Communication

Ultrasensitive Ambient Mass Spectrometry Immunoassays: Multiplexed Detection of Proteins in Serum and on Cell Surfaces

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Ultrasensitive Ambient Mass Spectrometry Immunoassays: Multiplexed Detection of Proteins in Serum and on Cell Surfaces

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ABSTRACT: The sensitive and accurate determination of multiple protein biomarkers for clinical diagnosis is in high demand. Here, an ambient mass spectrometry immunoassay platform that possesses advantages of high sensitivity, multiplexed quantitation, low sample consumption and convenient operation was established. A series of extensible rhodamine-based mass tags that ensured quantification of multiple proteins with ultrahigh sensitivity through two-stage signal amplification were developed. Thrombin was detected at zeptomole sensitivity in 2 μL serum/plasma, as was free cancer antigen 125 (CA125). Three protein biomarkers (CA125, carcinoembryonic antigen, and epithelial cell adhesion molecule) were simultaneously detected *in situ* on ca. 20 cells. This platform is promising for multiple protein detection in a single drop of sample or at the single-cell scale for clinical diagnosis and therapy.

Most clinical biomarkers and therapeutic targets are membrane proteins or secreted proteins; thus, monitoring their expression levels in bodily fluids or on cell surfaces is of great physiological value.[1,2] Immunoassays have been successfully used in protein detection for decades,[3] evolving from enzyme-linked immunosorbent assays (ELISA)[4] to fluorescence-,[5] electrochemistry-,[6] and mass spectrometry (MS)[7]-based assays. Immunofluorescence assays are generally applicable due to their ultrahigh sensitivity, but band overlap and unavoidable background interference limit multiplexed detection. Although the latest iterative staining strategies have increased their throughput,[8] efficient multiplexed measurements on one spot are still needed. Electrochemical immunosensors also suffer from restricted electrochemical probes and electrodes.

As a readout of immunoassays, MS overcomes the multiplexed detection limitation because of its high mass resolution. Direct MS analysis of proteins provide sequence and quantitative information, but the detection of large biomolecules is often hampered by limited sensitivity.[9] Indirect protein measurement through labeling strategies in MS immunoassays has become a topic of interest in recent years. Multiple proteins tagged with elements have been detected by inductively coupled plasma MS.[10,11] However, the specialized instrumentation and labeling elements greatly increase the operating costs and limit their application. Compared with elements, organic molecules enable considerably more possibilities for tag design.[12] Combining organic tags with ambient mass spectrometry (AMS) can enable simple, patient-friendly and low cost detection.[13] Thus far, stable and extensible tag development, sensitive and multiple-protein detection, and flexible and convenient operation remain challenging in MS immunoassays.

Herein, an ultrasensitive and multiplexed AMS immunoassay (Figure 1a) was developed based on the new design of mass tags and immune-recognition on chips. New series of rhodamine-based mass tags (RMTs, Figure 1b) were designed with two-stage MS signal amplification and excellent distinguishability. The use of multiple mass tags, signal amplification and a convenient and high-throughput

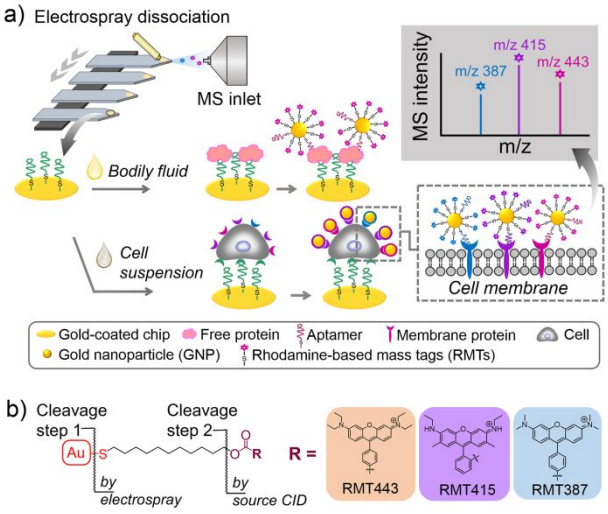

Figure 1. (a) Schematic of an AMS immunoassay platform and its workflow for protein detection in bodily fluids and on cell surfaces: protein or cell capture, protein recognition in a sandwich-type structure, mass tag dissociation and MS analysis. (b) Chemical

structure of three RMTs (RMT443, RMT415, and RMT387) and the two-step cleavage of mass tags during MS detection.

AMS technique allowed the ultrasensitive and efficient detection of multiple protein biomarkers in single drops of bodily fluids or on a few cells *in situ*.

In detail, the AMS immunoassay platform was established based on an array-type electrospray accelerated chip spray ionization (eCSI) setup (Figure S2). The target protein capturing and labeling and the mass tag dissociation and ionization were all realized on an indium tin oxide glass chip (Figure 1a). Briefly, gold was deposited on the front of a chip and modified with recognition units such as aptamers/antibodies for protein or cell capture. 2 μL sample was sufficient for detection, which is patient-friendly for clinical diagnosis. Target proteins/cells were captured on the chips and an immune sandwich were formed after the addition of gold nanoparticle (GNP) probes functionalized with self-assembly recognition aptamers and RMTs. After incubation and washing, the *in situ* dissociation and detection of multiple RMTs were achieved directly by eCSI MS within 2 minutes. Samples can be screened efficiently using an automatic sampling rail (Figure S5). The platform was successfully applied to the detection of zeptomole proteins in serum/plasma and the simultaneous detection of multiple membrane proteins on approximately 20 cells *in situ*.

Mass tags, acting as MS reporters and signal amplifiers, play key roles in ultrasensitive and multiplexed protein quantification. Serial and homologous molecules with high dissociation efficiency and good MS response are the best candidates of mass tags for multiplexed detection. Three analogous molecules, named RMT443, RMT415, and RMT387 according to the different terminals (Figure 1b), were synthesized as mass tags for the first time through universal synthesis methods (Figure S1). Stable storage for more than 6 months after synthesis was proven which is crucial for their practical application. Rhodamine derivatives were selected due to their good response and stability in MS[14] and their fluorescence which could be exploited in imaging.[15] The thiol group and undecyl chain enabled their self-assembly on GNPs and efficient dissociation during MS detection. Unlike other tags that have to be dissociated with lasers, high temperature or special reagents,[12,13,16,17] these mass tags can be dissociated and handled easily under atmospheric conditions with general electrospray solvent.

The MS detection of RMTs underwent two-step cleavages (Figure 1b), which resulted in the two-stage signal amplification and spectral simplification. The efficient dissociation of RMTs from GNPs during eCSI obtained the primary amplification, and the subsequent fragmentation by the in-source collision-induced dissociation (CID) provided the secondary signal amplification and the simplification of the MS spectrum. In detail, abundant mass tag derivatives (Table S1) were identified in the mass spectra of RMT443 after primary cleavage of the Au-S bonds (Figure 2a). Experiments investigating the eCSI parameters (Figure S3) suggested that chemical/electrochemical regulation and nanoparticle catalysis (Figures S12-S14) contributed to the efficient tag dissociation. The second amplification came from the cleavage beside the ester bond induced by in-source CID, resulting in the accumulation of the distributed signals from various RMT derivatives. All RMT443 derivatives generated the same fragment at m/z 443.2 (Figure S7), corresponding to a conjugated structure of rhodamine terminal. An in-source CID energy of 100 V afforded the maximum 3-fold enhancement of signal intensities (Figure 2a and 2b and Figure S8). Benefiting from the noise reduction after fragmentation, an approximate 6-fold higher signal-to-noise (S/N) ratio was achieved for m/z 443.2 than for m/z 611.2 before fragmentation (Figure 2c). Similar cleavages and S/N gains were achieved for RMT415 (Table S2, Figure S10) and RMT387 (Table S3, Figure S11) with final MS reporters at m/z 415.2 and m/z 387.2, respectively. These three peaks were easily and clearly identified in mass spectra (Figure 2d), demonstrating the advantage of MS in overcoming the spectral band overlap. The two-stage signal amplification and high mass resolution make the highly sensitive and multiplexed protein detection possible and extendable.

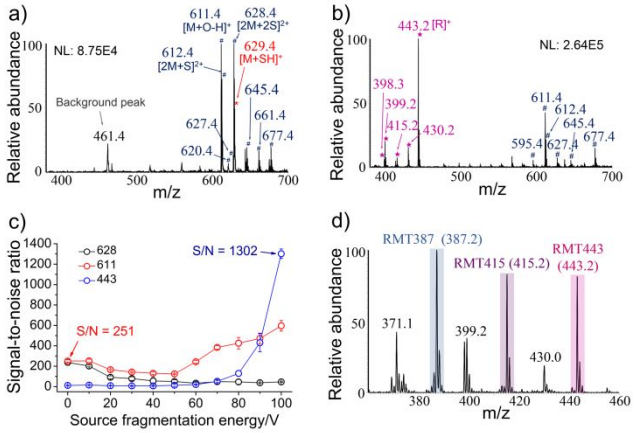

Figure 2. Mass tag dissociation. (a) Mass spectrum of RMT443 dissociation under eCSI (M: tag residue of $C_{28}H_{30}N_2O_3(CH_2)_{11}$). (b) Mass spectrum of dissociated RMT443 with the source fragmentation energy of 100 V (R: tag residue of $C_{28}H_{31}N_2O_3$). (c) Signal-to-noise ratios of m/z 628.4, m/z 611.4, and m/z 443.2 under different fragmentation energy (0-100 V). (d) MS spectrum of three RMTs after dissociation and fragmentation, with m/z 443.2, m/z 415.2, m/z 387.2 as the final MS reporters.

Quantitation of proteins at ultralow concentrations in biofluids and on cell surfaces was achieved with the help of crystal violet (CV, Figure S15) dissolved in the electrospray solvent at certain concentrations as internal standard (IS). The intensity ratios of the MS reporter (m/z 443.2, Figure 3b, red bar) and fragment ion of CV (m/z 340.2, Figure 3b, blue bar) were calculated for quantitation. The IS showed good signal stability during the whole detection (Figure 3a, RSD = 6.64%, *n = 15*). Standard thrombin was quantified over a wide range with ultrahigh sensitivity when spiked in PBS and serum (Figure 3c). The LOD for thrombin spiked in PBS was 5.45 fmol/L (S/N = 3, 2 μL sample), corresponding to 10.9 zmol, and an LOD of 35.1 zmol was obtained for thrombin spiked in serum, which is superior to most of the sandwich-type thrombin detection assays with either

electrochemical or optical signal amplifiers.[18,19] Compared to the LOD of 0.4 pmol for direct detecting thrombin using eCSI-MS, 10^7 times higher sensitivity was achieved (Figure 3b) which attributed to the new RMT design and the signal amplification strategy. Quantitative accuracy was confirmed by the recoveries (95.9%-118%, RSD < 11.8%, *n = 6*, Table S4). Good selectivity was proven under the optimized molar ratio of GNPs/aptamers/RMTs (1/120/5000, Figure S19) by almost no signals of MS reporters from either negative control (Figure 3b) or nontarget proteins (Figure S23), and good interday repeatability was achieved (RSD = 10.1%, *n = 6*, Figure 3d).

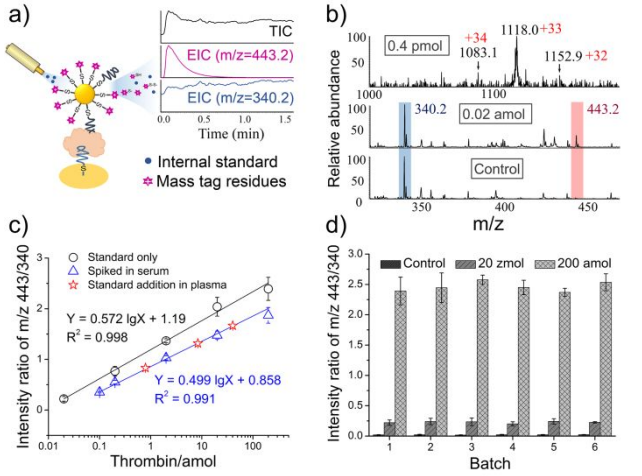

Figure 3. Thrombin analysis. (a) Schematic of thrombin detection and the total ion chromatogram (TIC) and extracted ion chromatograms (EICs) of the MS reporter at m/z 443.2 and the IS at m/z 340.2 for thrombin detection. (b) MS spectra of the direct detection of thrombin (LOD: 0.4 pmol, S/N = 3) and the detection of 0.02 amol thrombin and a blank control using RMT443 and the IS. (c) Calibration curves of thrombin spiked in PBS (20 zmol to 200 amol) and serum (100 zmol to 200 amol), and standard addition results in plasma. (*n = 3*, error bar: standard deviation, SD) (d) Repeatability of thrombin detection (interday RSD = 10.1%, *n = 6*).

The application of this versatile AMS immunoassay was expanded to the detection of cancer antigen 125 (CA125) biomarker in serum (LOD of 0.2 U/mL, Figures S24-27, Table S5). Antibodies were immobilized on chips for recognition, confirming that this assay can be flexibly extended to other recognition units. Serum samples from ovarian/breast cancer patients were examined using this assay. Consistent results with human ELISA kit (Table S6) demonstrated the high possibility for clinical diagnosis.

Since most biomarkers are localized on the cell surface,[1] the mass tag and AMS immunoassay were assessed for the *in situ* detection of multiple proteins on living cells. Ovarian cancer cells (OVCAR-3) and breast cancer cells (MCF-7) were chosen as models. These cells were captured on chips by mucin 1 protein aptamer[20] when 2 μL cell suspension solutions were deposited on chips, followed by the incubation with GNP probes for target protein recognition *in situ*. The incubation time was optimized and controlled to ensure the efficient labeling of surface protein and minimize the interference from cell internalization of probes (Figure S29). Target proteins on cell surface were observed under a microscope through the fluorescent signal from RMTs (Figures S28, S38 and S39), which helped to guide the MS analysis. Since fluorescent imaging did not damage the mass tags, subsequent MS quantitation was obtained on the same chip. CA125 on cell surfaces was successfully detected alone with high sensitivity (Figure S31). An obvious MS signal from the mass tags was observed in 25 OVCAR-3 cells.[21] Few signals were detected from a large number of MCF-7 cells,[22] indicating the high selectivity and low nonspecific adsorption of GNP probes for cell analysis. A good linear response was found between MS intensity ratios and cell numbers, and the LOD was 13 OVCAR-3 cells (S/N = 3, Figure S33).

Three important cancer biomarkers localized on the cell surface—CA125, carcinoembryonic antigen (CEA), and epithelial cell adhesion molecule (EpCAM)—were analyzed simultaneously *in situ*. Three individual GNPs modified with distinct mass tag-aptamer pairs were prepared for each protein, mixed and incubated with cells together. After incubation and washing, obvious red light was observed under a fluorescence microscope for both cell lines (Figure 4a, inset). Then, the three labeled mass tags were dissociated and analyzed simultaneously by eCSI MS, and the corresponding reporters clearly appeared in the spectrum without any cross-interference (Figure 4a). The MS results revealed that CA125 and EpCAM were overexpressed on OVCAR-3 cell surfaces, while CEA and EpCAM were overexpressed on MCF-7 cell surfaces (Figure 4b).[23-25] These results were confirmed at cell numbers as low as 25 (Figure S37), illustrating the ultrasensitivity and multiplexed detection of these platforms.

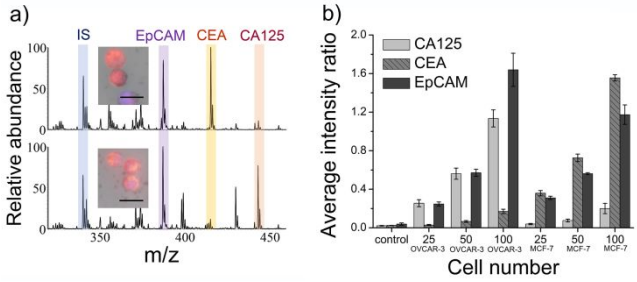

Figure 4. *In situ* membrane protein analysis on cell surfaces. (a) Mass spectra of EpCAM (m/z 387.2), CEA (m/z 415.2) and CA125 (m/z 443.2) on MCF-7 (up) and OVCAR-3 (bottom) cells. Inset: merged fluorescence and bright-field photographs of captured MCF-7 and OVCAR-3 cells incubated with three GNP probes (scale of 20 μm). (b) Multiplexed detection of CA125, CEA, and EpCAM on different numbers of OVCAR-3 and MCF-7 cells captured on the chips. (*n = 3*, error bar: SD)

In summary, an ultrasensitive and multiplexed AMS immunoassay was established for the measurement of proteins in single drops of bodily fluids or on a few cells *in situ*. Rhodamine-based molecules were verified to be ideal mass tags that presented high MS intensities and excellent distinguishability. Compared with expensive inorganic MS tags and fluorescent tags with unavoidable band overlap, organic mass tags will provide abundant possibilities for immunoassays. Representative proteins were detected with a 10^7-fold sensitivity enhancement, with a zeptomole LOD in a 2 μL sample, and three membrane proteins were

simultaneously detected *in situ* without cross-interference on ca. 20 cells. In addition, fluorescent imaging of labeled cells enables the guidance of MS detection by RMTs. All of these characteristics make this versatile platform a promising technique for the multiplexed detection of dozens of protein biomarkers in a single drop of sample or at the single-cell scale for clinical diagnosis and therapy.

ASSOCIATED CONTENT

Supporting Information

This information is available free of charge via the Internet at http://pubs.acs.org.
Reagents, instruments, mass tag synthesis (Figure S1), chips preparation and eCSI mechanism (Figures S2-S6), mass tags dissociation (Tables S1-S3, Figures S7-S14), protein detection (Tables S4-S6, Figures S15-S39).

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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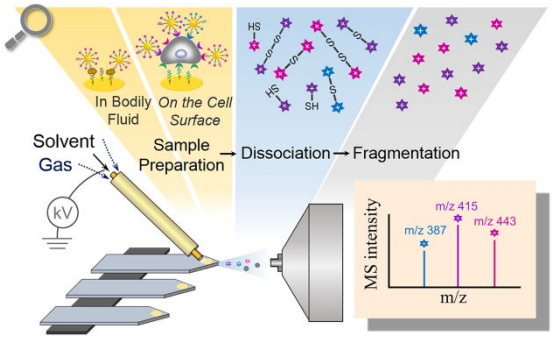
In Bodily Fluid
On the Cell Surface
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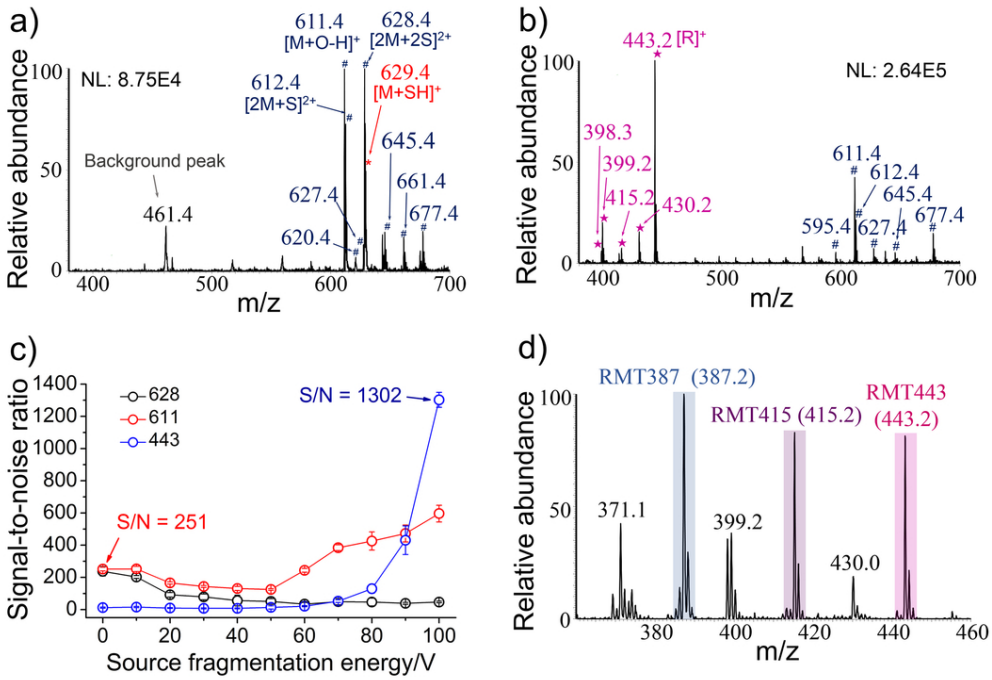

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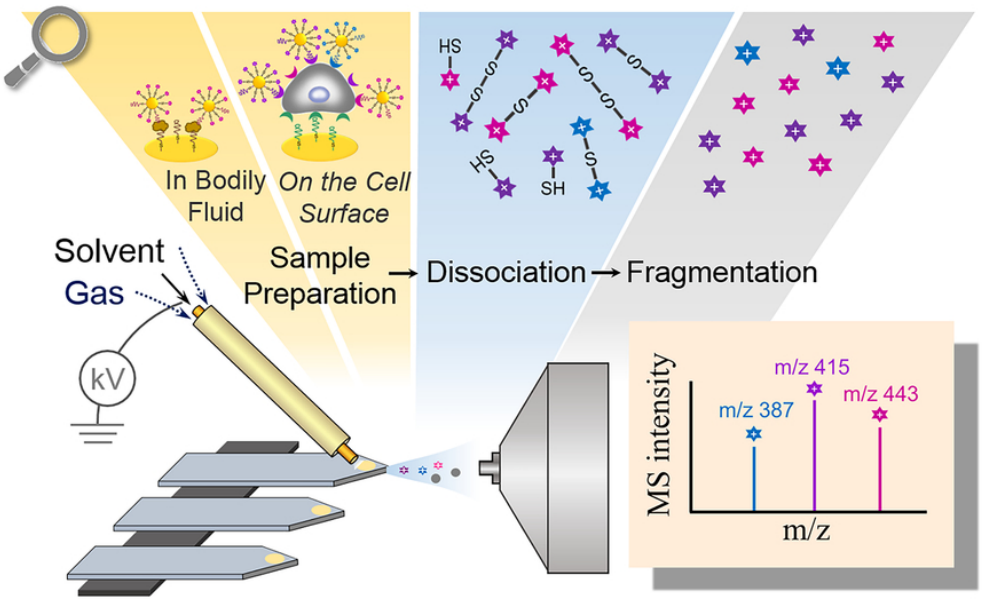

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