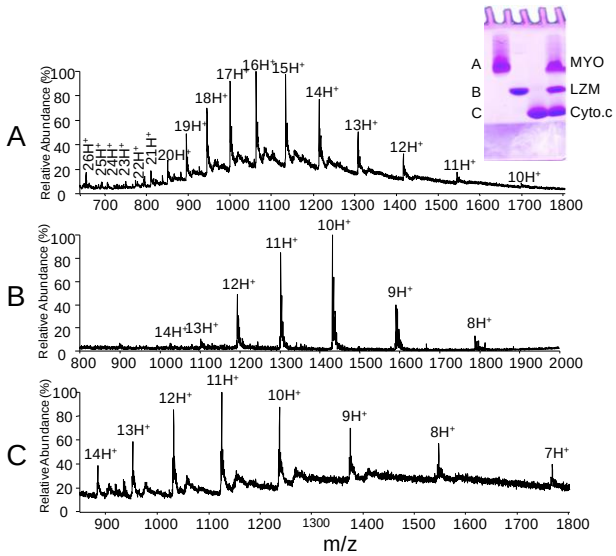

Figure 7. Mass spectra of myoglobin (A), lysozyme (B) and cytochrome c (C) in gel after PAGE.

Conclusions

In summary, the direct extraction, desorption and ionization of in-gel intact proteins after electrophoresis has been achieved by CNTs-modified paper spray MS at ambient conditions. Via modification of the paper substrate, the characteristics of CNTs can be endowed to the porous filter paper, including the larger surface area, smaller pore diameter and high conductivity. As a result, in the applied electric field, proteins could be extracted from the gel and subsequently migrate to the tip of the filter paper by electrophoresis-like behavior for paper spray ionization. Without any pre-treatment, the present strategy offers a new pathway for direct detection of in-gel intact proteins at ambient conditions, which shows potential for application in top-down proteomics.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See

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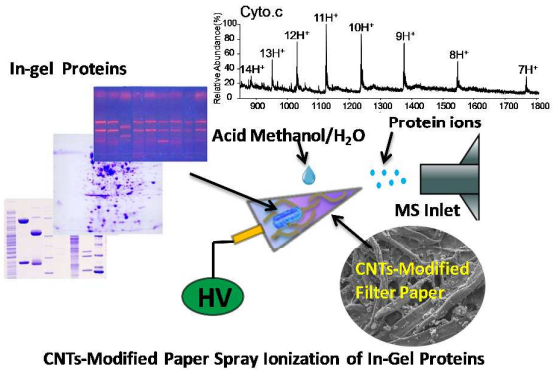
TOC:



Direct extraction, desorption and ionization of in gel-intact proteins after electrophoresis have been achieved by a CNTs-modified paper spray MS at ambient conditions.