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Cite as: APL Mater. 5, 053403 (2017); https://doi.org/10.1063/1.4976020
Submitted: 29 November 2016 . Accepted: 19 January 2017 . Published Online: 14 February 2017

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Pulse laser-induced generation of cluster codes from metal nanoparticles for immunoassay applications

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(Received 29 November 2016; accepted 19 January 2017; published online 14 February 2017)

In this work, we have developed an assay for the detection of proteins by functionalized nanomaterials coupled with laser-induced desorption/ionization mass spectrometry (LDI-MS) by monitoring the generation of metal cluster ions. We achieved selective detection of three proteins [thrombin, vascular endothelial growth factor-A165 (VEGF-A165), and platelet-derived growth factor-BB (PDGF-BB)] by modifying nanoparticles (NPs) of three different metals (Au, Ag, and Pt) with the corresponding aptamer or antibody in one assay. The Au, Ag, and Pt acted as metal bio-codes for the analysis of thrombin, VEGF-A165, and PDGF-BB, respectively, and a microporous cellulose acetate membrane (CAM) served as a medium for an in situ separation of target protein-bound and -unbound NPs. The functionalized metal nanoparticles bound to their specific proteins were subjected to LDI-MS on the CAM. The functional nanoparticles/CAM system can function as a signal transducer and amplifier by transforming the protein concentration into an intense metal cluster ion signal during LDI-MS analysis. This system can selectively detect proteins at picomolar concentrations. Most importantly, the system has great potential for the detection of multiple proteins without any pre-concentration, separation, or purification process because LDI-MS coupled with CAM effectively removes all signals except for those from the metal cluster ions. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). [http://dx.doi.org/10.1063/1.4976020]

The greatest challenge in the detection of specific proteins or tumor markers for the diagnosis of cancer is their low concentrations in human plasma.1,2 Interferences due to other proteins with similar properties also cause difficulties in their selective detection by conventional methods.2 Therefore, to achieve high selectivity for the identification of specific proteins in biological fluids, such as plasma, immunoassays based on aptamer (Apt)-protein-specific and antigen-antibody (Ab)-specific interactions are widely used in both clinical and medical research.3 Currently, the enzyme-linked immunosorbent assay (ELISA) has demonstrated reasonable sensitivity and specificity; however, it fails in the analysis of multiple proteins in a single well, which limits its application.4 Matrix-assisted laser-induced desorption/ionization (MALDI) time-of-flight mass spectrometry (MS) is an effective tool for identifying biomacromolecules such as proteins, nucleic acids, and polysaccharides with great selectivity.5 However, non-uniform matrix-analyte co-crystallization and interference signals from organic matrices significantly decrease the reproducibility and sensitivity of this method.6 Recent reports show that surface-assisted laser-induced desorption/ionization mass
Spectrometry (SALDI-MS) using nanomaterials, such as a matrix (substrate), can improve reproducibility and reduce matrix interference.\(^7\)\(^-\)\(^10\) SALDI-MS has been successfully used in the analysis of a wide variety of analytes such as proteins, DNA, microbes, and tumor cells. However, in the case of multiple-target analysis and quantitation by SALDI-MS, the fragmentation of several analytes, background molecules, and their adducts is unpredictable. Thus, the comprehensive detection of complex samples such as plasma containing complicated proteins, small molecules, and salts, is difficult. To resolve the interference from background proteins and the unpredictable fragmentation of analytes encountered in SALDI-MS, we have developed a simple immunoassay that exhibits significant potential for the simultaneous detection of different proteins in a single analysis by monitoring the cluster ion signals generated from the metal nanoparticles (NPs) under pulse laser irradiation.

In this work, instead of observing the various intact or fragmented protein ions, we measure the metal-codes (specific metal cluster ions) from the NPs itself for the quantitative detection of the three analytes: thrombin, vascular endothelial growth factor-A\(_{165}\) (VEGF-A\(_{165}\)), and platelet-derived growth factor-BB (PDGF-BB). These proteins play critical roles in angiogenesis and tumor progression.\(^1\)\(^-\)\(^3\) Thrombin promotes angiogenesis by activating PAR1 receptors in platelet and endothelial cells.\(^1\) VEGF and PDGF are signal proteins, which are highly expressed by tumor cells to stimulate tumor angiogenesis and vascular remodeling by binding to specific receptors on endothelial cells.\(^1\)\(^2\)\(^,\)\(^3\) Therefore, the determination of the concentration of these three cytokines in plasma and in tumor environments is very important for the diagnosis of tumor growth and metastasis.\(^1\)\(^-\)\(^3\)

The functionalization of different metal NPs with their respective aptamers or antibodies enables specific targeting of thrombin, VEGF-A\(_{165}\), and PDGF-BB (Fig. 1). Here, three metal (Au, Ag, and Pt) NPs are used as mass tags for the proteins, rather than a SALDI matrix, to enhance the ionization of the analyte molecules. The metal NPs absorb pulsed laser energy and undergo photothermal evaporation and/or Coulombic explosion, which produces a substantial amount of cluster ions useful for signal amplification.\(^4\)\(^-\)\(^7\) We used cellulose acetate membrane (CAM), which can be directly mounted onto a plate for LDI-MS analysis, to serve as a medium (substrate) to separate the antibody (Ab)- or aptamer (Apt)-modified NPs and their conjugates formed with their targeting proteins \textit{in situ}. This assay does not require any additional processing steps such as separation, preconcentration, or washing. Because of the increased particle weight or decreased affinity towards CAM upon interaction with the target proteins, target-bound nanoparticles penetrate deeper into the CAM. Consistent with our hypothesis, the intensity of metal cluster ions (\([M_n]^+; M = \text{Au and Ag}, n = 1–3\)) is observed to decrease with increasing concentration of target protein. Platinum ions ([Pt]\(^+\)) exhibit the opposite trend: their intensity increases as the concentration of target protein increases. The target protein-induced aggregation of Pt NPs leads to the deposition of NPs onto the upper layer of the CAM.

Details of the syntheses of Au NPs, Ag NPs, and Pt NPs are given in the experimental section of the supplementary material. The transmission electron microscopy (TEM) images of the metal NPs

**FIG. 1.** Schematic graphical representation of the strategy for the fabrication and detection of a nanoparticle-based probe for the simultaneous detection of thrombin, VEGF-A\(_{165}\), and PDGF-BB by LDI-MS.
indicate that the as-prepared Au NPs, Ag NPs, and Pt NPs have average particle sizes of ~13, ~26, and ~24 nm, respectively (Fig. S1, supplementary material). The UV-Vis absorption and X-ray diffraction (XRD) spectra show the surface plasmon resonance band and crystal structures, respectively, which further confirm the formation of corresponding metal nanoparticles (Fig. S2, supplementary material). From dynamic light scattering (DLS) measurements (Fig. S3, supplementary material), the increased hydrodynamic size (~20 nm) of the NPs observed after aptamer- or antibody-modification supports that the aptamer or antibody ligands are anchored on the NPs’ surfaces. To demonstrate our detection strategy, the fabrication of a probe [aptamer-modified gold nanoparticles (Apt-Au NPs)] for the detection of thrombin is described in detail. To achieve specificity, Au NPs were functionalized with two types of thiold-modified thrombin-binding aptamers (TBAs): a 15-base-long aptamer (TBA15), which interacts with exosite I of thrombin, and a 29-base-long aptamer (TBA29), which binds with exosite II of thrombin. TBA15/TBA29-modified Au NPs (TBA15/TBA29-Au NPs) have been demonstrated to have multivalent interactions with thrombin with an ultra-strong binding affinity (dissociation constant ($K_d$) of ~$10^{-11}$ M) in our previous study. The average number of TBA molecules on the surface of membranes was counted and found to be approximately a factor of 2 lower for TBA15/TBA29-Au NPs thrombin on CAM are shown in Fig. S6 (supplementary material). The average number of TBA molecules on the surface of membranes was counted and found to be approximately a factor of 2 lower for TBA15/TBA29-Au NPs thrombin on CAM are shown in Fig. S6 (supplementary material). The decrease in the zeta potential of TBA15/TBA29-Au NPs is insensitive to their surface properties. As the thrombin concentration increased, the TBA15/TBA29-Au NPs bound more thrombin. As a result, the heavier TBA15/TBA29-Au NPs/thrombin conjugates
FIG. 2. Detection of thrombin by TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM coupled with LDI-MS. (a) Mass spectra of TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM as a probe for (A) 0 nM, (B) 0.01 nM, (C) 0.1 nM, (D) 1.0 nM, and (E) 10 nM thrombin in physiological buffer in the presence of 100 µM BSA. (b) MS signal intensity changes of [Au\textsubscript{1}]\textsuperscript{+} ions (I\textsubscript{Au} - I\textsubscript{Au0}) plotted as a function of the concentration of thrombin. I\textsubscript{Au0} and I\textsubscript{Au} represent the signal intensities of [Au\textsubscript{1}]\textsuperscript{+} in the absence and presence of thrombin. Signals at m/z 196.97, 393.93, and 590.90 are assigned to [Au\textsubscript{1}]\textsuperscript{+}, [Au\textsubscript{2}]\textsuperscript{+}, and [Au\textsubscript{3}]\textsuperscript{+} ions, respectively. A total of 1000 pulsed laser shots were applied to accumulate the signals from five LDI-targeted positions at a laser power density of 3.8 \times 10\textsuperscript{4} W cm\textsuperscript{-2}. The error bars represent the values obtained from three experiments.

penetrated faster into the CAM. Therefore, as the concentration of thrombin increased, the number of TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs on the surface of the CAM decreased. Additionally, thrombin bound to the surfaces of TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs may compromise the interaction between the highly dense TBA ligands and the cellulose acetate fiber and may also contribute to its faster migration (penetration). Substituting CAM with nitrocellulose membrane (NCM) and mixed cellulose ester membrane [MCEM; composed of cellulose nitrate (80%) and cellulose acetate (20%)] produced similar results for the sensing of thrombin (Fig. S7, supplementary material), whereas a positively charged nylon membrane (N\textsuperscript{+}M) did not work in this system. The porous N\textsuperscript{+}M has a high binding capacity for nucleic acids—as high as to 600 µg cm\textsuperscript{-2}—because of its high number of positively charged quaternary ammonium groups. Thus, the TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs cannot easily penetrate into the pores because of the strong electrostatic interaction between the N\textsuperscript{+}M fiber and the nanoparticles.

Compared to the noisy spectra typically obtained when using nanoparticle-assisted LDI-MS\textsuperscript{7–10} or our previously described TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs (Fig. S4(a)), the MS spectra of the TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM system are very clean (Fig. 2(a)), presumably because the negatively charged porous cellulose acetate fiber effectively binds the interfering cationic molecules produced under LDI.\textsuperscript{21} We cannot rule out the possibility that the clean MS spectra are due to small molecules and salt ions depositing not on the top but rather on the bottom of the CAM. In addition, the relative standard deviation (RSD) of the MS signals of [Au\textsubscript{1}]\textsuperscript{+} obtained from the same TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM substrate, collected from 50 different mass spectra, was less than 10%, revealing a high homogeneity of nanoparticle distribution on the CAM. In our previous study, we have demonstrated that the microporous membrane is an ideal substrate for homogenous deposition of Au NPs.\textsuperscript{24} We conducted control experiments under similar conditions; however, instead used a random oligonucleotide (base number same as TBA\textsubscript{29})-capped Au NPs for the analysis of thrombin. As expected, the addition of thrombin (10 nM) did not induce any substantial changes in the signals of the [Au\textsubscript{n}]\textsuperscript{+} cluster ions (Fig. S8, supplementary material). We also evaluated the selectivity of the TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM as an LDI-MS substrate for the analysis of various proteins (10 nM for thrombin, 1.0 µM for each of the other proteins) in the presence of BSA (100 µM). A plot of the relative signal intensity changes of the [Au\textsubscript{n-1}]\textsuperscript{+} revealed that this system was highly selective (1000-fold or more) toward thrombin over the other proteins (Fig. S9, supplementary material). Our TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM coupled with LDI-MS allows for detection of thrombin at concentrations as low as 10 pM (Fig. 2(b)) in the presence of 100 µM BSA (i.e., a 1 \times 10\textsuperscript{7}-fold higher concentration), further demonstrating the system’s high selectivity. The high selectivity of the TBA\textsubscript{15}/TBA\textsubscript{29}-Au NPs/CAM probe is due to the high specificity and strong binding between TBAs and thrombin. Moreover, background proteins
FIG. 3. Detection of VEGF-A165 by AbVEGF-Ag NPs/CAM coupled with LDI-MS. (a) Mass spectra of AbVEGF-Ag NPs/CAM as a probe for the detection of (A) 0 nM, (B) 0.01 nM, (C) 0.1 nM, (D) 1.0 nM, and (E) 10 nM VEGF-A165 in physiological buffer solutions in the presence of 100 µM BSA. (b) MS signal intensity changes of [Ag1+] ions \((I_{Ag^+}) - I_{Ag^+})\) plotted with respect to the concentration of VEGF-A165. (c) Selectivity of the AbVEGF-Ag NPs/CAM substrate coupled with LDI-MS measurements of various proteins. The concentrations for VEGF-A165 and all other proteins were 10 nM and 1.0 µM each, respectively. \(I_{Ag^+0}\) and \(I_{Ag^+}\) represent the signal intensities of \([Ag_{1-3}]^+\) in the absence and presence, respectively, of VEGF-A165 or other proteins. Other conditions were the same as those described in Figure 2. 

are bound by CAM, resulting in low interferences. In comparison with other methods (Table S1, supplementary material), our sensing platform for thrombin is relatively simple, rapid, and sensitive. Note that most other methods require tedious labeling of nanoparticles and complicated separation, preconcentration, and/or washing processes during sensing.

We further used our LDI-MS-based sensing system to detect other proangiogenic factors. Ag NPs and Pt NPs were modified with VEGF-A165 antibody (AbVEGF) and PDGF-BB antibody (AbPDGF) to form functional AbVEGF-Ag NPs and AbPDGF-Pt NPs for the detection of VEGF-A165 and PDGF-BB, respectively (details regarding the preparation of antibody-Ag or -Pt NPs are included in the experimental section of the supplementary material). Flocculation assay studies suggest that the average numbers of antibodies modified per Ag NP and Pt NP are approximately 70 and 80 molecules (data not shown), respectively (see details in supplementary material). Both the as-prepared AbVEGF-Ag NPs and AbPDGF-Pt NPs are stable (no aggregation) in a physiological solution containing 100 µM BSA. Similarly, AbVEGF-Ag NPs/CAM and AbPDGF-Pt NPs/CAM coupled with LDI-MS exhibit high sensitivity [limits of detection (LODs) of approximately 5 and 50 pM (based on a signal-to-noise (S/N) ratio of 3), respectively] and selectivity (>1000-fold relative to other proteins) toward their target proteins, VEGF-A165, and PDGF-BB (Fig. 3 and Fig. 4), respectively. The homodimeric characteristic of PDGF-BB induces significant crosslinking aggregation of AbPDGF-Pt NPs (Fig. S10, supplementary material). As a result, the aggregated Pt NPs cannot penetrate into the porous CAM and instead on the surface of the CAM (Fig. S11, supplementary material). Therefore, the mass signal

FIG. 4. Detection of PDGF-BB by AbPDGF-Pt NPs/CAM coupled with LDI-MS. (a) Mass spectra of AbPDGF-Pt NPs/CAM as a probe for (A) 0 nM, (B) 0.01 nM, (C) 0.1 nM, (D) 1.0 nM, and (E) 10 nM PDGF-BB in physiological buffer solutions in the presence of 100 µM BSA. (b) MS signal intensity changes of [Pt1+] ions \((I_{Pt^+}) - I_{Pt^+0})\) plotted with respect to the concentration of PDGF-BB. (c) Selectivity of the AbVEGF-Pt NPs/CAM substrate coupled with LDI-MS measurements of various proteins. The concentrations of PDGF-BB and other proteins were 10 nM and 1.0 µM each, respectively. \(I_{Pt^+0}\) and \(I_{Pt^+}\) represent the signal intensities of \([Pt_{1-3}]^+\) in the absence and presence, respectively, of PDGF-BB or other proteins. Other conditions were the same as those described in Figure 2.
FIG. 5. Simultaneous detection of thrombin and VEGF-A$_{165}$ by functional NPs/CAM coupled with LDI-MS analysis. (a) Mass spectra of TBA$_{15}$/TBA$_{29}$-Au NPs and Ab-VEGF-Ag NPs coupled with CAM as probes in the (A) absence and ((B)–(D)) presence of (B) thrombin (100 nM), (C) VEGF-A$_{165}$ (100 nM), (D) thrombin (100 nM), and VEGF-A$_{165}$ (100 nM) in physiological buffer solution containing 100 µM BSA. (b) Relative MS signal intensities ($I_{M+0}/I_{M+}$) of $[M_1]^+$ ($M = $ Au or Ag) with respect to the protein samples. $I_{M+0}$ and $I_{M+}$ represent the signal intensities of $[M_1]^+$ in the absence and presence of thrombin or VEGF-A$_{165}$. Other conditions were the same as those described in Fig. 2.

of $[\text{Pt}_1]^+$ ions increases upon increasing the concentration of PDGF-BB (Fig. 4(a)). The LOD of the detection of PDGF-BB (50 pM) is relatively higher than that of VEGF-A$_{165}$ (5 pM) and thrombin (10 pM), mainly because it fails to induce large degree of aggregation of Ab-PDGF-Pt NPs, at very low concentrations.

By measuring their respective metallic cluster ions ($[\text{Au}_1]^+$ or $[\text{Ag}_1]^+$), we also demonstrated that TBA$_{15}$/TBA$_{29}$-Au NP and Ab-VEGF-Ag NP probes enable the selective simultaneous detection of thrombin and VEGF-A$_{165}$. As shown in Fig. 5, the intensities of the signals of the metallic cluster ions ($[\text{Au}_1]^+$ and $[\text{Ag}_1]^+$) decreased when only their corresponding protein was present. This result demonstrates that the system we developed is superior to ELISA for the detection of multiple proteins in a single assay. We also attempted to use the three NP probes for simultaneously detecting thrombin, VEGF-A$_{165}$, and PDGF-BB (Fig. S12, supplementary material). Unfortunately, the TBA$_{15}$/TBA$_{29}$-Au NP and Ab-VEGF-Ag NP probes exhibit nonspecific binding to PDGF-BB. TBA$_{15}$/TBA$_{29}$-Au NPs interact with PDGF-BB probably due to the strong electrostatic interaction between the highly dense, negatively charged aptamer ligands on Au NPs, and the positively charged PDGF-BB (isoelectric point $\sim 9.8$). On the other hand, similarity in protein structure (subfamily) of VEGF and PDGF may also result in cross talk of their antibody-modified NPs. 27

To test the practicality of the newly developed sensing system, we analyzed proangiogenic factors (thrombin, VEGF-A$_{165}$, and PDGF-BB) in human plasma. The relative signals of the metal cluster ions of $[\text{Ag}_1]^+$ and $[\text{Pt}_1]^+$ increased linearly with increasing concentrations of the spiked VEGF-A$_{165}$ and PDGF-BB, respectively (Figs. 6(b) and 6(c)). The LODs ($S/N = 3$) for VEGF-A$_{165}$ and PDGF-BB in plasma are approximately 25 and 200 pM, respectively. The recoveries for the spiked VEGF-A$_{165}$ and PDGF-BB are determined to be 94%–106% and 95%–109%, respectively. Even

FIG. 6. Validation of the use of functional NPs/CAM coupled with LDI-MS for the detection of proteins in plasma samples. Relative signal intensities of (a) $[\text{Au}_1]^+$ ions ($[I_{\text{Au}+0} - I_{\text{Au}+}] / I_{\text{Au}+}]$; (b) $[\text{Ag}_1]^+$ ions ($[I_{\text{Ag}+0} - I_{\text{Ag}+}] / I_{\text{Ag}+}]$; and (c) $[\text{Pt}_1]^+$ ions ($[I_{\text{Pt}+} - I_{\text{Pt}+}] / I_{\text{Pt}+}$) plotted with respect to the concentrations of (a) thrombin, (b) VEGF-A$_{165}$, and (c) PDGF-BB spiked in 10-fold-diluted plasma samples. Other conditions were the same as those described in Fig. 4.
though our proposed approach appears to be applicable to the practical screening of proangiogenic factors in complex biological samples, our assay unfortunately failed to detect thrombin in the plasma (Fig. 6(a)), primarily because of the nonspecific binding between basic proteins in the plasma and the original surface properties of the Au NPs being modified by TBA ligand. Antibody-modified Au NPs may function as an alternative to TBA in the future to improve the specificity for the detection of thrombin in plasma.

In summary, we have demonstrated a simple nanomaterial-assisted method using LDI-MS coupled with CAM-mediated separation for the detection of proteins. The CAM employed in this study not only acts as a separation matrix but also suppresses the fragmentation of ligands functionalized on the metal NPs and target proteins, which leads to a clean mass spectrum, especially in the low-molecular-weight region. Monitoring of the MS signal of metal cluster ions from metal NPs in LDI-MS provides greater sensitivity relative to that of intact proteins or surface ligands because of the poor ionization efficiency and easy fragmentation of proteins and surface ligands.28–32 Furthermore, monitoring the changes in the cluster ions’ intensity of metal bio-codes enables the quantification of different proteins using a single assay. We hope the principles applied in this work offer a new direction for the development of multiplex immunoassays.

See supplementary material for additional information (experimental section of materials and LDI-MS, Table S1, and Figures S1–S12) which is noted in the text. This material is available free of charge via the Internet at http://dx.doi.org/XXXX.

This study was supported by the Ministry of Science and Technology of Taiwan under the Contract Nos. 104-2628-M-019-001-MY3, 104-2622-M-019-001-CC2, and 103-2627-M-007-002-MY3. The assistance of Ms. Ya-Yun Yang and Ms. Ching-Yen Lin from the Instrument Center of National Taiwan University (NTU) for TEM and SEM measurements is appreciated. The authors declare no competing financial interest.

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