Aptamer Microarray

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# Development of a MALDI-TOF MS Strategy for the High-Throughput Analysis of Biomarkers: On-Target Aptamer Immobilization and Laser-Accelerated Proteolysis**

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Protein biomarkers are a group of macromolecules that provide important information about the pathological state of certain diseases. The detection of biomarkers can promote early diagnosis, monitor disease progression, and facilitate follow-up therapy.[1] However, many protein biomarkers of clinical currency are present at very low concentration in body fluids and the detection is very difficult.[2] Antibody-based immunoassays are currently the most popular method for the detection of biomarkers, because antibodies can extract target proteins from complex background with high specificity.[3] Recently, nucleic acid aptamers have emerged as a new class of affinity-based agents that rival antibodies in diagnostics.[4] Compared with antibodies, aptamers offer great advantages, which make them very promising in analytical and diagnostic applications.[5] First and most importantly, aptamers can be chemically synthesized with ease and accuracy at low cost. Second, aptamers have much wider target binding range from small molecules[6] to large proteins[7] or even whole cells.[8] Thirdly, the affinity is tuneable through manipulation of the selection process. Moreover, aptamers are more stable and easier to modify chemically compared with antibodies.[9]

MALDI-TOF MS is now widely applied to the analysis of biomolecules because of its high mass resolution, excellent sensitivity, high speed, and simplicity.[10] Since the first report by Brockman and Olando in 1995,[11] the modification of MALDI-TOF MS target plates with specific solid phases and antibodies, the so-called on-target immunoaffinity MALDI-TOF MS strategy,[12] has attracted much attention as a new approach for the selective detection of target proteins and peptides.[13] Using MALDI-TOF MS as the readout system has unique benefits over other methods (including ELISA): 1) the identity of the antigen can be confirmed by MS and MS/MS analysis, 2) antigenic protein species, caused by different isoforms or posttranslational modifications, can be differentiated, 3) MS allows the set-up of multiplexed assays and facilitates the analysis of up to hundreds of molecules simultaneously in a high-throughput manner.[12] However, because antibodies will be digested together with the captured target proteins, most of the reports are limited to the analysis of peptide targets,[14] or epitope-containing peptides from predigested target proteins.[15] Some groups also studied the detection and quantification of target proteins in linear mode.[13a,c,16] Although their results are good, the application is limited because the analysis of peptides through MALDI-TOF MS is more sensitive and the resolution is higher in reflective mode, and peptides can be further sequenced by MS/MS.

By using an aptamer as the affinity-based agent, the above-mentioned problems could be avoided, because the nucleic acid nature makes it impossible for aptamers to be digested by proteolysis enzymes, such as trypsin. Herein, we report a novel sensitive high-throughput approach that features an on-target aptamer microarray for affinity-based enrichment, laser-accelerated proteolysis for fast digestion, and MALDI-TOF MS for detection. Figure 1 shows a representation of the overall detection strategy. 1) The target plate was modified with a layer of gold in a simple but effective manner. A solution of gold colloids with an average diameter of about 13 nm was directly spotted onto the surface of the target plate. After calcination at 200 °C for 2 h, a stable layer

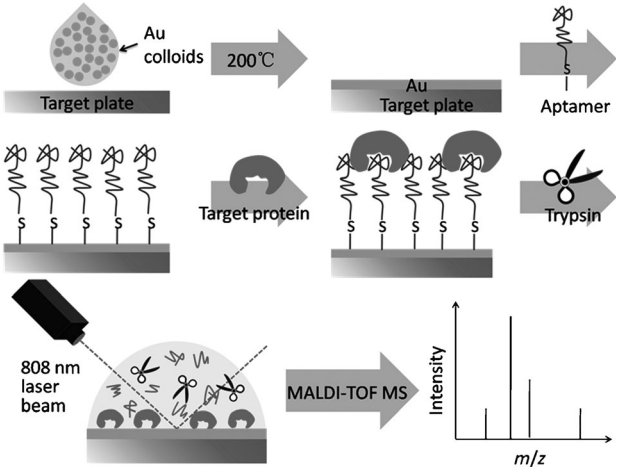


***Figure 1.*** Experimental design for the selective high-throughput detection of biomarkers, such as lysozyme: 1) modification of the target plate with a gold layer; 2) immobilization of the aptamer; 3) Enrichment of the target protein; 4) laser-accelerated digestion; 5) MALDI-TOF MS analysis.

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of gold was formed. 2) Thiol-group-modified aptamers were immobilized on the spotted surface through the easy formation of covalent bonds with the gold atoms. 3) Target protein was added, captured, and digested with laser-accelerated digestion. Previously, we have developed infrared-laser-assisted protein digestion for peptide mapping.[17] Laser-accelerated digestion greatly improved the efficiency of the digestion, especially when proteins were present at relatively low concentrations (Table S1 in the Supporting Information), yet the digestion time was significantly reduced to 30 s. 4) The sample was subjected to MALDI-TOF MS analysis for qualification and quantification.

Lysozyme, which has two tyrosine phosphorylation sites (Y56 and Y38),[18] was employed as the model protein in this experiment. Abnormal levels of this protein biomarker in urine and serum are related to diseases such as renal diseases,[19] meningitis,[20] and leukemia.[21] In 2010, Lammertyn and co-workers selected a new lysozyme-binding aptamer (LBA) with a dissociation constant of 2.8 nm.[22] The affinity of this aptamer is higher than the previously widely used DNA aptamer[5a,23] selected by Cox and Ellington with a dissociation constant of 31 nm.[24] Recently, Li and co-workers obtained better results with the aptamer of Lammertyn and co-workers,[25] which is why we used this aptamer in our experiments.

First, we tested the performance of our method for the detection of lysozyme in standard solutions. A solution (2 μL) of lysozyme was spotted onto the surface of the target plate. After 1 h, the plate was washed and trypsin was added followed with laser-accelerated digestion. A trypsin amount of 50 ng was enough for a satisfying digestion result (Figure S1). Then, MALDI-TOF MS analysis was performed, and the highest peak in the whole spectrum was found at $m/z =$ 1400.6. The sequence of this peptidewas determined to be STDYGIFQINSR by MS/MS analysis. It is wise to choose a peptide without posttranslational modifications (PTMs) for quantification. By database searching and MS/MS analysis, we didn't find any possible PTMs for the peptide at $m/z =$ 1400.6. Thus, in the following experiment, this peak was used for the determination and quantification of lysozyme and called "signal peptide peak".

The drying speed is very important to the uniformity of the gold surface on the target plate (Figure 2a). When the plate was dried quickly, the gold layer was thin and peeled off easily by washing. Slow drying led to a much more homogenous, stable, and denser gold layer, which was very important to the following enrichment. The concentration of gold colloids also had great influence on the properties of the gold layer (Figure 2b). We tested the lysozyme-enrichment efficiency of the gold layers formed at different concentrations, and found that a concentration of 92 nm gave the best results (Figure S2). The SEM image of a gold layer (Figure 2c) shows that coagulated gold nanoparticles were linked with each other to form porous three-dimensional networks that covered almost the entire surface of the target plate, thus significantly increasing the surface area of the target plate.

A calibration curve was derived for standard solutions. After enrichment and digestion, peptide SR-12 (STDYGIFQINTR, $m/z = 1414.6$), which differs in only one amino acid

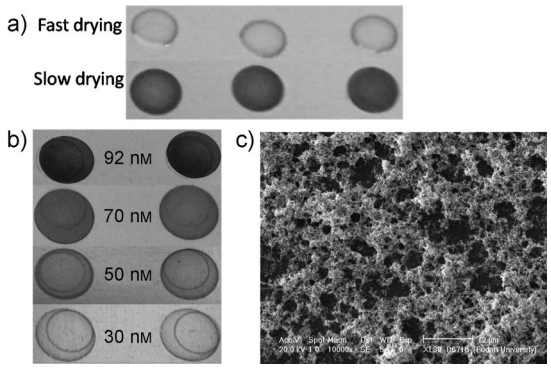


***Figure 2.*** Influence of a) the drying speed and b) the concentration of gold colloids on the formation of the gold layer. c) SEM image of the gold layer on the target plate (92 nm).

from the signal peptide (one serine was changed to threonine), was added onto the spot as internal standard. The ratio of signal intensity of the signal peptide to the internal standard was plotted against the concentration of lysozyme, and a perfect linear relationship was observed in the range from 20 ng mL$^{-1}$ to 10000 ng mL$^{-1}$ with $R^2 = 0.99$ (Figure 3b). By using the calibration curve and the standard deviation of blank samples, the limit of detection (LOD) was calculated to be 14 ng mL$^{-1}$, corresponding to a protein concentration of 1 nm, that is, 2 fmol of protein in 2 μL of original solution. Next, a lysozyme solution with a concentration of 14 ng mL$^{-1}$ was prepared, and the signal-to-noise (S/N) ratio of the signal peptide was found to be larger than 10 (Figure 3c). The LOD

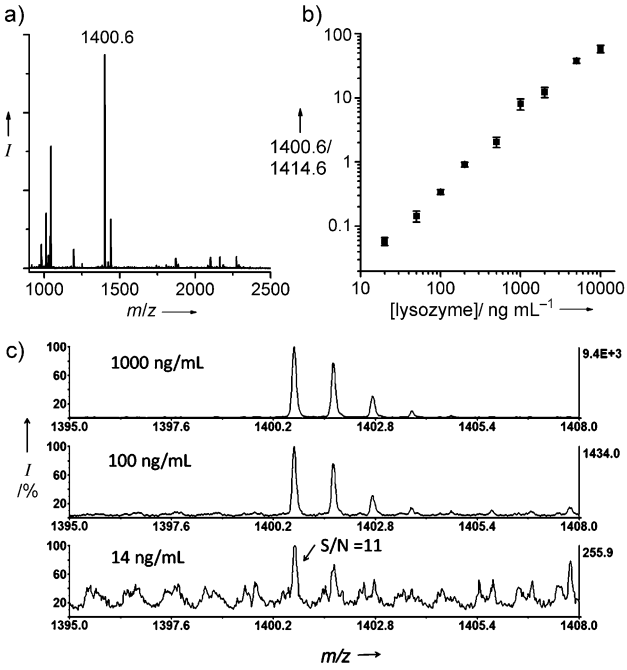


***Figure 3.*** Detection of lysozyme in a standard solution. a) MALDI-TOF mass spectrum of lysozyme at a concentration of 5000 ng mL$^{-1}$. Peak at $m/z = 1400.6$ is the highest peak in the whole spectrum. b) Linear calibration curve. The data were obtained from three independent experiments. c) Results of detection at three different concentrations.

of our method is comparable to those of most of the other methods (Table S2 in Supporting Information).

The selectivity of our method was also investigated. The analysis of five other proteins, including myoglobin (MYO), human serum albumin (HSA), cytochrome C (CYT C), thrombin, and transferrin, are shown in Figure S3. The concentrations of these five proteins and lysozyme were fixed at 100 nM. Although this was a relatively high concentration, no peak belonging to these five proteins was observed in the whole mass spectrum. However, when lysozyme was analyzed, the signal peptide peak appeared with high intensities. This demonstrated the great resistance of non-specific adsorption and excellent selectivity of the SH-LBA-modifed gold surface.

Analysis of urine from healthy human spiked with lysozyme was also performed. The analysis of lysozyme in undiluted human urine is a real challenge, because urine is a complex mixture of proteins, peptides, ions, and other metabolites. Thus, it usually has to be diluted to achieve satisfying results.[25–26] In our experiment, undiluted human urine was spiked with lysozyme at final concentrations ranging from 50 ng mL$^{-1}$ to 10000 ng mL$^{-1}$. The sample was analyzed in the same manner as that for standard proteins. The calibration curve is shown in Figure 4a. A linear relationship with $R^2 = 0.97$ was obtained, and the LOD in undiluted urine was calculated to be 66 ng mL$^{-1}$, corresponding to 4.7 nM. To the best of our knowledge, no LOD of lysozyme in a urine sample has been reported using other aptamer-based approaches. We have also identified a peak at $m/z = 1400.6$ in blank urine (without added lysozyme; Figure 4b, bottom). This peak was further subjected to MS/MS analysis (Figure 4c, bottom). It is compared with the fragment ions of the signal peptide (Figure 4c, top). As shown, the b ions and y ions, that is, the N- and C-terminal ions, respectively, generated by fragmentation at the amide bond of the peptide structure during MS/MS analysis, match pretty well. Therefore, it is reasonable to conclude that we have successfully identified the endogenous lysozyme in human urine. The level of endogenous lysozyme was calculated to be 125 ng mL$^{-1}$. It is in consistence with the reported normal lysozyme range in urine from healthy humans (0–2 μg mL$^{-1}$).[27]

Serum from healthy human was also spiked with lysozyme to further test the selectivity and specificity of our method. Both undiluted and diluted serum were spiked with 1000 ng mL$^{-1}$ of lysozyme and subsequently analyzed. The results are shown in Figure S4. When the serum was not diluted, there seem to be a lot of other peaks, but the intensities of these peaks are in fact rather low (less than 4000), considering that the total protein concentration in serum is usually approximately 50–80 mg mL$^{-1}$. When serum was diluted by a factor of 10, a distinct lysozyme peak at $m/z =$ 1400.6 can be observed. When serum was diluted by a factor of more than 50, this peak became the strongest peak in the whole spectrum (Figure S4). Through comparison with the control experiment (Figure S5) and MS/MS analysis (Figure S6 up), the identity of this peak can be further validated to belong to lysozyme. We also found a weak peak at $m/z =$ 1400.6 in the undiluted serum without addition of lysozyme.

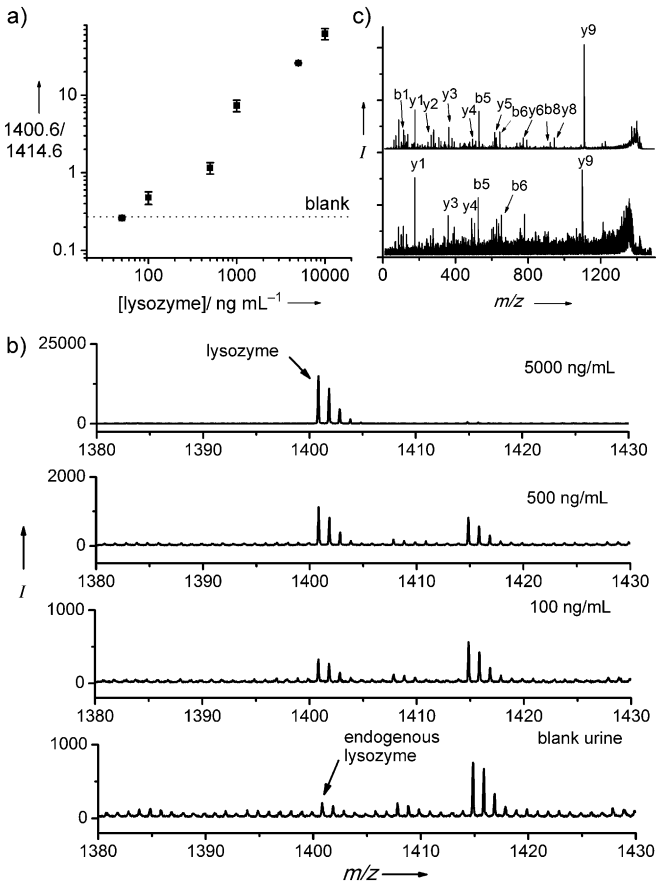


***Figure 4.*** Detection of lysozyme in spiked samples of human urine. a) Linear calibration curve. The data were obtained from three independent experiments. Dotted line represents results obtained from blank urine (without added lysozyme). b) Mass spectra of urine samples spiked with different concentrations of lysozyme. c) MS/MS spectrum of the standard signal peptide (top) and the peak with $m/z = 1400.6$ in blank unrine (bottom).

According to the MS/MS spectrum (Figure S6, bottom), it is highly probable that this peak belongs to the endogenous lysozyme in human serum. In all, these results demonstrate the excellent selectivity and specificity of our method.

In conclusion, we have developed a novel aptamer-based on-target MALDI-TOF MS strategy with laser-accelerated digestion for the high-throughput detection of lysozyme. High specificity and sensitivity were achieved by using as little as 2 μL of sample. Our method can be used for the analysis of other proteins, as long as corresponding aptamers exist. The fabrication of a platform for multiplexed protein detection will be much easier with our method than with other methods, such as fluorescence or electrochemical assays, because with MS it is possible to differentiate molecules by their masses. Because of the simplicity, high-speed, sensitiveness, and versatility of our method, we believe it has a bright future in the large-scale detection and analysis of proteins.

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