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REVIEW

Highly sensitive and multiplexed mass spectrometric immunoassay techniques and clinical applications

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Abstract

Immunoassay is one of the most important clinical techniques for disease/pathological diagnosis. Mass spectrometry (MS) has been a popular and powerful readout technique for immunoassays, generating the mass spectrometric immunoassays (MSIAs) with unbeatable channels for multiplexed detection. The sensitivity of MSIAs has been greatly improved with the development of mass labels from element labels to small-molecular labels. MSIAs are also expended from the representative element MS-based methods to the laser-based organic MS and latest ambient MS, improving in both technology and methodology. Various MSIAs present high potential for clinical applications, including the biomarker screening, the immunohistochemistry, and the advanced single-cell analysis. Here, we give an overall review of the development of MSIAs in recent years, highlighting the latest improvement of mass labels and MS techniques for clinical immunoassays.

Keywords Immunoassay · Mass spectrometry · Multiplexed detection · Mass label · Clinical application · Ambient mass spectrometry

Introduction

Immunoassay is described as the detection of antigens with complementary antibodies or antibody-related reagents and has become an important tool for clinical analysis and biological research with high selectivity and high sensitivity [1]. High selectivity of immunoassay is attributed to the high specificity and reactivity of the antigen–antibody interaction which allows the separation and purification of antigens in complex matrix, such as bodily fluids and tissues. Meanwhile, readily detectable labels are usually immunolabeled to antigens to convert and amplify antigen signals, which ensures the high sensitivity of immunoassays.

Nowadays, immunoassays with various labels and detectors have been developed, including radioimmunoassay (RIA) [2], enzyme-linked immunosorbent assay (ELISA) [3], fluorescence immunoassay (FIA) [4, 5], electrochemiluminescence immunoassay (ECLIA) [6], surface-enhanced Raman scattering immunoassay [7], and electrochemical immunoassay [8]. These methods usually possess extremely low limits of detection (LODs) (pg/mL–ng/mL) due to the sensitive labels and readout systems. Representative ECLIA and ELISA have been equipped with commercial instruments and kits, and play a major role for early cancer screening and disease diagnosis with protein biomarkers. Immunohistochemistry based on similar principle has been applied in tissue pathological examination [9]. However, these methods based on optical or electrical readout systems are often restricted in the one-spot multiplexed detection (limited to five targets) because of the overlap between label signals [10]. Strategies, such as the iterative labeling [11] and multi-channel detection [12], have been developed, but the consumption of sample and time is inevasible. Obtaining

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multiplexed signals from as few samples as possible is an important goal for immunoassays to facilitate the disease diagnosis and biological research.

Mass spectrometry (MS) has been employed as the readout instrument for immunoassays because of its theoretical unfettered resolution for multiplexed detection in a single test. MS also owns large dynamic range and has the ability of absolute quantification. Hutchens et al. [13] were the first to demonstrate the enhanced detection of multiplexed macromolecules directly from biological fluids by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) after affinity capture in 1993. Nelson et al. [14] further put forward the principle of mass spectrometric immunoassay (MSIA) in 1995. The MSIA allowed the microscale immunoaffinity capture, identification, and quantification of target antigens using MALDI-MS. Since the qualification with structural information is a unique advantage of MS, the early MSIA achieved the direct signals of antigens with their characteristic mass-to-charge ratios for accurate qualification and quantitation. Furthermore, multiple mass-resolved antigens (six antigens as a demonstration) were easily detected in a single test. However, the direct detection of macromolecules could only obtain the LODs at the level of ng/mL–μg/mL due to the limited MS performance for macromolecule detection [15], making the early MSIAs insufficient for protein biomarker screening in clinical analysis.

Efforts have been made to improve the sensitivity of MSIAs by making a compromise between achieving structural information of targets and increasing sensitivity. Mass labels have been developed and utilized to immunolabel targets to convert and amplify macromolecular signals [16]. The utilization of mass labels allows a simplified and controllable acquisition of multiplexed signals. The development of MSIAs with inductively coupled plasma promoting mass spectrometry (ICP-MS) and element labels is straightforward. Zhang et al. [17] reported the first ICP-based MSIA for the detection of thyroid-stimulating hormone with Eu^{3+} chelates in 2001. The simultaneous determination of proteins using Au/Ln labels coupled with ICP-MS was demonstrated in 2002 with LODs as low as sub ng/mL [18–20]. Since then, ICP-MS combined with element labels has been popular for immunoassay [21]. Besides element labels, a new branch of immune labels as small-molecular labels have been recently designed, which can be coupled to more flexible MS instruments, such as laser desorption ionization (LDI) MS and ambient MS, showing various unique performance in immunoassays. In this review, we discussed the recent advanced MSIAs with high sensitivity and multiplexed ability based on different MS systems and various mass labels. Specific design of the latest element labels and promising small-molecular labels coupled to different MS ionization modes to improve sensitivity, specificity, quantitative accuracy, and multiplexed detection for MSIA were highlighted. We further discussed the clinical applications of these recent MSIAs, such as multiplexed antigen screening, tissue imaging, and cell screening, to show the future prospects of MSIAs in the clinical usage.

ICP-MS immunoassays

ICP-MS has the advantages of high sensitivity (less than pg/mL level for most isotopes), large dynamic range (up to 9 orders of magnitude), absolute quantification, and little matrix interference for heavy-metal isotopes [22]. It is commonly acted as the mass detector for immunoassays with stable heavy-metal isotopes tagged to antibodies as immune labels. This type of immunoassay has been developed for a long period since the early 2000s, and has been a mature immunoassay technology with commercial instruments and kits. Metal ions, clusters, and nanoparticles have been coupled with traditional integral ICP-MS, single particle (SP)-ICP-MS, and laser ablation (LA)-ICP-MS for various immunoassays. Several reviews have already summarized the immunoassays in this field [21–24]. Here, we highlighted the latest progress of highly sensitive element tags and multiplexed ICP-MS-based immunoassays.

Element labels

Labels containing stable elements ranging from 75 to 209 Da are usually advised for ICP-based MSIAs due to their low interferences in biological matrix, high sensitivity, and good compatibility with the general channels of commercial instruments [25]. These labels can be partly inherited from the labels for FIA, ELISA, and ECLIA, such as recognition units linked to rare-earth complexes, quantum dots, nanoparticles, and nanoclusters [26, 27]. With no need to possess optical, electric, or any other special properties, the masses are directly detected as unique signals in MSIA. The sensitivity of ICP-based MSIA is depended on the number of atoms per label, and the stable and narrow distribution of the number of atoms in labels is the basic for the accurate and multiplexed quantitation of antigens. Considering the high stability, high sensitivity, low interferences in biological matrix, and easy preparation, polymer-based metal ion-chelating compounds, nanoparticles, and clusters based on noble-metal isotopes (Au, Ag, Pt, Pd, Rh, Ir), lanthanide isotopes, and some post-transition metals (In, Bi) are mainly used to link with recognition units (antibody, aptamer, etc.) as element labels. The performance of representative element labels and latest element labels with reference atom numbers per label and the label size is highlighted in Table 1.

Metal ions are chelated by bifunctional ligands, such as N,N',N'',N'''-tetra acetic acid (DOTA) and

Table 1 Representative and latest element labels and their performance

Element forms	Signal atoms per label	Label size (without recognition unit)	Antigens	Limit of detections	Ref
Polymer-Ln^{3+}	60–120 (Pr, Tb, Ho, Eu, and Tm)	~10 nm (line)	Cell surface antigens: CD33, CD34, CD38, CD45, and CD54	Copy numbers as 100	[28]
Polymer-Ln^{3+}	~160 (Gd, Tb, Dy, Ho, Er, etc.)	~10 nm (line)	Cell surface antigens: CD33, CD4, CD8, CD3, CD20, etc	Single cells	[29]
Eu^{3+}-DOTA-bacteriophage	965 ± 13 (Eu)	~30 nm	Folate receptor on KB cells	5 KB cells/mL	[30]
Pr/Eu/Ho^{3+}-DOTA-DNA	180 bp (Pr, Eu, and Ho)	~60 nm (line)	CEA, AFP, and FER	3.31 pg/mL, 5.31 pg/mL, 2.02 pg/mL	[31]
Ln^{3+}-polymer dots	1100–2000 (Nd, Eu, Tb, Ho, and Er)	10–30 nm	Cell surface antigens: CD326	Single cells	[32]
AuNPs	~6×10^6 (Au)	60 nm	p53	2.6 ng/mL	[33]
Silver enhanced AuNPs	~10^9 (Ag)*	> 100 nm	CEA	30 pg/mL	[34]
LnNPs	~10^3 (Eu and Lu)	8 nm	p-$p53^{15}$ and p-$p53^{392}$	200 pg/mL 20 pg/mL	[35]
LnNPs	~1.4×10^6 (Ce, Pr, Nd, etc.)	50 nm	HER2, EGFR, etc	Single cells	[36]
ZnSe QDs	~10^3 (Zn)*	1.6–4.0 nm	CEA	6 pg/mL	[37]
UCNPs	5.54×10^6 (Y)/1.48×10^6 (Yb)/1.74×10^5 (Er)	40–55 nm	AFP	0.3 ng/mL (^{89}Y)/0.22 (^{174}Yb) ng/mL	[38]
TaO_2 NPs	~2700 (Ta)	5.7 nm	Cell surface antigens: CD20, CD25, and CD196	Single cells	[39]
Zr-NMOF	~10^5 (Zr)	33 nm	Cell surface antigens: CD19 and CD45	Single cells	[40]
$Au_{22}(GSH)_{18}$	22 (Au)	1.48 nm	AFP	5.43 nmol/L	[41]
$Au_5Peptide_3$	5 (Au)	< 1 nm	EGFR	Single cells	[42]
Bismuth oxido cluster	6 (Bi)	0.8–1.5 nm*	-	-	[43]

*Data were not given in the original work. Estimated data were referred to [44–46]

diethylenetriaminepentaacetic acid (DTPA), as the early single-metal-chelating labels that only have one signal atom per label [47]. Polymer-based metal ion-chelating compounds were subsequently developed to increase the number of atoms in each label. However, the increasing of label size and structure complexity bring more uncertainty in the number of labeling atoms and the efficiency of specific recognition, which requires special design. Many polymeric reaction methods and agents have been designed to ensure the large signal amplification, good uniformity, water solubility, and biocompatibility of polymeric labels. Lou et al. [28] firstly introduced a water-soluble polymer that contained 33 metal-DTPA ligands and finally contributed to 60 to 120 signal atoms on one antibody. These polymer-based agents have been commercially available, such as Maxpar polymer agents to bind 38 isotopes of lanthanides, two isotopes of In, one isotope of Bi, and one isotope of Y [25]. More atoms in each label are still pursued with a new DTPA polymer developed by Majonis et al. [29], achieving 161 ± 4 Tb atoms to each antibody. Grenier et al. [48] developed the [In(cb-te2pa)]$^+$ chelate functionalized in polylysine polymer with a polymerization degree of 52. Hu et al. [31] introduced a new click-chemistry strategy to couple rare-earth element-DOTA to the facilely prepared alkyne-DNA chains. Thirty lanthanide ions were loaded on a 180 bp alkyne-DNA, and longer alkyne-DNAs with hundreds of ions could be prepared via polymerase chain reaction. Yuan et al. [30] developed element-tagged virus-like nanoparticles to provide 965 ± 13 Eu atoms for one label. Wu et al. [32] developed lanthanide-coordinated semiconducting polymer dots with 1100 to 2000 lanthanide atoms per dot, obtaining at least seven times higher sensitivity than the commercial probe. The polymer dots were also demonstrated to have low non-specific binding with polyethylene glycol structures. These polymeric labels have been widely used, but they have high non-metallic proportions which limit the further improvement of the detectable atom ratio per label size.

Nanoparticles (NPs) with large amount of detectable metal atoms per label size are also popular for ICP-based MSIAs. The fast development of nanoscience brings various NP labels [24]. Noble-metal NPs (AuNPs [33, 49, 50], PtNPs [51, 52], and AgNPs [34, 53]), quantum dots (QDs;

ZnSe [37], CdSe [45], and PbS [54]), metal oxidel NPs (CuO [55], TaO_2 [39, 56]), lanthanide NPs [35, 36], and lanthanide-doped upconversion nanoparticles (UCNPs) [38] have all been conjugated to the antibodies for MSIA. High sensitivity is ensured by large amount of atoms in each NP. For example, one AuNP in 10–100 nm diameter is supposed to have ~ 3×10^4 to ~ 3×10^7 atoms [57]. Ngamcherdtrakul et al. [36] developed phosphonated mesoporous silica NPs (~ 50 nm) that could load lanthanides for up to 1.4×10^6 atoms per particle. However, the homogeneity of NPs is difficult to be controlled and receive widespread attention. Preparation with dispersant, precise condition control, and dialysis after preparation are common strategies to control homogeneity of NPs. Oh et al. [56] used a microemulsion method to synthesize TaO_x NPs in controllable diameter from 5 to 15 nm with very narrow size distribution ($\sigma_r \leq 5\%$). Zhang et al. [39] employed 5.7 nm TaO_2 NPs (~ 2700 tantalum atoms per NP) for ICP-based MSIA. Dang et al. [40] introduced nanometal organic frameworks (NMOFs) as element labels, and synthesized a uniform-sized Zr-NMOF (33 nm; polydispersity index < 1) carrying 10^5 metal ions under modulator/reaction time co-regulation. NPs with abundant active sites on surfaces sometimes bring nonspecific adsorption, stability, and biocompatibility problems during immunoreaction. Blocking with bovine serum albumin [33] or polyethylene glycol [58, 59], controlling the size of NPs, and effectively washing are often used to reduce the nonspecific adsorption. Yin et al. prepared two apoferritin templated NPs that overcame the limitations of homogeneity, nonspecific adsorption, and stability with the apoferritin spherical shell [35].

Metal clusters are another type of element labels. Compared with NPs that are difficult to be perfectly uniform, clusters with precise molecular formula and good biocompatibility are better for quantitative analysis. Although the atoms are less in each cluster with sizes of 0.2–3 nm than NPs, the detection limits using clusters are still acceptable for quantifying 10^4 to 10^7 biomarkers [21]. Gold nanoclusters are the most common cluster labels, such as $Au_{22}(GSH)_{18}$ [41]. Gold clusters are easily synthesized and functionalized. Zhai et al. [42] developed a new peptide-Au cluster probe ($Au_5Peptide_3$) that was relatively cheap and easily synthesized. Peptide sequences in the probe were specially designed for specific recognition and Au binding. Li et al. [60] further developed metal clusters modified with rationally designed peptides ($Au_{24}Peptide_8$ and $Au_{22}Peptide_8$), termed clusterbodies, though a controlled one-step biomimetic mineralization. Bauer and Remmler et al. [43] presented a general approach for efficient functionalization of a well-defined bismuth oxido cluster (6 Bi atoms in each cluster) with peptide sequences for sensitive and specific immunoassays.

Integral ICP-MS for immunoassays

Regular mode of ICP-MS is the continuous measurement of homogeneous solution to obtain a constant signal of elements for quantitative analysis. The readout of element labels by ICP-MS requires to release the elements from conjugations into a homogeneous solution after immunoassay process (immune capture, immune labeling, separation, and washing). HNO_3 solution (1%, v/v) is efficient to release ions from polymer-based element tags, and it is less likely to form polyatomic substances that may cause interference in the element determination [20]. Other acidic solutions, such as formic acid solution and the mixture of HCl and HF, are also used to dissolve NPs, including noble-metal NPs, QDs, and UCNPs [18, 19, 38].

Several advantages of ICP-MS and element labels have contributed to the absolute quantification of target proteins in ICP-MS-based immunoassays [61], including (i) the special elements in labels with low matrix interference, (ii) the uniform size and good reproducibility of labels, (iii) known quantitative relationships for elements per antibody, and (iv) high sensitivity and element-independent response in ICP-MS. Benefitted from these inherent advantages, absolute quantification of target antigens by element labels and ICP-MS can be obtained following the typical immunoassay principles as (i) the complete labeling or known labeling relationship between labels and targets and (ii) the complete separation of label-target conjugations with excess free labels.

The separation of label-target conjugations from free labels can be easily achieved for the immunoreactions in heterogeneous system, such as sandwich immunoassays [62, 63] and competitive immunoassays [64] that can be conducted on solid plates or magnetic particles. In sandwich immunoassays, antigens are simultaneously recognized by capture antibody and detection antibody to form sandwich-type complex, so this immune type is often used to analyze proteins with two or more recognition sites. Labeled antibodies are usually used as detection antibody to provide signals and added excessively during immune recognition to ensure the nearly complete labeling. The sandwich mode can achieve the signal positively correlated with the amount of target, and has high sensitivity. Yin et al. [35] developed an ICP-MS-based sandwich-type immunoassay in 96-well plate, and prepared EuNPs and LuNPs as labels for the quantification of two phosphorylated p53 proteins at serine 15 and serine 392 sites. This method achieved the linear ranges of 0.5–20 and 0.05–20 ng/mL and the LODs of 200 and 20 pg/mL for p-$p53^{15}$ and p-$p53^{392}$, respectively. For competitive immunoassays, antigens and labeled antigens are competitively combined to antibody to form antigen–antibody complex. This mode is suitable for analytes with only one binding site. Labeled antigen needs an optimal

amount for accurate quantification, and the signals are negatively correlated with the amount of target. The sensitivity of competitive immunoassays is usually lower than sandwich immunoassays. Sun et al. [65] demonstrated a competitive ICP-MS-based immunoassay for the accurate quantification of β2-MG antigen with an LOD of 0.17 μg/mL. A ratiometric quantification was employed in this method via using Y-labeled capture antibody as internal standard probe and Sm-labeled antigen used as report probe. The signal ratio Sm/Y could compensate for the particle loss and suppress the signal fluctuation to improve the quantitation precision. Zhang et al. [66] designed the simultaneous competitive and sandwich immunoassays on magnetic beads to quantify both small and large molecules (Au-labeled thyrotropin and Eu-labeled free thyroid hormone) at the single test.

More targets can be quantified together using a series of element labels. The acquisition of each element takes milliseconds, and dozens of elements can be monitored rapidly. Element labels that can be dissolved with the same condition and have similar MS response and no cross interference are used for multiplexed detection [21, 22]. Polymer-based element tags and NPs doped with various metals are commonly employed for multiplexed detection. For polymer-based element tags, available elements and isotopes are potentially greater than 79. Sun et al. [67] demonstrated a multiplexed immunoassay of 12 proteins labeled with isotopes of ^{141}Pr, ^{151}Eu, ^{152}Sm, ^{153}Eu, ^{158}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{168}Er, ^{169}Tm, ^{170}Er, and ^{175}Lu (Fig. 1A). These labels present similar signal response and detection condition with LODs at ng/mL level. NPs are now not comparable to polymer-based element tag series in the universality of preparation strategies and the multiplexed channels, but they can also be easily employed for the simultaneous detection of several targets. Ko et al. [69] developed metal-doped inorganic NPs for the simultaneous detection of three antigens with the LODs from 0.35 to 77 ng/mL.

SP-ICP-MS for immunoassays

SP-ICP-MS is a time resolved mode of ICP-MS for the detection of single particles. When the ICP-MS instrument works at very high reading frequencies over ~ 100 Hz, elements in particles that have high spatial aggregation can be atomized and detected directly within 300–1000 μs to present a series of events corresponding to individual particles over a continuous baseline [70]. For immunoassays by this mode, element labels are detected with minimal dilution, which ensures high sensitivity.

Both competitive immunoassays and sandwich immunoassays have been demonstrated by SP-ICP-MS with AuNPs and some other NPs [71, 72]. The frequency of the transient signals is proportional to the concentration of NP labels, and large particles linked to many element tags via sandwich immunoreaction are preferred to better recognize transient signals for quantification. For example, Cao et al. [37] developed a highly sensitive sandwich-type magnetic immunoassay based on SP-ICP-MS using ZnSe QDs as labels, and carcinoembryonic antigen (CEA) was detected with an LOD of 6 pg/mL. They [73] further employed three labels (AuNPs, ZnSe QDs, and AgNPs) and magnetic beads as core for the sandwich immunoassays of three antigens, cytokeratin fragment antigen 21–1 (CYFRA21-1), CEA, and carbohydrate antigen 15–3 (CA15-3). The multiplexed detection could achieve the same performance as single target detection with 6 pg/mL LOD for CEA, 0.02 ng/mL for CYFRA21-1, and 0.25 mU/mL for CA15-3.

SP-ICP-MS provides an opportunity for simple homogeneous immunoassays that requires only the mixing of samples and immune reagents before detection. The protocols without separation minimize the time-/lab-consumption. Huang et al. [74] validated the SP-ICP-MS for homogeneous immunoassay of CEA using AuNPs-antibody conjugations as probes. The binding of probes with CEA causes the aggregation of AuNPs and leads to the change of both the frequency and the intensity of a single nanoparticle aggregate. The frequency and mean intensity of the transient signals could both be used for quantification. The frequency presented a negative correlation with CEA concentration with an LOD of 0.21 ng/mL, while the mean intensity had a positive correlation with CEA concentration with an LOD of 0.68 ng/mL. Multiplexed detection was then realized for the simultaneous quantification of three antigens (Fig. 1B) [51]. The multiplexed homogeneous immunoassay achieved the LOD of sub ng/mL for CEA and sub U/mL LODs for CA125 and CA19-9.

Single bacterial cells labeled with elements have also been detected by the SP-ICP-MS when single cells are recognized as large particles. Liang et al. [75] prepared five Ln-coded antibodies, serving for specific bacterial identification and counting. The SP mode works like the mass cytometry for single-cell analysis. Mass cytometry is a fusion of flow cytometry and ICP-MS, which has been a mature instrumentation called Cytometry by Time-Of-Flight (CyTOF) to simultaneously detect many targets in single cells [25, 76]. Single-cell droplets are introduced to the ICP torch through specific spray chamber and splitter [77]. The technological core of mass cytometry consists of the multiplexed element detection based on ICP-MS and the immunoassay for cellular proteins using element labels, so it can be considered as an important part of SP-ICP-MS immunoassays. Polymer-based element label series are most commonly used for single-cell analysis in mass cytometry. More than 40 antigens, especially cluster of differentiation (CD) antigens in single cells, can be monitored together, such as the ^{89}Y, $^{102-110}$Pd, ^{113}In, and ^{115}In for antigens with relative high abundance,

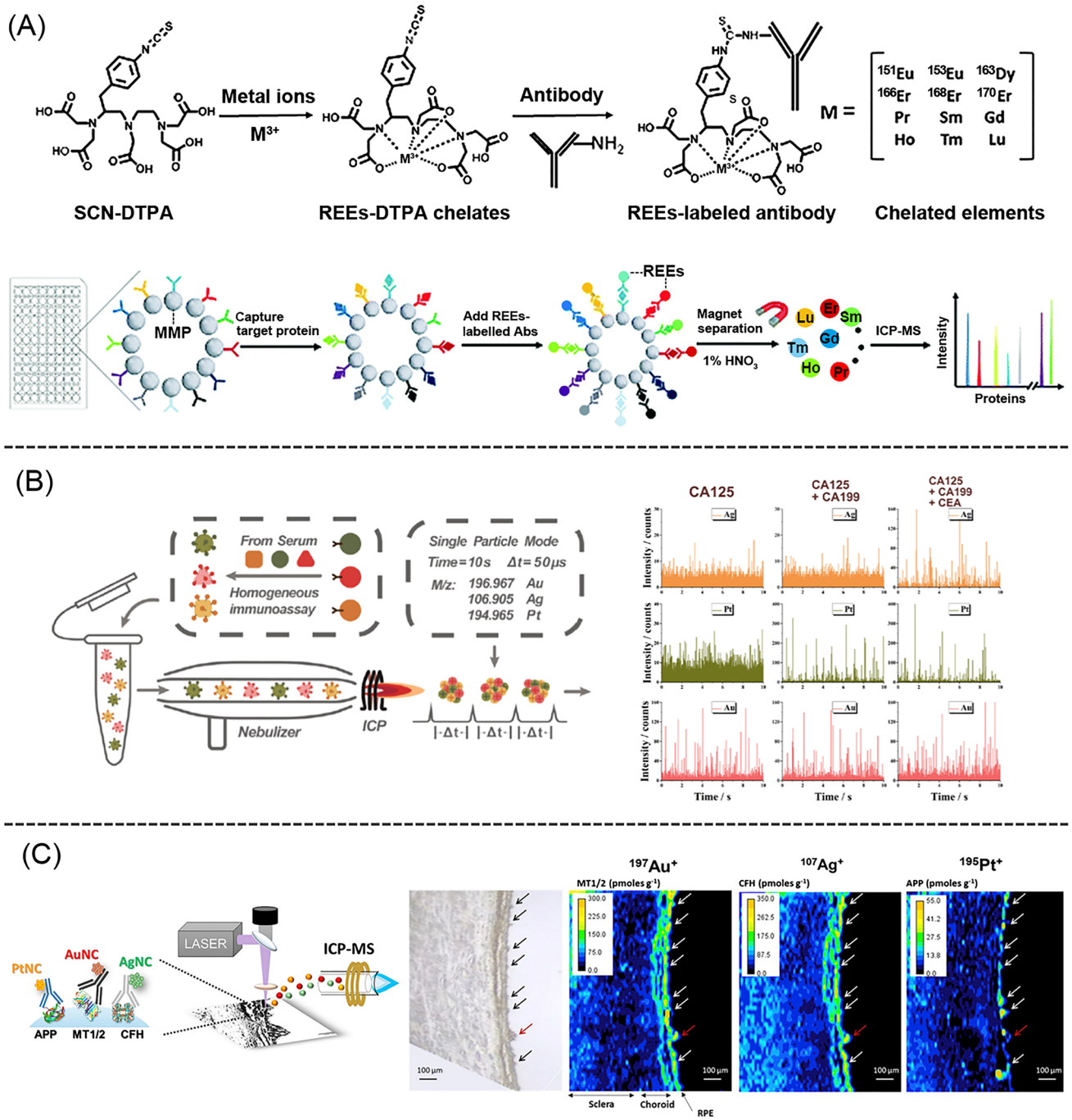

Fig. 1 Representative ICP-based MSIAs: **A** integral ICP-MS-based immunoassay of 12 proteins labeled with 12 isotope labels (^{141}Pr, ^{151}Eu, ^{152}Sm, ^{153}Eu, ^{158}Gd, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{168}Er, ^{169}Tm, ^{170}Er, and ^{175}Lu). Adapted with permission from Ref [67], permission conveyed through Copyright Clearance Center; **B** SP-ICP-MS-based immunoassay of three proteins labeled with three labels (AuNPs, ZnSe QDs, and AgNPs). Adapted with permission from Ref [51], Copyright © 2020, American Chemical Society; **C** LA-ICP-MS the multiplexed quantitative mapping of three proteins in eye sections. Adapted with permission from Ref [68], Copyright © 2021, Elsevier

and $^{153-176}$Ln and ^{209}Bi for low abundance antigens during multiplexed detection. The advantage of ICP-MS to increase the number of cellular parameters has been fully demonstrated in mass cytometry.

LA-ICP-MS for immunoassays

The above techniques are suitable for the targets dissolved and dispersed in solution by introducing solution sample into the nebulization of ICP-MS. LA-ICP-MS is developed

to analyze targets in situ on solid surface, such as capture substrates and biological tissues [78]. A sample aerosol is generated by LA, and introduced into the plasma for vaporization, atomization, and ionization. The elements on the solid surface are analyzed by LA-ICP-MS without sample pretreatment.

Labeled proteins loaded on several common substrates, such as the glass and polyethylene substrates, can be concurrently quantified for sandwich immunoassays [79], and the membrane after Western blotting can be imaged directly [80]. Sampling by LA is appropriate to detect immunoassays on microarrays to obtain high-throughput detection with micro-volume sample. Hu et al. [79] developed a method to detect proteins on an immuno-microarray by LA-ICP-MS. Three antigens were tagged with three labels (Eu^{3+}, Sm^{3+}, and Au) by sandwich-type immunoassay and detected on one spot with sub ng/mL LODs for three antigens. Waentig et al. [81] further achieved simultaneous analysis of 8 different cytochromes on microarrays (Fig. 1C). This method showed excellent detection limits in the lower amol level.

Using LA for sampling usually suffers the problems in spot homogeneity, matrix effect, and laser stability during sampling, which might affect the absolute quantification results. Internal standardization using elements on substrates can partly solve these problems and improve the accuracy for quantification and semi-quantification. Hoesl and Neumann et al. [82] developed a method to add metal spiked inks onto the solid thin layer samples using conventional ink-jet printer for simultaneous internal standardization and calibration of LA-ICP-MS. Isotope dilution analysis (IDA) technique has also been utilized in complex system, such as the detection of gel plates after gel electrophoresis, to ensure the accurate quantification [83, 84].

LA-ICP-MS possesses the ability for sample screening with high spatial resolution, which has been developed for in situ tissue imaging, like the immunohistochemistry. LA-ICP-MS-based tissue imaging can acquire much more information than the typical immunohistochemistry during one test with acceptable about 1–200 μm resolution [85]. LA-ICP-MS also provides a possibility for quantitative mapping based on the basic principles of ICP-MS-based immunoassays, and additional requirements are raised for immunohistochemistry [61]. Small label size and better dispersibility are preferred for labels. Polymer-based element tags and clusters with uniform sizes are often utilized for tissue imaging to ensure the good reproducibility on each spot. The labeled antibody is general in excess and the labeling process is maintained for sufficient time during immunohistochemistry. Giesen et al. [86] introduced the first multi-parametric LA-ICP-MS immunohistochemical approach using three lanthanide-tagged antibodies for the simultaneous imaging of three antigens in a breast cancer tissue section with a laser spot size of 200 μm. Recently, LA-ICP-MS has been applied for the multiplexed quantitative mapping of proteins in eye sections, especially the retina. The micrometric resolution (laser spot size of 10 μm) and high sensitivity of LA-ICP-MS are ideal for the imaging of such structures. Valencia et al. [87] demonstrated the simultaneous imaging of metallothioneins 1 and 2 (MT1/2) and superoxide dismutase 1 in human retinal layers using silver of natural isotopic composition and isotopically enriched silver. Lores-Padín et al. [68] further applied Pt, Au, and Ag nanoclusters for the simultaneous determination of three proteins (MT1/2, complement factor H, and amyloid precursor protein) in several human cadaveric eye sections.

The combination of LA with CyTOF mass cytometry generated another similar technique for immunohistochemistry named imaging mass cytometry (IMC) [88]. The IMC employed a high-resolution laser ablation system and a low-dispersion laser ablation chamber at a cellular resolution of 1 μm. Using polymer-based element tags, IMC has been demonstrated for the imaging of about 40 targets in a tissue section [89].

The existing ICP-MS-based immunoassays possess sensitivity comparable to the commercial ELISA and ECLIA, and take the unique advantage as multiplexed quantification for more than 40 targets. Various element tags, including the polymer-based element tag series, NPs, and clusters, have been designed. Although some tags with pure isotopes are relative expansive, these tags have served the sensitive, accurate, and multiplexed quantification, and new labels are springing up for larger amplification, better stability, and expandability. Optional modes as integral mode, SP-mode, and LA-mode have been developed for almost all kinds of immunoassays, including homogeneous immunoassay, sandwich immunoassay, competitive immunoassay, and in situ micrometric resolution immune-imaging in cells and tissues. These ICP-MS-based techniques are powerful tools, and some of them have been commercially available for clinical applications.

LDI-MS immunoassays

Typical MALDI-MS is the first technique to be combined with immunoassays [13], but the early methods suffered from the very limited sensitivity. The introducing of mass labels is effective to increase the sensitivity for LDI-MS-based immunoassays and immunohistochemistry, and mass labels have been expended from elements to more diverse small molecules. Various small molecules are designed according to the LDI condition for the sensitive and multiplexed immunoassays on substrates, in cells and in tissues, opening the door of a new branch of MSIA.

From element labels to small-molecular labels

Some element labels can be fragmented and ionized under laser irradiation, providing specific signals for LDI-based MSIAs [90]. AuNP is a popular type of element label, and the AuNPs can produce Au cluster ions ($[Au_n]^+$) under LDI-MS [91]. However, the form of clusters is sometimes uncertainty, which depends on the laser power density, pulse width, and surface properties of AuNPs. So, the intensity of Au clusters under stable LDI acquisition is sometimes averaged for semi- or relative quantification [92]. Different from the large particles used for ICP-MS, studies on AuNPs (2–10 nm) under LDI-MS suggested that the smallest 2 nm AuNPs have a higher LDI efficiency due to the quantum confinement effect, and smaller AuNPs are preferred for LDI-based MSIAs [93].

Although element labels have some compatibility and application value for LDI-based MSIAs, their types and performance are limited. Taking the unique advantages as high compatibility with organic MS, almost unlimited structural expansion, flexible labeling chemistry, easy ionization and specific molecular ions and fragment ions for qualitative analysis, organic small molecules including peptides (with molecular weights less than 2000), and other organic small molecules (mostly with molecular weights less than 1000) have been developed recently as the labels for MSIAs (Table 2).

For sensitive and multiplexed immunoassay, small-molecular labels need to have expandable structures and high MS response. In addition, photocleavable sites are necessary in the labels to generate reporter signals under LDI-MS. Inspired by existing biomolecules, Lemaire and Stauber et al. [94] proposed the concept of "Tag-Mass" with short peptides (usually less than 2000 Da after cleavage) for indirect detection of proteins. Peptides were employed with a photocleavable linker that presented a specific absorption band (340 nm) closed to that of MALDI lasers (337–355 nm). Peptides were easily expanded with different sequences for multiplexed detection, and presented excellent biocompatibility. However, fast fragmentation of peptides was observed during MALDI-MS detection, which suppressed the sensitivity. Smaller-molecular labels with molecular weights less than 1000 and stable structures under LDI offer more focused and specific MS signals. Triphenylmethanethiol derivatives become efficient labels because the carbon–sulfur bond is photocleavable to yield the very sensitive and stable carbocation [95].

To further increase sensitivity, many combination labels have been developed to modify abundant small molecules on NPs. Baird et al. [96] designed an amplification probe using particles functionalized with a large number of peptides for the immune labeling and detection of cells, demonstrating significant improvement of LODs with higher amplification factor. Among various NPs, AuNPs play important role as the carriers for LDI-based immunoassays. Small molecules with end of thiol groups are mostly used due to their self-assembling on AuNPs and efficient cleavage from AuNP surface under LDI. Polyethylene glycol (PEG)-thiol derivatives are classic ligands on GNPs because of their high assembling efficiency. It has been demonstrated that ~ 2600 PEG thiol molecules can assemble on each 15–20 nm GNP [97]. The PEG structure can also reduce the nonspecific adsorption in biological system [98–101], and is easy to expand via using different degree polymerization to provide

Table 2 Representative and latest small-molecular labels and their performance

Small molecules in labels	Dissociation	Antigens	Limit of detections	Ref
Peptides	Laser	Serine hydrolases	35 nmol/L	[103]
Triphenylmethanethiol derivatives	Laser	PSA	200 pg/mL	[95]
Triphenylmethanethiol derivatives	Laser	Cell surface sialoglycoconjugates	5 fmol	[104]
Peptides-NPs	Laser	IC9-1	50 ng/mL	[96]
PEG-AuNPs	Laser	Bcr/Abl chimeric protein	-	[99]
PEG-AuNPs	Laser	PSA	2 fmol/L	[100]
PEG-AuNPs	Laser	Thrombin	100 amol/L	[101]
PEG-AuNPs	Laser	IgE antibodies	~ pg/mL	[102]
PEG-AuNPs	Laser	Fructose, xylose, and galactose on cells	200 cells/mL	[105]
PEG-AuNPs	Laser	EpCAM, CK19, and MUC-1 on single cells	~ zmol	[106]
Ionic quaternary ammonium derivatives	Hydrolysis	*Pf*HRP2 antigen; CA125 and CEA	2.8 ng/mL; < 0.25 kU/mL, < 100 ng/mL	[107]
RDM-AuNPs	Electrospray	Thrombin	2.5 pg/mL	[108]
RDM-AuNPs	Electrospray	CA15-3, CA19-9, CEA, CA125, HE4, and AFP	0.903 U/mL, 0.825 U/mL, 0.154 ng/mL, 1.612 U/mL, 0.00830 nmol/L, and 1.56 ng/mL	[109]
TMTP-AuNPs	Electrospray	PSA	3.0 pg/mL	[110]

multiple labels for MSIAs [102]. Lee et al. [98] modified a large amount of triethylene glycolterminated alkanethiol molecules on large 2 μm gold particles as labels, achieving ultrahigh amol sensitivity. However, the large particles can suffer from dispersion and stability problems. Small particles are more stable and better dispersed during functionalization and immunoreaction. GNPs with the diameter less than 20 nm have further been found to assist efficiently LDI as special inorganic matrix [101]. As a result, PEG-thiol derivatives on GNPs (less than 20 nm) are now the mostly used combination labels for LDI-based immunoassays. Since these combination labels can only be controlled within a certain range of uniformity, their average signals are often used for quantification.

LDI-MS-based microarray immunoassays

MALDI-MS offers array-detection mode for high-throughput sample screening. It is suitable for the sandwich immunoassays on microarrays with low sample consumption. However, compared to the absolute quantification ability of ICP-MS, LDI-MS methods often have problems for quantification with limited uniformity of labels, laser fluctuation, heterogeneous sample deposition, rough sample surface, and high matrix effects. Since signals from continuous detection are more reliable for quantitative analysis, semi-quantification or relative quantification is more commonly achieved with average label signals for LDI-based MSIAs. Lorey et al. [95] developed an array-type sandwich mass-tag analysis method for prostate specific antigen (PSA) using triphenylmethanethiol derivatives linked to antibodies as labels. This method obtained an LOD below 200 pg/mL for a 10 μL plasma sample. Du et al. [101] developed a sandwich immunoassay with PEG-thiol derivatives-AuNP-aptamers as labeling probe on MALDI plate (Fig. 2A). This method consumed 2 μL sample each time, achieving the LOD of 100 amol/L for thrombin.

Other strategies have been adopted to improve the quantification of LDI-MS immunoassays. Some extra steps are usually needed after typical immunoassays to achieve a satisfied LDI condition, such as eluting labels and adding internal standard. For example, preparing a homogeneous sample is useful to reduce the matrix and surface effect. Liu et al. [92] eluted the AuNP labels after immunoreaction, and loaded the homogeneous labels on LDI plate to achieve an average and uniform signals. Internal standardization is essential to achieve reliable signal intensity for quantification. Hong et al. [99] used PEG-thiol derivatives-AuNP-antibodies as the probe with deuterated alkanethiols as the internal standard for the quantification of the Bcr/Abl chimeric protein on biochips. Similar probes and internal standard have been further used for the quantification of PSA in serum [100]. Zhong et al. [102] employed two PEG-thiol derivatives-AuNP-antibodies probes for the simultaneous

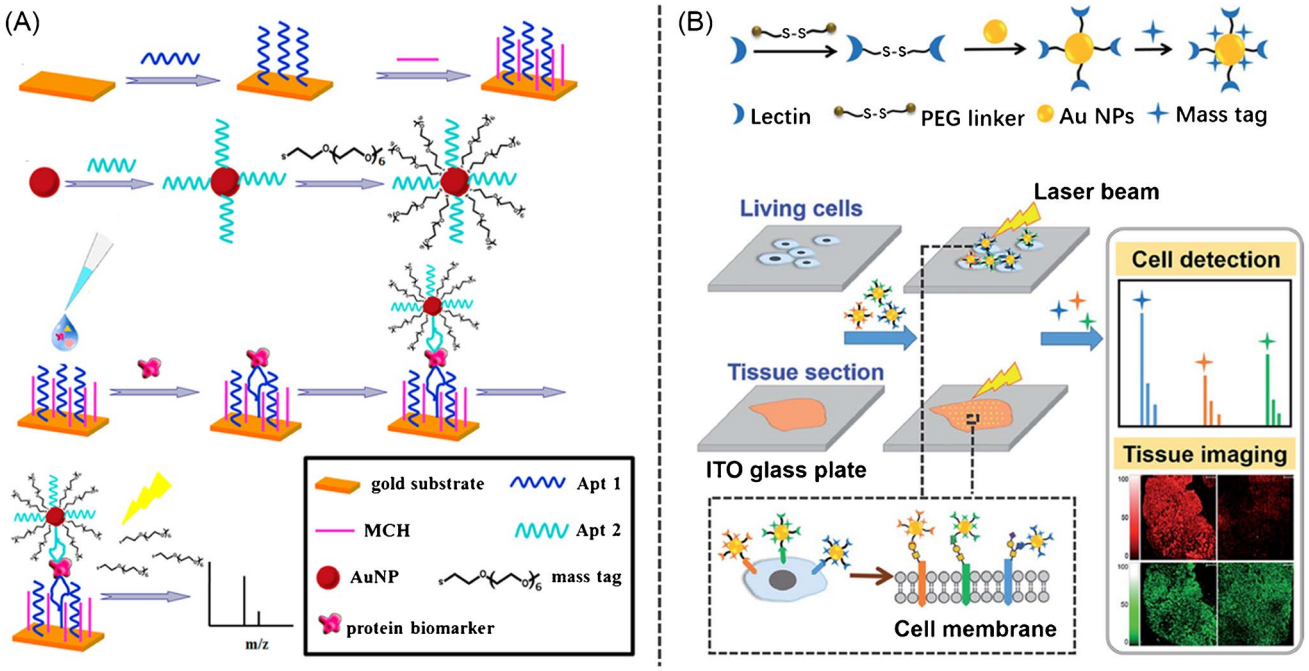

Fig. 2 Representative LDI-based MSIAs: **A** LDI-MS-based immunoassay of thrombin labeled with PEG-AuNPs. Adapted with permission from Ref [101], Copyright © 2016, American Chemical Society; **B** LDI-MS-based immunoassay for in situ multiplexed glycan detection and imaging using PEG-AuNPs as labels [105]

detection of two specific IgE Abs, achieving the LODs of pg/mL. They utilized immunomagnetic separation to obtain uniform immune complex after sandwich immunoreaction, and one more PEG-thiol derivatives-AuNP probe was deposited together with the sample on a MALDI-MS target as internal standard to reduce the errors from the fluctuation during LDI.

LDI-MS-based immunoimaging

High spatial resolution from the LDI-MS-based immunoassays makes it efficient for immunoimaging of tissues, as an alternative assay of immunohistochemistry. The continuous detection of a flat tissue section is needed to ensure the semi-quantification, decreasing the impact from complex biological matrix and inherent heterogeneous tissue surface and present the distribution of targets in a section. Comparison between sections needs to be achieved with internal standard loaded on the sections. Carboxypeptidase D protein in rat brain tissue section and serine hydrolase enzymes in mammalian tissue were imaged with peptides as labels [94, 103]. When using peptides as the labels, organic matrix, such as 3-hydroxypicolinic acid (3-HPA), are usually needed to promote desorption and ionization of labels in the tissue. However, the addition of organic matrix sometimes brings more heterogeneity and signal interference. Matrix-free imaging was then realized by small-molecule-AuNP labels using small size AuNPs as matrix, which can simplify the preparation processing for LDI-MS-based immunohistochemistry. Ma et al. [105] designed the PEG-thiol derivatives-AuNP-lectin, and demonstrated the AuNPs as excellent matrix in LDI-MS imaging (Fig. 2B). Three probes were prepared via a simple and generic synthetic route for multiplexed immunoimaging. Two probes were used for the simultaneous imaging of two glycans in human cancer and paracancerous tissues, whereas the rest one probe worked as internal standard.

Immune analysis is also helpful for the detection of antigens on cells. Baird et al. [96] used the peptide-particle probes for the immune detection of 690 circulating tumor cells isolated from a 10 mL blood sample. Adherent cells can be semi-quantified in situ. Wang et al. [106] developed three PEG-thiol derivatives-AuNP probes for the simultaneous detection of three antigens, including epithelial cell adhesion molecule (EpCAM), cytokeratin 19 (CK19), and mucin 1 protein (MUC-1) in cells. These probes have been demonstrated to reach zmol sensitivity for these targets, and the three antigens in single cells were relatively quantified by this method. During the LDI-based MSIAs, the LDI-MS presents an inherent ability for the simultaneous detection of endogenous molecules, which can hardly be detected via optical- or even ICP-MS-based immunoassays. Sun et al. [104] detected the cell surface sialoglycoconjugates in situ with triphenylmethanethiol derivatives as labels, achieving an LOD of 5 fmol. At the same time, metabolites related to sialoglycoconjugates including lipids were achieved, providing more information for the pharmacological mechanism study.

The sensitivity improvement of LDI-based MSIAs with small-molecular labels makes LDI-MS popular for high-throughput sandwich immunoassays and immunoimaging of tissues and cells. The PEG-thiol derivatives coated on AuNPs are efficient labels with high sensitivity, easy expansion, low nonspecific adsorption, and free of matrix, and the pg/mL sensitivity can be achieved by most of the LDI-based MSIAs for clinical applications. The LDI-based detection has a potential to obtain endogenous information simultaneously for complex biological research. However, the accuracy for quantification via LDI-MS needs further improvement, especially by reducing the impact from heterogeneous sample deposition and biological matrix.

Ambient MS immunoassays

For immunoassays based on either ICP-MS or LDI-MS, specialized and expensive instruments are necessary, and professional sample preparation is needed to take the good performance of MS analysis. Ambient MS, ionizing compounds in the open air with minimum or no additional sample preparation, has been rapidly developed to analyze complex samples with low matrix effect and high salt tolerance [111, 112]. Furthermore, some flexible ionization modes for ambient MS, such as desorption electrospray ionization (DESI), paper spray ionization (PSI), and chip spray ionization (CSI), greatly reduced the instrument cost and operational complexity. DESI-MS has been successfully used for the tissue imaging based on the lipids and amino acids [113]. PSI has been applied to directly detect drugs and metabolites in micro-volume biological samples, such as blood, urine, and saliva [114]. Compared to MALDI-MS, these ambient MS methods are suitable for the semi-quantification or sometimes quantification of small molecules.

The detection of small molecules after immunoreaction has been conducted with ambient MS by Joshi et al. [115]. As a proof-of-principle, deoxynivalenol was captured using a surface plasmon resonance gold chip containing monoclonal antibodies against the toxin, and then analyzed by direct spray MS of the biosensor chip. The molecules conjugated with antibodies through noncovalent interaction are easily dissociated during spray ionization processing. This method can be applied to any MS-amenable analyte with available antibodies, and many conjugated targets can be profiled simultaneously. Zhang and Wang et al. [116] developed the interfacing bio-affinity arrays to capture biomolecules via affinity interactions. These biomolecules were desorbed

and detected with nano-DESI-MS. This method enabled the high-throughput identification and quantification of peptides from biofluids in a label-free manner, including the peptides from tryptic digests of casein, cytchrome *c*, and horseradish peroxidase. However, large molecules, especially intact proteins, are still difficult to be detected directly with the LODs at μg/mL level by PSI-MS [15]. As a straightforward strategy, several organic small-molecular labels mostly with molecular weights less than 1000 have recently been expanded to the ambient MS methods to improve the sensitive of ambient MS-based immunoassays.

Small-molecular labels and dissociation techniques

Several definite requirements are specific for small-molecular labels to serve ambient MS-based methods. Structures that are highly competitive during ionization and easy to be expanded are preferred as labels. It is more important for labels to be efficiently dissociated during ambient ionization. Unlike the ICP or LDI with high energy, spray-based ambient ionization modes are lack of specific power to dissociate labels from the immune conjugations. Other label dissociation approaches need to be introduced as the key step for the ambient MS-based immunoassays.

Chen and Wan et al. [107] developed chemical cleavable ionic probes for immunoassays based on PSI-MS. These probes contained the ionic quaternary ammonium structure that provided high MS response, and pH-sensitive ester bond that could be rapidly hydrolyzed within seconds or minutes by the addition of NH_4OH solution. The isothiocyanate terminal of probes was covalently connected to the amino of antibodies. The hydrolyzed small molecules on paper were then detected by PSI-MS. Two homologue molecules were prepared for multiplexed detection.

Xu et al. [108] discovered a unique dissociation mechanism for Au–S bonds during electrospray ionization with no need of extra actions. The cleavage of Au–S bonds is preformed when electro-droplets sprayed to the AuNP surface, which may undergo chemically or electrochemically controlled processes during electrospray ionization. As a result, the dissociated small molecules are detected by MS. Based on this mechanism, the ideal thiol-AuNP combination labels for LDI-MS can be expended to spray-based MS, but the thiol structures need to be re-designed for spray-based ionization. Rhodamine-based mass tags (RMTs) coated on AuNPs were designed and prepared as immune labels. The RMTs contained the thiol group and undecyl chain that enabled their self-assembly on AuNPs and efficient dissociation during ionization. The ionic rhodamine derivative structures were specially designed for spray-based ionization to provide high sensitivity. A large signal amplification was achieved based on the large numbers of RMTs on one AuNP (material molar ratio of 5000:1). The rhodamine derivative structures were simply expanded, leading to six label series for multiplexed immunoassays. Chen et al. [110] prepared an immune probe by coating trimethylolpropane tris(3-mercaptopropionate) (TMTP) on AuNPs. Three spray solvent, including reductive, oxidative, and acid, were compared to obtain the optimized MS signal of labels, and the results indicated that the addition of appropriate amount of NH_4OH to the spray solvent improved the detection efficiency of this mass labels.

Spray-based MS for sandwich immunoassays

The sensitive and multiplexed sandwich immunoassays based on spray-based ambient MS have been demonstrated with the aforementioned small-molecular labels. Sandwich immunoreactions are prepared on simple substrates, such as paper, membrane, and glass chips, and labels were dissociated, ionized, and detected by PSI-MS, CSI-MS, etc. The ionization conducted under ambient environment using lab-made ionization sources might suffer from serious signal fluctuations. Good reproducibility of the lab-made sources and detection conditions is important to ensure the quantification analysis, and internal standard is needed on paper or chip along with the immune conjugations to improve the performance of quantification and semi-quantification.

Chen and Wan et al. [107] created a new PSI method for the stable and high-throughput on-surface MS detection with low interference (Fig. 3A). The paper used for the immunoreaction was touched to a second wax-printed paper substrate with matching hydrophilic strips. Dissociated tags on the upper paper layer were transferred to the bottom paper spray strips to form the spray and then detected by MS, which reduced the matrix interference and enabled the reproducible MS detection for quantification. Similar ionic molecule was added on the paper as internal standard, and specific fragments were generated with tandem MS to further reduce the background interference and improve the accuracy of qualitative signals. The intensity ratio of tag fragment and internal standard fragment was employed for the quantification of P*f*HRP2 antigen, achieving the LOD of 2.8 ng/mL for serum sample diagnosis. CA-125 and CEA in one spot were detected simultaneously by this method. Xu et al. [108] established an array-type CSI setup for immunoassays (Fig. 3B). Internal standard added to the spray solvent and the tandem MS was both employed for accurate quantification. Since large amplification was achieved by RMTs-AuNP probes, this immunoassay achieved an LOD of 2.5 pg/mL for thrombin spiked in 2 μL serum. Three labels were employed for the simultaneous immune detection of three antigens, including CA125, CEA, and EpCAM. They further expanded six RMTs, and developed the one-step hexaplex immunoassays on paper substrate for the simultaneous quantification

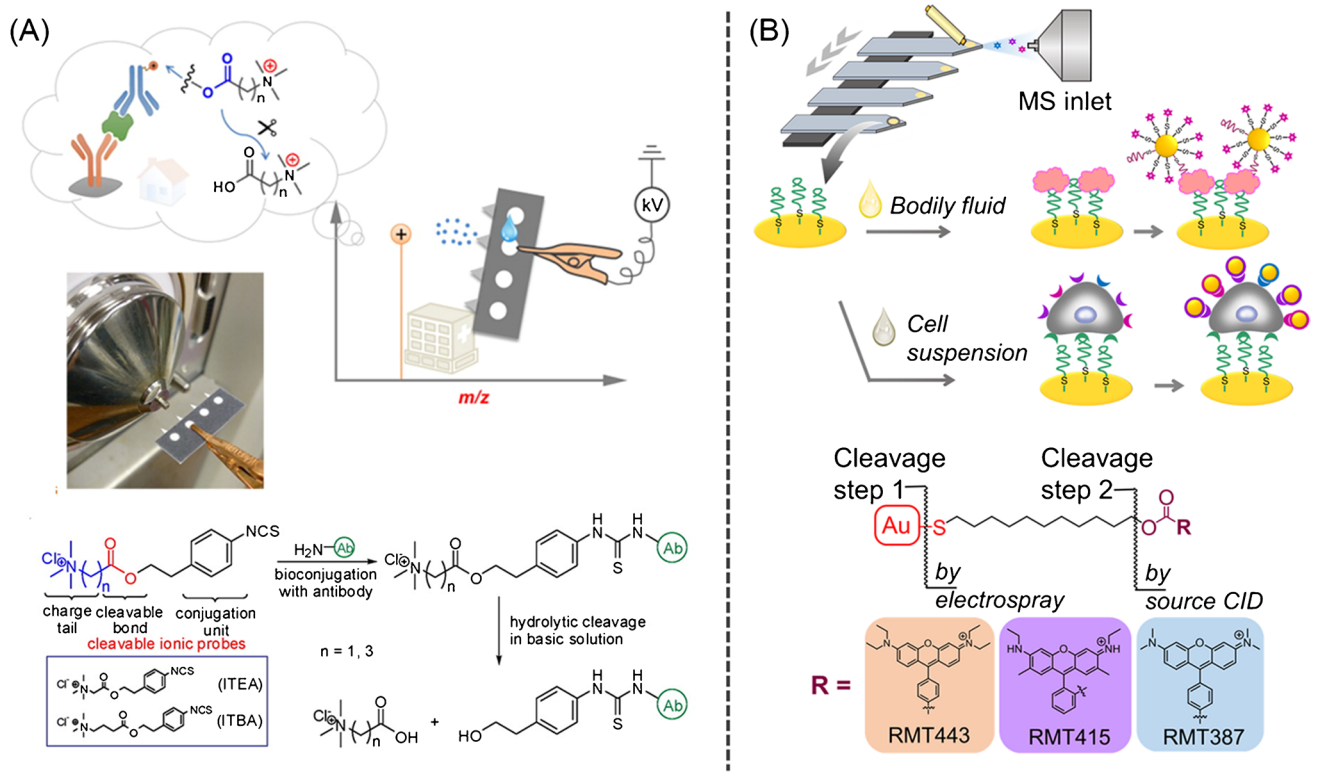

Fig. 3 Representative ambient MS-based immunoassays: **A** paper-MS-based immunoassays with chemical cleavable quaternary ammonium probes. Adapted with permission from Ref [107], Copyright © 2016, American Chemical Society; **B** chip spray-MS-based immunoassays for sensitive and multiplexed protein biomarkers using rhodamine-based mass tags-AuNPs as labels. Adapted with permission from Ref [108], Copyright © 2019, American Chemical Society

of six antigens in serum, including CA15-3, CA19-9, CEA, CA125, human epididymis protein 4 (HE4), and alpha fetoprotein (AFP) [109]. Chen et al. [110] developed a lab-on-membrane immunoassay platform combined with PSI-MS for the detection of PSA. Besides the signal amplification by labels, the biotin-streptavidin scaffold in this system caused an increase in the capturing efficiency of PSA by a factor of five. PSA was quantified with an LOD of 3.0 pg/mL, and of course internal standard was added to help the quantification.

Immunoassays based on spray-based ambient MS and small-molecular labels are under the early stage of development, and high potential of these methods for sensitive and multiplexed immunoassays has been preliminarily verified by recent works. The sensitivity at pg/mL level with only micro-volume sample is sufficient for clinical applications. An obvious advantage of these ambient MS-based immunoassays is the requirement reduction of specific MS instrument by using paper or glass chip as ionization source. Using small molecules as mass labels, the instrument will be further simplified when coupling the paper ionization sources with hand-held mass spectrometers. The simplification of the MS instrument facilitates the future applications of multiplexed ambient MS-based immunoassays for clinical use, and more small-molecule labels and more mature methodology are still needed as technical support.

Future clinical applications

Immunoassay is one of the most important clinical techniques for biomarker detection and tissue imaging in disease/pathological diagnosis. Although MS-based immunoassays are mostly at the stage of laboratory research, they show great potential for the efficient, accurate, and in-depth clinical analysis. Various MS-based immunoassays with attractive advantages (summarized in Table 3) are capable of the basic clinical applications, such as the biomarker screening and immunohistochemistry, as well as the cutting-edge clinical research based on single cells.

Multiplexed biomarker screening

Biomarker screening is of great importance in the diagnosis of cancer and other diseases, because it is an efficient way to reflect the occurrence and development of diseases

Table 3 Summary of immunoassay performance based on different MS instruments

MS instruments	Detectable singles in label	Advantages	Disadvantages	Applications
Integral ICP-MS	Element	Absolute quantification; multiplexed detection (12-plex); high sensitivity (pg/mL–ng/mL); commercial labeling kit	High cost instrument; high cost reagent	Sandwich immunoassay; competitive immunoassay
SP-ICP-MS	Element	Accurate quantification; multiplexed detection (> 40-plex); high sensitivity (pg/mL–ng/mL); single particle resolution	High cost instrument; high cost reagent	Sandwich immunoassay; competitive immunoassay; homogeneous immunoassay; single-cell analysis
LA-ICP-MS	Element	Accurate quantification; multiplexed detection (> 40-plex); high sensitivity (< ng/mL); spatial information	High cost instrument; high cost reagent	Immunohistochemistry; sandwich immunoassay; competitive immunoassay
LDI-MS	Element/small molecule	Multiplexed detection (> 3-plex); high sensitivity (~ pg/mL); spatial information; endogenous information	High cost instrument; limited quantification accuracy	Immunohistochemistry; sandwich immunoassay; competitive immunoassay
Paper/chip spray MS	Small molecule	Multiplexed detection (> 6-plex); high sensitivity (pg/mL–ng/mL); simplified instrument; endogenous information	Limited quantification accuracy; limited stability of lab-made devices	Sandwich immunoassay; competitive immunoassay; POCT

and monitor the response to treatment. It has been more and more popularized in regular physical exams. MSIAs provide the possibility for multiplexed quantification of biomarkers with micro-volume sample, making the clinical diagnosis more patient-friendly and more efficient for broad disease screening and accurate diagnosis. The standard evaluation and clinical sample verification are ongoing for many MSIAs, especially for the mature ICP-based MSIAs. Jiang and sun et al. [117] evaluated the ICP-MS-based duplex immunoassay for CEA and AFP with $Eu3^+$/$Sm3^+$-labeled antibodies according to guidelines from the Clinical and Laboratory Standards Institute, and compared their method with ECLIA. The measurement range, sensitivity, inter-assay and intra-assay imprecision, and correlation with ECLIA of the ICP-based assay all meet the requirements for clinical analysis based on the results of 329 clinical samples. A 12-plex ICP-based MSIA has been developed and validated by comparing with time-resolved FIA [67]. The ICP-based MSIAs are potential for personalized medicine based on accurate diagnostic of a broad series of biomarkers.

The promising ambient MS-based immunoassays can be conducted with simplified and flexible instruments, which have been demonstrated to be potential methods for rapid biomarker screening and point-of-care testing (POCT). Clinical application has been preliminarily verified for an ambient paper-based MSIAs and small-molecular labels with 12 clinical samples, and good correlations have been achieved between the ambient MSIA with the clinical ECLIA [109].

Tissue imaging

Immunohistochemistry is the gold standard for histopathology examination, and the LA-based MSIAs, including LA-ICP-MS and LDI-MS, are powerful techniques for immunohistochemistry. Since the multiplexed imaging ability and the relative high analysis cost, LA-based MSIAs are applied for the basic medical research of many complex tissue environments more than the rapid diagnosis. Representative tissue samples, such as breast cancer sections [86, 118], pancreas sections [119, 120], and human central nervous system sections [121], have been analyzed based on the LA-ICP-MS-based imaging (IMC technique) with polymer-based element labels. A 40-marker panel for high dimensional characterization of tissue composition and microenvironment has been easily achieved and measurement of over 100 markers will be possible [89]. The IMC enables the basic studies of tissue heterogeneity, and supports the transition of medicine towards individualized molecularly targeted diagnosis and therapies. The labeling specificity and precision imaging measurement of IMC have been demonstrated after compared to the fluorescence-based IHC [86]. LDI-MS-based imaging with small-molecular labels has also been applied to the imaging of colorectal cancer sections to

analyze the cancer and paracancerous heterogeneity [105]. Based on the technique of LDI-MS imaging under ambient pressure, live tissues are observed close to their real physiological state.

Single-cell screening

Single-cell analysis provides the heterogeneous biological information of individual cells among populations. Based on several MSIAs that have the sensitivity at sub-amol level, antigens in single cells can be labeled and semi-quantified for basic medical research, such as cell lineage reconstruction and tumor immune microenvironment profiling, and clinical diagnosis [76]. The commercial mass cytometry instrument and polymer-based element labels enabled the quantification of more than 60 protein parameters on single cells with a throughput of ~ 300 cells/s. It has been applied for cell population identification, cellular behavior characterization (signaling pathways or dynamic changes), and cellular progression and diversity analysis for various cell types, especially myeloid and lymphoid cells.

Recently, the highly sensitive RMT-based tags have been expanded for the single-cell immunoassay. Six cell surface antigens have been successfully semi-quantified for single cancer cells, which was applied for cell identification and typing [122]. Furthermore, the organic MS-based single-cell technique presents its unique advantage for the simultaneous of antigens and metabolites in single cells. The cell typing with combined antigen and metabolite information has been demonstrated with higher sensitivity and specificity, and it is potential for the analysis of signaling pathways in single cells for the study of pathogenic mechanism.

Conclusions and perspectives

MSIAs take the advantages of high sensitivity (mostly pg/mL level), multiplexed detection (theoretically more than 100-plex), and accurate quantification or semi-quantification. Various immune labels, from element labels to small-molecular labels, have been coupled to readout instruments from element MS to organic MS. The performance of MSIAs for biomarker screening, tissue imaging, and single-cell analysis has been demonstrated and validated following the standard evaluation guidance for the final clinical use. These MSIAs based on different technology and methodology present a division of labor for clinical analysis, thus the ICP/LDI-MS-based methods for basic medical research and the ambient MS-based methods for general and rapid clinical diagnosis. In our opinions, the MSIAs could be promoted in the following areas for clinical applications:

(i) ICP-based MSIAs have relative mature technology and methodology, and the commercial labeling kits, specialized MS instruments, and standard operating procedures have been developed for a long time. Clinical validation is accelerating to promote these MSIA techniques for clinical application. Higher sensitivity and throughput methods are pursued for more advanced clinical use, such as the antigen monitoring in single exosomes and synaptosomes.

(ii) Small-molecular labels are still at the early stage, and more types of labels, standard and stable preparation protocols are required to be developed and validated. New labels can be designed based on abundant MS enhancement structures, such as pyridine, dimethylamino, phenylamine, and fluothane [123]. Labels can be expanded based on homologous series and stable isotope labeling. The reproducibility and uniformity of small-molecular labels need to be improved, and clear stoichiometric relationships need to be established between labels with targets to improve the quantification of targets by LDI-MS and ambient MS detection. Ideally, commercial small-molecular labeling kits are produced and employed for the MSIAs.

(iii) For ambient MS-based immunoassays, besides the improvement of small-molecular labels, the combination of ambient ionization sources with miniaturized MS should be evaluated to establish a standard immunoassay protocol. Furthermore, the typical immunoassay process usually takes nearly hours for immunoreaction, so small-molecular labels and related immunoassays aim to be simple, rapid, and efficient for the rapid biomarker screening for POCT.

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Declarations

Conflict of interest The authors declare no competing interests.

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