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Mass Spectrometry for Paper-Based Immunoassays: Toward On-Demand Diagnosis

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Supporting Information

ABSTRACT: Current analytical methods, either point-of-care or centralized detection, are not able to meet recent demands of patient-friendly testing and increased reliability of results. Here, we describe a two-point separation on-demand diagnostic strategy based on a paper-based mass spectrometry immunoassay platform that adopts stable and cleavable ionic probes as mass reporter; these probes make possible sensitive, interruptible, storable, and restorable on-demand detection. In addition, a new touch paper spray method was developed for on-chip, sensitive, and cost-effective analyte detection. This concept is successfully demonstrated via (i) the detection of *Plasmodium falciparum* histidine-rich protein 2 antigen and (ii) multiplexed and simultaneous detection of cancer antigen 125 and carcinoembryonic antigen.

Direct-to-consumer (DTC) diagnostic tests[1] and point-of-care (POC) testing[2] have emerged as two of the most prominent forms of "personalized" healthcare.[3] Compared with the processes of standardization and centralization in the clinical laboratory, which guarantee reliability and accuracy of determinations, POC tests are performed by different clinical staff on multiple devices in different locations. Moreover, the functional limitation of portable instruments has compromised the quantitative performance of POC testing.[4] Current colorimetric detection methods based on enzymatic reactions or gold nanoparticles often employed for POC and DTC tests add further complications by requiring the test results to be read within a specified time interval to ensure their validity.[5] To overcome these challenges, an on-demand, two-point separation diagnostic approach that has capacity to empower on-site assay and off-site centralized signal detection, and that which can establish reliability and accuracy in DTC and POC testing, is a promising solution. This approach will not only benefit patient-friendly testing and avoid the degradation of samples but could also allow detection at a later time in a centralized manner/location to improve the reliability of detected results.

Mass spectrometry (MS) has the advantages of low detection limits, large dynamic range, multiplexing potential and better spectral resolution;[6] state-of-the-art mass spectrometers provide sensitive and reliable detection for both large biomolecules and small molecules. Instead of direct analysis of intact large biomolecules,[7] strategies that enable the detecting of tagged small molecules have the advantages of higher sensitivity and lower requirements for the instrumentation.[6a,b,8] Development of such an alternative approach is essential for the emerging paradigm shift in analytical instrumentation in which performance is characterized according to speed, simplicity of operation, and capabilities for field testing.

Here, we developed a new paper-based MS immunoassay platform (Figure 1) that employs cleavable ionic probes to enable the new approach of on-demand analysis of biomarkers. Two pH-sensitive probes 2-(4-isothiocyanatophenethoxy)-*N,N,N*-trimethyl-2-oxoethanaminium chloride (ITEA) and 4-(4-isothiocyanatophenethoxy)-*N,N,N*-trimethyl-4-oxobutan-1-aminium chloride (ITBA), were synthesized (Figure S1) as mass reporters for the first time. These stable ionic probes

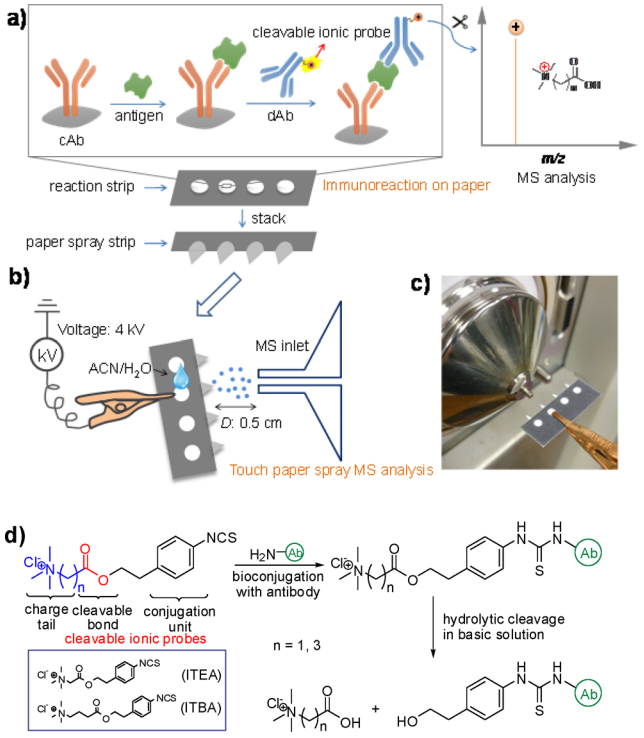

Figure 1. TPS-MS immunoassays with ionic probes. (a) Schematic illustration of the experimental procedure, including immobilization of cAb, capture of antigen, complexation of dAb conjugated with cleavable charge-tag, and MS detection [cAb, capture antibody; dAb, detection antibody]. (b) Schematic illustration of the TPS-MS analysis. (c) Photograph of the TPS-MS analysis. (d) Structures of the probes, their bioconjugation, and the hydrolysis.

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make it possible to interrupt, store, and restore the detection of antigens, after sample loading and immunoreaction, thereby allowing quantitative analysis at a later time. To facilitate future POC applications with hand-held mass spectrometers, the probes were rationally designed to produce small ions (m/z < 200) upon stimulation. To enable on-chip detection, we also proposed a new paper spray method,[9] which we term touch paper spray (TPS, Figure 1b,c), for ambient MS.[10] This paper-based analytical device, on which the assay is performed and signal detected, is easy to fabricate and transport, and is degradable.[11] This proposed methodology is demonstrated through (i) the detection of malaria antigen *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) and (ii) multiplexed detection of two cancer antigens.

The experimental workflow consists of four steps: (i) fabrication of paper substrate and immobilization of capture antibody (cAb) by the reaction of an amino group on cAb with aldehyde-functionalized paper (Figures S2–S4), (ii) blocking of excess aldehydes in the paper substrate with Tris-buffered saline, (iii) immunoreaction on the prepared paper surface, and (iv) MS analysis. For example, to detect *Pf*HRP2 antigen, a paper surface on which anti-*Pf*HRP2 antibodies had previously been immobilized was used. Typically, a solution (e.g., serum) containing *Pf*HRP2 is added to the paper surface, and is selectively captured (Figure 1a). The next immunoreaction step is to add a solution containing the detection antibody (dAb) conjugated to the cleavable probes (Figure 1d) for complexation. We have flexibility in the subsequent step, which is quite different from the traditional enzyme-linked immunoassays; the next analytical step need not be completed immediately because stable chemical probes are used.

The positive charge tags, (carboxymethyl)trimethylammonium chloride (CMTA) and (3-carboxypropyl)trimethylammonium chloride (CPTA), can be easily released by the addition of NH_4OH solution to the antigen/antibody complex (Figure 1d; also see Figure S5 for the effect of different basic solutions and Figures S6–S9 for MS characterization).[12] Further experiments showed that ITBA has higher hydrolytic rate than ITEA under the same pH value, which may be influenced by the proximity of the electron-withdrawing quaternary ammonium (QUAT) cations to the ester bond (see the detailed results and discussion in Figure S10). The hydrolysis times are less than 1 min for ITEA at 100 mM NH_4OH solution and 20 min for ITBA at 1 M NH_4OH solution. Delightfully, however, both probes are stable under neutral conditions, even after 30 days of storage (Figure S11).

The use of the probes for disease diagnosis via MS was first optimized for the detection of malaria *Pf*HRP2 antigen in PBS solution, followed by analysis in human serum. ITEA- and ITBA-conjugated anti-*Pf*HRP2 antibodies (ITEA-dAb and ITBA-dAb; see Figure 2a,b and Figures S12–S14 for the MS and spectroscopy characterization) were used for complexation and reporting, whereas nESI-MS/MS was first employed for detection. The fragment ions m/z 59 from CMTA and m/z 87 from CPTA (Figure 2c,d) were chosen for quantification because their intensities were directly proportional to concentration (Figure S9). Good linearity was obtained for analytes spiked into PBS (Figures S15 and S16), with limit of detection (LOD) 500 pM and 50 pM using ITEA-dAb and ITBA-dAb, respectively, which correspond to only 10 fmol and 1 fmol in each test zone. This high sensitivity is ascribed to the MS method and the use of the charge tags. In serum, the LODs were 1 nM (37 ng/mL) and 75 pM (2.8 ng/mL) for ITEA-dAb and ITBA-dAb, respectively; corresponding absolute amounts were 50 fmol/zone and 1.5 fmol/zone, respectively. The sensitivity of the proposed method with ITBA-dAb is comparable to that which we have recorded with enzyme-amplified ELISA methods (ELISA LOD = 1 ng/mL for *Pf*HRP2 in serum, Figure S17), although no amplification is adopted in the MS method. These results indicate that the proposed MS immunoassay can be used to diagnose malaria infection for blood parasite densities of 200 parasites/μL (mean antigen concentration, 9.1 ng/mL), which is the WHO-recommended lowest density for diagnosis.[13]

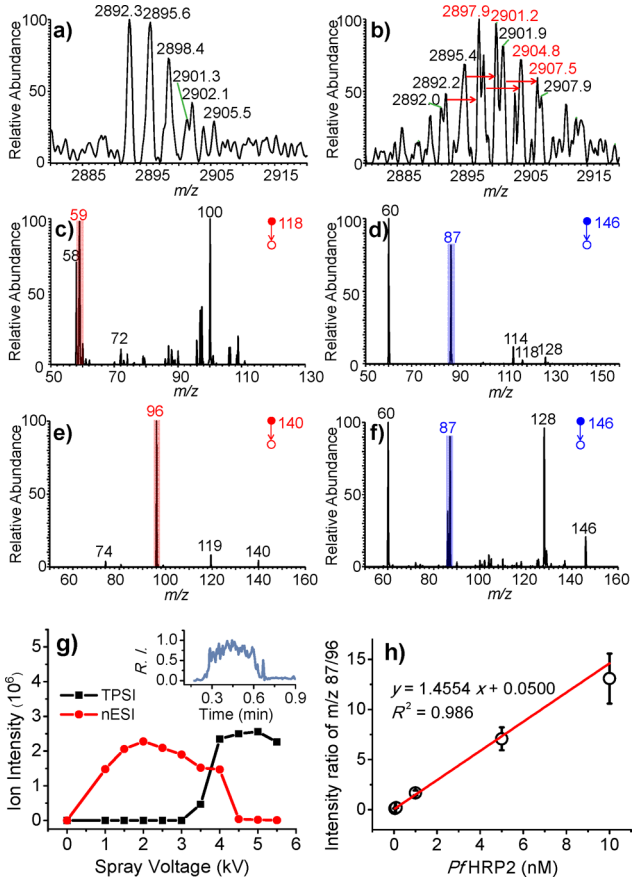

Figure 2. Detection of *Pf*HRP2 antigen and spray voltage comparison. (a,b) MS characterization of (a) dAb and (b) ITBA-dAb. Charge state is 52+. The peaks labeled with red numbers come from the conjugated antibodies. (c,d) MS/MS of (c) m/z 118 in the detection of 100 nM of antigen with ITEA-dAb and (d) m/z 146 in the detection of 50 nM of antigen with ITBA-cAb using nESI. (e,f) MS/MS of (e) m/z 140 in the detection of 50 nM of antigen with ITEA-dAb and (f) m/z 146 in the detection of 10 nM of antigen with ITBA-cAb using TPS. (g) TPS and nESI show different ion intensity (CPTA, 1 μM, m/z 146) with spray voltage. The inset shows the duration of the TPS-MS for CPTA (1 μM, 5 μL ACN–H_2O). (h) Calibration curve for *Pf*HRP2 (0.05–10 nM) spiked in serum using TPS-MS with ITBA-dAb. Intensity ratios of product ions from CPTA (m/z 146→87) and CMTA (internal standard, 10 nM, m/z 140→96) were calculated for quantification.

The nESI-MS/MS detection strategy used here features low sample consumption and high sensitivity; however, we further wished for a more convenient method which does not require transfer of analyte solution from the paper test zones to the nESI glass capillary.

Here we created a new TPS method (Figure 1b,c) to enable on-surface MS detection. This objective was achieved by simply

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touching the paper device previously used for the immunoreaction to a second wax-printed paper substrate (i.e., the paper spray strip, Figure 1a) with matching hydrophilic channels designed to aid paper spray ionization.[14] Upon the addition of spray solution to this 3D paper scaffold (Figure 1b,c), analytes present on the upper paper layer were extracted and transferred to the bottom paper spray strip[9b] to form the spray and then detected by MS. TPS mass spectra recorded for ITEA, ITBA, and CPTA samples were similar to those obtained from nESI (Figures S18 and S19), except for CMTA, which produced sodiated (m/z 140) and potassiated (m/z 156) adducts in TPS-MS. The intensities of fragment ions m/z 96 (from $[CMTA+Na-H]^+$) and m/z 87 (from $[CPTA]^+$, Figure 2d,f) were directly proportional to solution-phase concentrations of CMTA and CPTA, respectively (Figure S20). We further studied the effect of spray voltage for TPS and nESI. As observed in Figure 2g, TPS required a much higher spray voltage (>3.5 kV) compared with nESI (>1 kV), possibly because of differences in the effective size of the capillary channels. Notably, the ion intensities of TPS (4−5.5 kV) were comparable to intensities generated from the optimum nESI voltages (1.5−3 kV), which imply comparable sensitivity.

The elution/extraction efficiency, from the immunoassay reaction paper strip to the paper spray strip, was characterized for CMTA and CPTA (Figure S21). A predominant fraction (~90%) of the probes eluted during the first extraction step of the paper spray process. This high efficiency is presumably due to the high hydrophilicity of these two probes, which facilitates their transfer to the paper spray tip for TPS-MS analysis when using aqueous-base spray solvent. No significant difference in extraction efficiency was observed between the two probes, which may be ascribed to their similar structures. The results for comparing the ion intensity of CMTA/CPTA in solution and that pre-deposited onto paper also indicate only ~10% reduction in sensitivity during this transfer process (Figure S22).

The performance of the new TPS-MS detection method was also tested by analyzing *Pf*HRP2 antigen. Good linearity was obtained for both buffer solution and human serum using fragment ions m/z 96 of CMTA and m/z 87 of CPTA produced from ITEA-dAb and ITBA-dAb (Figure 2h and Figure S23). The LODs in serum are 1 nM (37 ng/mL) and 100 pM (3.7 ng/mL), respectively. These results indicate that, when using ITBA-dAb, the on-surface TPS-MS/MS method is sufficient to meet WHO sensitivity requirements for the diagnosis of malaria infection.

To examine the robustness and stability of this assay, we investigated the reliability of the test results after storage in two ways. First, the paper strips were stored after the complexation of the probe-conjugated dAb. The stored test zones were subjected to hydrolysis and then analyzed by nESI-MS after the specified duration (1−30 days). The strong agreement and reproducibility among the detected MS signals (RSD = 4.7%, ITBA-dAb) indicate that the reaction system is tolerant to long storage times (Figure 3a and Figure S24a,b), which is attributed to the high stability of ITEA and ITBA in ambient air. This stability was confirmed in a second experiment (Figure S24c,d), where the test surfaces were subjected to hydrolysis immediately after the immunoassay and hydrolyzed products were stored. Here, similar precision results were recorded (RSD = 3.3%, ITBA-dAb), showing the high stability of the hydrolyzed products CMTA and CPTA. These stabilities were also confirmed by on-surface TPS-MS detection (Figure S25). In contrast, we observed that horseradish peroxidase (HRP) enzyme typically used in ELISA is vulnerable and easily loses activity during storage.

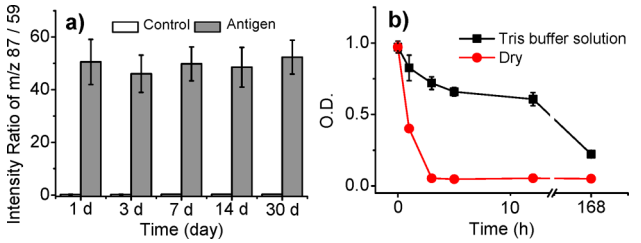

Figure 3. Stability of the probe and enzyme involved in immunoassay. (a) MS analysis results of positive (*Pf*HRP2, 10 nM) and negative control test zones stored before the hydrolysis reaction with ITBA-dAb. (b) Optical density values of ELISA assay of *Pf*HRP2 (2.7 nM) after storage under Tris buffer solution (black) or dry (red) conditions before the addition of substrate. HRP-conjugated dAb and 3,3′,5,5′-tetramethylbenzidine substrate were used. Each data point is an average of eight replicates, and error bars indicate standard deviation.

This result is summarized in Figure 3b, where the optical density (OD) value dropped to zero after the test strips were stored under dry conditions for just 2 h (red line), indicating a loss of enzymatic activity. In buffer solutions, the OD dropped to 23% of the initial value after 7 days of storage (black line). Degradation of the colorimetric product (Figure S26a) and the dynamic enzymatic reaction (Figure S26b) add further difficulty to the readout reliability. Collectively, the above experiments demonstrate that, by using the proposed enzyme-free MS immunoassay protocol, the assay can be interrupted, stored, and restored.

As many clinical outcomes are multidimensional, a single measure of biomarker may miss domains of interest or even cause misleading diagnosis. For traditional colorimetric detection methods, several test zones are needed for multiplexed assay, requiring large sample volumes. Here we applied the paper-based MS immunoassays platform, via the simultaneous use of the two cleavable probes, for multiplexed detection of two related cancer antigens: CA-125 and CEA. In this experiment, anti-CA 125 and anti-CEA capture antibodies were immobilized in the same test zone (i.e., one spot). As expected, both of the product ions from CMTA and CTPA can be clearly detected by MS/MS, and the ion intensities increase over the concentration range of antigens tested (Figure 4a), illustrating the multiplexed detection capabilities of the method. Moreover, no significant differences were observed between the one-spot assay and experiments in which the two antigens were detected separately in two different test zones (Figure 4b,c). The slight decrease in signal intensity for the one-spot assay may be due to the minor cross-reactivity and interference during the immunoreactions.[15]

In summary, we have demonstrated a new paper-based enzyme-free MS immunoassay platform. The design of cleavable ionic probes to replace enzymatic reactions in immunoassay enabled two-point separation and on-demand quantitative analysis. This approach has the advantages of both patient-friendly sample collection and reliable centralized detection. The proof-of-concept application in malaria and cancer-related antigens analysis gave evidence that combining the paper-based immunoassays with touch paper spray MS detection can provide an environmentally friendly, highly sensitive analytical approach for biomarker quantification. We believe this protocol provides a promising option to the

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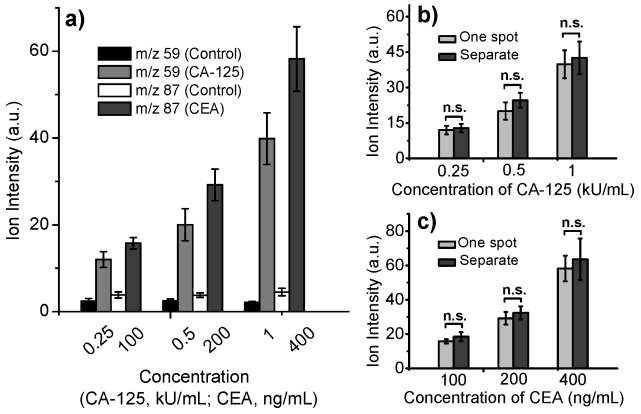

Figure 4. Multiplexed detection of CA-125 and CEA antigens in human serum. (a) Ion intensity of product ions from CMTA (m/z 118→59) and CTPA (m/z 146→87) in the detection of CA-125 and CEA in one spot. The controls mean that no antigen was added. (b,c) Comparison of the ion intensity when detecting (b) CA-125 and (c) CEA in one spot and separately; "n.s." indicates no significant difference ($P > 0.05$, two-tailed t test). ITEA-dAb of anti-CA-125 and ITBA-dAb of anti-CEA were used for the detection of CA-125 and CEA, respectively. Each data point is an average of eight replicates, and error bars indicate standard deviation.

traditional in-hospital testing and point-of-care testing by creating opportunities for self-testing, followed by signal development and diagnosis after sending the test to a central facility. Although moderately high initial capital may be required to establish this approach, we believe the low-cost paper-based consumable devices will enable sustainable implementation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b02232.

Materials, full experimental details, additional Figures S1−S26, synthesis, and NMR characterization of the probes (Figures S27−S30) (PDF)

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Author Contributions

‡S.C. and Q.W. contributed equally.

Notes

The authors declare no competing financial interest.

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