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RESEARCH ARTICLE

A Surface Pattern on MALDI Steel Plate for One-Step In-Situ Self-Desalting and Enrichment of Peptides/Proteins

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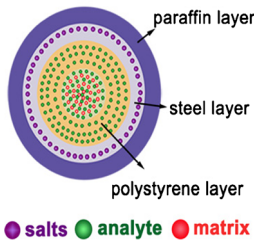

Abstract. We report a novel strategy to achieve simultaneous one-step in-situ self-desalting and enrichment (OISE) of peptides/proteins on a facilely fabricated patterned MALDI steel plate with a circular paraffin-steel-polystyrene structure. The OISE plate could efficiently segregate salts from both analytes and matrices while retaining both analyte and matrix concentrate, and facilitating them to form homogeneous co-crystals on the centrally located polystyrene pattern. With the OISE plate, high quality and reproducible spectra could be obtained for low abundance peptides even in the presence of high salt concentrations (200 mM NH_4HCO_3, 1 M NaCl, or 400 mM urea). Using this strategy, a significant sensitivity enhancement was gained over traditional MALDI plate. The practical utility of this method was further demonstrated by the successful profiling of BSA digests and human serum.

Keywords: Surface pattern, Desalting, Enrichment, MALDI

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Introduction

Since its introduction in the late 1980s, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become an indispensable tool for proteome research because of its characteristics of rapid, sensitive, and high-throughput analysis [1]. However, detection of trace amounts of peptides/proteins in complex biological samples is still challenging because of coexisting compounds, such as salts or other contaminants, which often interfere with the analysis of target analyte and lead to a drastic decrease in spectra quality and reproducibility [2, 3].

To address these issues, many off-target and on-target sample preparation strategies have been developed [4–15]. Among them, hydrophobic coatings for on-target sample preparations have received much attention [7–14]. By using these on-target coatings, a concentrated analyte spot along with homogeneous matrix-analyte co-crystal can be readily achieved, leading to significantly enhanced detection sensitivity and improved spectrum reproducibility [7, 11]. For example, Teflon-coated MALDI plates, such as Anchorchips and μFocus, have been commercially applied for sensitive MALDI analysis [15]. In this case, aqueous washing steps are often needed to remove salts from the sample spot. Along with the improvement in ion signals, some sample loss inevitably occurs during the washing steps because of the water solubility of bio-samples [16]. Moreover, manual washing also requires skillful techniques to ensure reproducibility and avoid excessive loss or destruction of analyte/matrix crystals, which creates difficulties for high throughput sample analysis [7].

Recently, we reported a novel hydrophobic-hydrophilic-hydrophobic patterned silicon wafer, which was able to achieve on-plate self-desalting and concentration of peptides/proteins without any washing steps [17]. However, the procedures for preparing surface patterns on the silicon wafer are somewhat laborious, time-consuming (≧4 h), and costly. Also, the patterned silicon wafer needs to be carefully attached on the target holder. Therefore, we launched this study to develop a new method for simultaneous one-step in-situ self-desalting and enrichment (OISE) of peptides/proteins based on a simple on-target surface patterning technique. This technique involves a sequential deposition of glycerol, paraffin, and polystyrene on the surface of MALDI steel plate to generate a hydrophobic paraffin-hydrophilic steel-hydrophobic polystyrene pattern. It

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is interesting to note that the entire surface patterning can be completed within 10 min without using any complex operations or chemical reactions. Additionally, the optimized OISE plate was able to efficiently segregate salts from both analyte and matrix while retaining both analyte and matrix concentrates, and allowing them to form homogeneous co-crystals on the centrally located polystyrene pattern. As a result, the detection sensitivity and signal reproducibility of analytes are significantly improved and high-quality MS signals can be obtained even in the presence of high concentration of salt contaminants. Furthermore, the successful analysis of bovine serum albumin (BSA) digests and human serum with this novel method is also demonstrated.

Experimental

Fabrication of OISE Plate

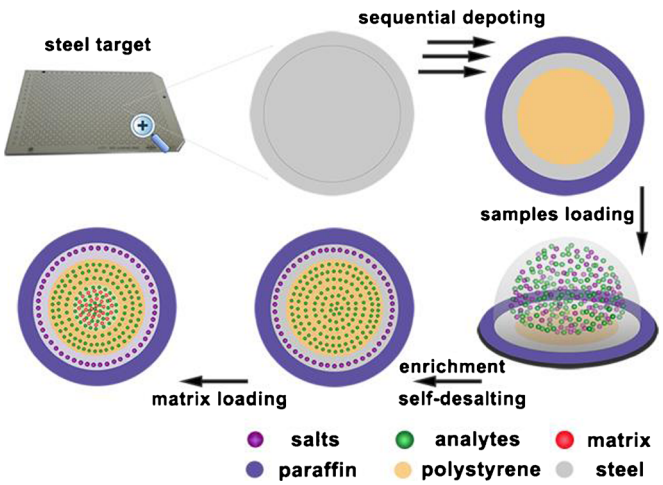
Standard MALDI steel plate (MTP 384 polished steel target, Bruker, Germany, Karlsruhe) was thoroughly washed (with ACN, water, and methanol) before use. A 2.5 μL aliquot of glycerol was first spotted on the center of target well, forming a fixed-shape droplet without spreading due to its high viscosity. Subsequently, 2 μL of paraffin wax solution (25 g/L in n-hexane) was spotted onto the glycerol droplet, quickly forming a homogeneous paraffin layer at the outer edge of glycerol spot. Alternatively, paraffin wax solution could also be sprayed onto the glycerol spots loaded target with an airbrush if large numbers of patterned spots need to be fabricated. After complete drying of the paraffin layer (~1 min), the glycerol droplet was washed away with deionized water, leaving a circular bare steel region (~2500 μm diameter) surrounded by a paraffin layer. Finally, 0.3 μL of polystyrene (PS) solution (15 g/L in chloroform) was spotted at the center of the bare steel region to form a hydrophobic PS pattern with size of about 1600 μm diameter. After drying for 5 min, the prepared patterned sample plate could be used for MALDI analysis. No interfering signals could be detected from the blank patterned plate (Supplementary Figure S1). After use, the patterned coating can be easily removed by washing with n-hexane and chloroform.

Results and Discussion

Optimization of the On-Plate Surface Pattern and Sample Preparation

The preparation of OISE plate was achieved by a simple sequence of glycerol spotting, paraffin spotting, washing away of glycerol and PS spotting, which thus formed a hydrophobic paraffin-hydrophilic steel-hydrophobic polystyrene pattern on the plate well. The outermost layer of the OISE target spot is highly hydrophobic paraffin coating, and the internal layer is a PS coating (Scheme 1). The gap between the paraffin and PS layer is hydrophilic bare steel region. When the sample solution is loaded on the OISE plate, the outermost hydrophobic paraffin not only efficiently prevents the sample solution from sliding on the target, but also enables the sample solution to be confined in the plate well without spreading (see digital photos in Supplementary Figure S2) [17]. During the subsequent sample drying process, the peptides/proteins can be captured on the center of PS polymer coating owing to their strong hydrophobic interaction with PS adsorbent material [17, 18], whereas the water-soluble salt contaminants are transferred to the hydrophilic bare steel gap because of the repulsion of hydrophobic PS material [13].

A pivotal aspect in MALDI-MS analysis is the development of a suitable sample preparation method for better analyte-matrix co-crystal formation, which is crucial for the desorption/ionization of analyte [19]. At first, an unoptimized OISE plate composed of a PS polymer coating about 600 μm in diameter and a bare steel layer about 800 μm in diameter was fabricated. The pattern diameter used here was similar to that of a previously reported silicon support [17]. In the MALDI analysis, however, an outward diffusion of matrix was observed along with the outward repulsion of salts by the



Scheme 1. Schematic presentation of the fabrication process of OISE plate, and its use for peptides/proteins enrichment and self-desalting

PS coating. As shown in Supplementary Figure S3c, after matrix deposition, a large number of matrix crystals aggregated on the bare steel area while the matrix was sparsely distributed on the PS layer. The crystal coverage on PS layer was calculated to be to 42.1%, whereas it was 65.3% on a traditional MALDI (TM) plate (Supplementary Figure S4). The poor formation of analyte-matrix co-crystals on the OISE plate would lead to a decrease in signal intensity and sensitivity [19]. For example, for the analysis of angiotensin I solution (100 fmol μL^{-1}) containing 200 mM NH_4HCO_3, a $[M + H]^+$ angiotensin I signal was detected with a medium S/N ratio of 63.5 (Supplementary Figure S5a).

To resolve this issue, we optimized the parameters of the OISE plate and the sample preparation by enlarging the diameter of PS coating to 1600 μm and decreasing the matrix deposition volume to 0.3 μL to avoid the contact of matrix solution with hydrophilic steel surface. Consequently, a remarkable difference was observed. As shown in Supplementary Figure S2, after matrix deposition, the droplet could be anchored on the center of the PS coating without spreading and formed a small matrix depot with diameter of ~700 μm on the enlarged PS layer (1600 μm). It could be clearly observed that the outward flow of matrix was completely absent and the analyte-matrix co-crystals were denser and more homogeneous (Supplementary Figure S6). This should be attributed to the isolation of matrix solution and bare steel area, as well as the concentration effect of hydrophobic polymer coating for the matrix solution. The crystal coverage on PS polymer coating was as high as 97.8%. Additionally, compared with the un-optimized OISE plate, the sample preparation using the optimized OISE plate could eliminate the possibility of recombination of the polar matrix with salts in the bare steel area. This would ensure the efficient desalting effect by the patterned plate. As a result, a strong signal corresponding to angiotensin I was detected with an average S/N ratio of 899.8 (Supplementary Figure S5b), giving ~14 times increase compared with that using un-optimized plate.

It should also be noted that the sample loading volume used for optimized OISE plate was accordingly increased to 5 μL as the larger diameter of PS coating was applied. Due to the concentration effect by the paraffin coating, the sample amounts per unit area would thus increase significantly compared with that on the TM plate. This would help to improve the detection sensitivity of MALDI-MS. Additionally, loading of large-volume sample resulted in more sample incubation/drying time (about 15 min, see Supplementary Figure S2), leading to more complete capture of the peptides/proteins in the solution by the internal layer. This would reduce the amount of analyte that is driven into the bare steel layer by quick solution evaporation and thus further improve the sensitivity.

Enrichment Efficiency Using OISE Plate

With the optimized OISE plate in hand, the enrichment efficiency was first evaluated for the analysis of a mixture of seven low abundance peptides (Pep1–Pep7, each at 1 fmol μL^{-1}). As shown in Supplementary Figure S7a and b, weak peptide signals corresponding to five of the seven peptides were detected with TM plate. In contrast, using the OISE plate, all seven peptides could be detected with stronger signals. The average S/N ratio of detectable peptides obtained with the OISE plate showed as much as a 10-fold improvement over the TM plate (Supplementary Table S1). Additionally, it can be seen that the improvements in the S/N ratio were not dependent on the hydrophobicity of tested peptides, which suggests that the OISE plate had enrichment ability for both hydrophilic and hydrophobic peptides. Moreover, a mixture containing trace amounts of four proteins (each at 50 fmol μL^{-1}) was also analyzed using TM plate and OISE plate separately (Supplementary Figure S7c and d). Only weak signals corresponding to $[\text{cytochrome } c]^{2+}$ and $[\text{ubiquitin I}]^+$ were observed with the TM plate. On the other hand, with the OISE plate, signals with excellent S/N ratios corresponding to all four proteins were detected, and the S/N ratios were increased from 2.4 to 64.8 for $[\text{cytochrome } c]^{2+}$ signal and from 9.1 to 91.6 for $[\text{ubiquitin I}]^+$ signal, respectively (Supplementary Table S1). In addition to the concentration effect from paraffin coating and enrichment effect from PS coating, the signal enhancement may also be attributed to the improved homogeneity of analyte-matrix co-crystals. Serial dilution tests demonstrated that the detection limit for the analysis of angiotensin I was at 100 amol μL^{-1} with the OISE plate, which is a 10-fold sensitivity improvement over that with TM plate (Supplementary Figure S8). Our findings clearly suggest a high enrichment efficiency of the OISE plate for the analysis of low abundance peptides/proteins.

Desalting Efficiency Using OISE Plate

Relatively high concentrations of salts and buffers are usually present in biological samples to stabilize their molecular structure and maintain their activities. However, these contaminants often disrupt the co-crystallization process, resulting in weak ion signal or even no detectable signal in MALDI-MS analysis [8, 18]. To investigate the desalting efficiency of the OISE plate, the seven peptides mixture (Pep1–Pep7, each at 50 fmol μL^{-1}) were evaluated in the presence of different salts (Supplementary Figure S9). In the presence of 200 mM NH_4HCO_3, no assignable signal could be observed with TM plate, whereas ion peaks with excellent signal quality corresponding to the seven peptides could be detected using the OISE plate (Supplementary Figure S9a and b; and Figure 1). The S/N ratios of these seven peptides, Pep1–Pep7, were 616.7, 250.3, 590.3, 611.0, 233.9, 60.0, and 629.0, respectively. In order to further evaluate the desalting efficiency using OISE plate, desalting of the peptides mixture was carried out with a commercial ZiptipC18 cartridge. All the S/N ratios obtained with ZiptipC18 are only 5%~18% of those using OISE plate (Figure 1; and Supplementary Figure S10). Insufficient time for peptide binding and sample loss during the washing steps are mainly responsible for the relatively poor signals with ZipTipC18 pretreatment [14]. As for the OISE

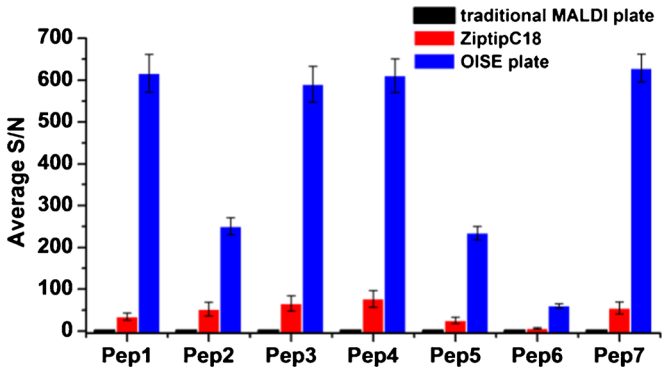


Figure 1. Average S/N ratios of peptide mixture (each at 50 fmol μL^{-1}): bradykinin 1-7 (Pep1, *m/z*: 756.86), angiotensin II (Pep2, *m/z*: 1046.19), angiotensin I (Pep3, *m/z*: 1296.49), substance P (Pep4, *m/z*: 1347.64), bombesin (Pep5, *m/z*: 1619.86), renin substrate (Pep6, *m/z*: 1759.03), ACTH 1-17 (Pep7, *m/z*: 2093.43) in the presence of 200 mM NH_4HCO_3 using the TM plate, ZiptipC18 on the TM plate and the OISE plate. Error bars represent the SDs of S/N from five independent measurements

plate, the strong retention ability of PS materials enables sufficient peptide binding, and the wash-free self-desalting procedure prevents excessive sample loss, thus resulting in more detectable peptide amounts. Additionally, the on-plate desalting strategy with OISE plate is much simpler and easier for high-throughput analysis compared with the laborious multi-step sample processing by ZipTipC18. Furthermore, signal deterioration was also observed in the presence of nonvolatile salts (such as NaCl and urea) because these contaminants severely impeded the UV absorption and energy transfer process. As shown in Supplementary Figure S9c–f and Supplementary Figure S11, weak signals and no signals were, respectively, detected on the TM plate in the presence of 1 M NaCl and 400 mM urea. After desalting on the OISE plate, signals for all the seven peptides were clearly detected and the sensitivity was markedly improved.

Reproducibility and Stability Using OISE Plate

The spectrum to spectrum reproducibility of peptides on the traditional MALDI plate in the absence of salts and on the OISE plate in the presence of high salt concentrations (1 M NaCl, 200 mM NH_4HCO_3, and 400 mM urea) was investigated. As listed in supplementary Table S2, the relative standard deviations (RSDs) of the spectra of three peptides using traditional MALDI plate are 34%~43% (without salts), whereas the corresponding RSDs on the OISE plate are all less than 13.3% even in the concentrated salt solutions. The OISE provides high reproducibility for three reasons: (1) the interference by high salt concentrations was efficiently eliminated by complete isolation of salts from analyte/matrix; (2) analyte-matrix co-crystals are formed densely and evenly on the OISE plate without outward flow effect; and (3) the smooth PS coating helps with both the adsorption of samples and the deposition of matrix (for

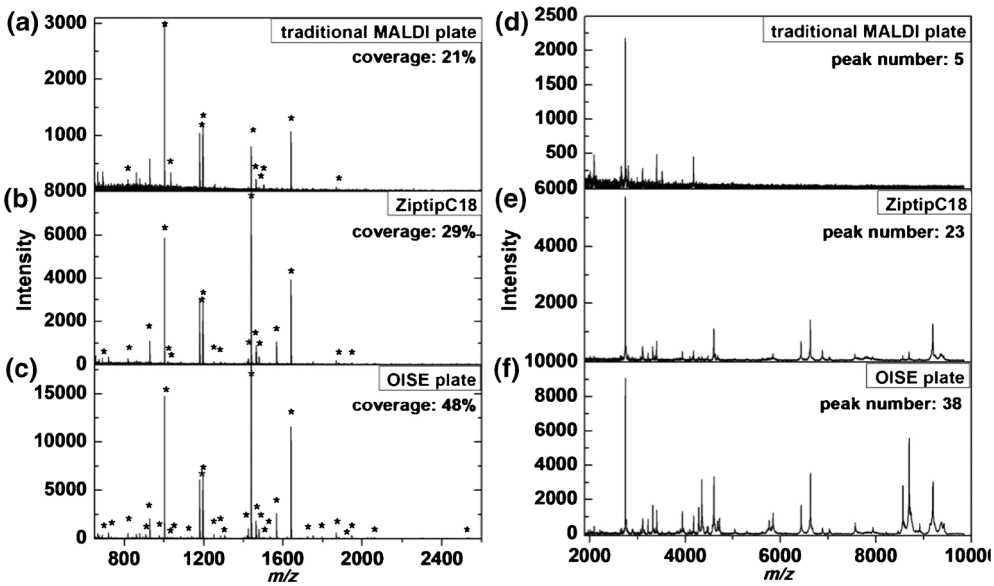


Figure 2. MALDI-MS spectra of (**a**), (**b**), (**c**) the BSA digests (31 fmol μL^{-1}), and (**d**), (**e**), (**f**) human serum (100-fold dilutions) with the TM plate, ZiptipC18 preparation on the TM plate and the OISE plate. Peptide peaks from BSA digests are noted with asterisks

more details, see Supplementary Figure S12). Additionally, we investigated the stability of the OISE plate. The OISE plate was stored at room temperature for 2 wk, and then used for MALDI-MS analysis of a peptides mixture. As shown in Supplementary Figure S13, there was almost no difference between the signal intensities of the spectra obtained using the stored OISE plate and the freshly fabricated OISE plate, indicating that the OISE plate fabricated with inert paraffin and PS materials remains stable during storage.

Analysis of BSA Tryptic Digest and Human Serum

The practical application of the OISE method was further evaluated by analysis of BSA digests and human serum. Figure 2a–c present the mass spectra of BSA digests (31 fmol μL^{-1}) with the TM plate, ZiptipC18 preparation, and the OISE plate. Ion peaks corresponding to digests of interest are marked with asterisks and listed in Supplementary Table S3. In the case of the TM plate, signals of the digests were suppressed because of the existence of salts [20, 21], and only 11 peptides could be detected with a sequence coverage at 21%. After desalting with ZiptipC18, improved signals with 18 peaks were identified, and the peptide sequence coverage increased to 29%. By contrast, the OISE plate provides the highest mass spectra quality with the number of detectable peptides and sequence coverage increased to 30% and 48%, respectively. Figure 2d–f show the results for the analysis of human serum. The human serum was first pretreated with DTT to remove large proteins. Although the concentration of salts and other contaminants was largely reduced by dilution of the sample solution, the remaining interfering substances in the serum solution still resulted in significant signal suppression with TM plate [22]. Only a few peptides/proteins (5 peaks) could be detected on the TM plate. The signals were significantly enhanced after desalting by ZiptipC18 method, and 23 peaks were identified. Most interestingly, the mass profile generated using the OISE plate allows us to recover 15 more signal peaks that could not be observed with the traditional ZiptipC18 method (Supplementary Table S4 and Figure 2f). Our results demonstrated the novel OISE technique for one-step peptides/proteins enrichment and self-desalting would be a highly useful strategy in real proteomics analysis.

Conclusions

We developed a novel patterned MALDI plate with a circular paraffin-steel-polystyrene structure by a sequential deposition process. The fabricated OISE plate achieved on-plate simultaneous wash-free self-desalting and enrichment for low concentration peptides/proteins. Compared with the traditional MALDI plate, higher quality spectra and better reproducibility could be obtained by the OISE plate for low abundance peptides even in the presence of high concentration of salts. Successful application of the OISE plate was further demonstrated in analyses of BSA digests and human serum with a significantly improved sequence coverage and an increased number of detectable protein signals, respectively. Importantly, the fabrication of patterned OISE plate is straightforward and easily adapted to common MALDI steel plate with low-cost commercial materials. These characteristics make it an attractive and potentially alternative enrichment/desalting method for various applications, especially for high-throughput MALDI-MS analysis.

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