Wash-free *in-situ* self-desalting and peptide enrichment by block copolymer analyzed with MALDI-TOFMS†

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A novel technique of simultaneous peptide enrichment and wash-free *in-situ* self-desalting for MALDI analysis is reported, where a newly synthesized block copolymer with a microphase-separated configuration is applied to embed salts with its hydrophilic domain of poly(ethylene oxide) and concentrate peptides with its hydrophobic domain of polysulfone.

With the number of human genes known to be ≃24,000,[1] most of their expression products still face a serious challenge in being detected globally at the proteome level.[2] Salts/buffers used in sample preparation may interfere with protein identification in subsequent mass spectrometry (MS) analysis, especially for those of middle to low or low abundance.[3] A sample cleanup procedure, therefore, must be implemented prior to MS analysis, even for matrix-assisted laser desorption/ionization (MALDI), which is relatively tolerant to salts compared with the electrospray ionization (ESI) method.[4] C18Ziptip as an off-target desalting method is still a traditionally used method.[5] However, C18Ziptip is used as an adsorbent solid phase which depends on a high affinity to proteins/peptides and a weak interaction between contaminants and the stationary phase. Therefore, both washing and elution steps are needed which result in irreversible sample loss. Many studies of the on-target desalting method, in which a number of membranes or coatings were patterned onto the target surface, have been reported recently. The coatings used include NC,[6] polyethylene,[7] PVDF,[8] polyurethane,[9] Nylon,[10] self-assembled monolayers (SAMs) containing C18,[11] Parafilm,[12] Teflon,[13] polyacrylamide and Nafion,[14] silicone polymer,[15] porous polymer monolith[16] and anodic alumina.[17] For the reported polymer coating and membrane methods, all these kinds of polymer coating and membrane layers are used as an adsorbent solid phase.[18] These coating methods are capable of extracting proteins or peptides selectively from complex sample solutions so as to suppress the noise from salts and surfactants in a complicated system. An effective method using small hydrophilic spots patterned in a hydrophobic field has been reported recently.[19] Thus, the small hydrophilic spots concentrate proteins or peptides to improve reproducibility and sensitivity for MALDI signals. However, during solvent evaporation, salts instead of peptides would be concentrated with more content onto the hydrophilic spot due to the stronger interaction between peptides and the hydrophobic polymer. Therefore, a washing step is unavoidable to remove the salts. This washing process requires significant technical expertise and skill to ensure reproducibility and avoid the excessive loss of peptides in the rinsing step. Additionally, the rinsing step prevents this method from being easily adapted to a high-throughput method. Recently we explored a rapid and automatic on-plate desalting method for MALDI-MS by the approach of templating the hydrophobic polymer on a small central spot.[20] Therefore, desalting and peptide enrichment can be performed on a MALDI target plate in one step with no additional water-rinsing step. However a superfluous volume of matrix solution was needed to make the salt redissolve and flow outside. We also developed a strategy to remove salt on the target-plate as well as to enhance the sensitivity in MALDI-TOFMS.[21] By applying pre-designed nano-materials, we enriched desired low-abundance peptides/proteins and alleviated the effect of contaminants simultaneously. However, the use of nano-materials required several steps including incubation and centrifugation.

Herein, we report a novel wash-free *in-situ* self-desalting and enrichment (WISE) method for preparation of MALDI samples. Remarkably, the sensitivity towards peptides in middle to low or low abundance has been effectively improved, since neither an off- nor an on-target washing procedure is incorporated. In this study, we synthesized a polysulfone-poly(ethylene oxide) block copolymer (PSF-b-PEO, PEO content of 60 wt%) (the synthesis method and validation results are detailed in ESI† section 1), which has high affinity towards salts but almost none towards peptides, and is stable in solvents (water–acetonitrile) with good film-forming ability. The PSF-b-PEO is highly soluble in several highly-volatile organic solvents such as tetrahydrofuran (THF) and chloroform. A uniform film can form quickly on the surface of a stainless steel target after the PSF-b-PEO solution has been applied. The PSF-b-PEO has a microphase-separated structure, which contains a hard and hydrophobic domain of polysulfone (PSF) to maintain film mechanical strength and prevent polymer dissolution in water–acetonitrile (ACN), and a soft and hydrophilic domain of poly(ethylene oxide) (PEO) to strongly absorb salts, resulting in effective desalting and peptide enrichment.

Depicted in Fig. 1 are the MS spectra of 50 fmol μL^{-1} myoglobin (MYO) digests without (upper panel) and with (lower panel) PSF-b-PEO substrate on a MALDI target in the presence of different kinds of contaminant. It can be seen that with a normal stainless steel MALDI target there were barely detectable signals in the presence of 100 mM ammonium bicarbonate (NH_4HCO_3), 25 mM sodium bicarbonate ($NaHCO_3$), 0.2% sodium dodecyl sulfate

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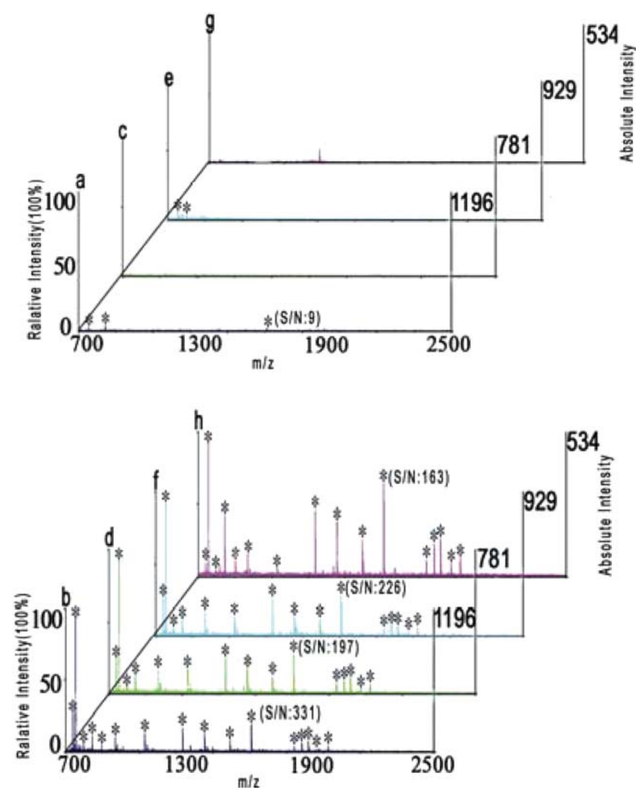

Fig. 1 MALDI-TOFMS spectra of 50 fmol μL^{-1} MYO digests in the presence of contaminant salts: (a) 100 mM NH_4HCO_3, (c) 25 mM $NaHCO_3$, (e) 0.2% SDS, (g) 0.1% CHAPS, spotted on a stainless steel target respectively; and (b) 100 mM NH_4HCO_3, (d) 25 mM $NaHCO_3$, (f) 0.2% SDS, (h) 0.1% CHAPS, spotted on PSF-b-PEO film respectively. Asterisks (*) represent peaks assigned to peptides of MYO digests.

(SDS), and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (as spectra a, c, e, g in the upper panel), respectively. In contrast, with the PSF-b-PEO substrate, the peptide signals became much stronger (lower panel). For example, in the presence of 100 mM NH_4HCO_3 (Fig. 1b), the signal-to-noise ratios (S/N) of the top three peaks were enhanced by 34- (peptide at *m*/*z* of 748.45), 18- (*m*/*z* 1271.69) and 28-fold (*m*/*z* 1606.88), respectively. For the cases of $NaHCO_3$, SDS and CHAPS contaminants, the S/N enhancement factors with PSF-b-PEO are 20, 25, and 15, respectively (Fig. 1d, f, and h), based on the peak signal at *m*/*z* of 1606.88.

The observed enhancement in S/N ratio with the proposed WISE method is attributed to the *in-situ* self-desalting capability of PSF-b-PEO as confirmed by validation experiments shown in Fig. 2. It is well known that PEO has high affinity towards many inorganic salts due to the strong interaction forces between oxygen atoms on PEO and metallic cations.[22] When salt-contaminated MYO digests are applied onto the surface of PSF-b-PEO, the PEO domain will absorb the salts due to its hydrophilicity and the strong PEO-salt affinity. Therefore contaminants are trapped within the hydrophilic domain of the polymer substrate and are unable to enter the hydrophobic domain where peptides are enriched when matrix solution is added later, as illustrated in Fig. 2a.

The strong interaction between contaminants and the PFS-b-PEO film has been elucidated by MS and ATR-FT-IR experiments. For 0.5 μL MYO digests of 1.0 ng μL^{-1} in the presence of

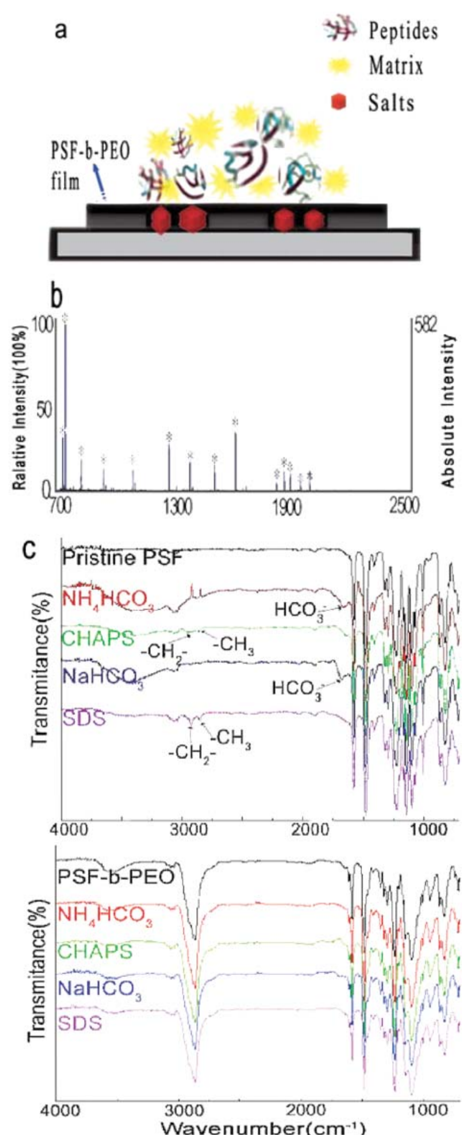

Fig. 2 Mechanism illustration and validation of WISE method in MALDI-TOFMS. (a) Schematic illustration of the sample desalting and peptide enrichment by PSF-b-PEO; (b) MALDI-TOF mass spectrum of rinsed solution of 0.5 μL 1 ng μL^{-1} MYO digests with 100 mM NH_4HCO_3 respotted on the stainless steel target (right) after being washed from PSF-b-PEO film using 1.0 μL 50% aqueous ACN solution containing 0.1% trifluoroacetic acid. Asterisks (*) represent peaks assigned to peptides of MYO digests; (c) ATR-FT-IR spectra of the surface of pristine PSF (upper) and PSF-b-PEO (lower) after addition of 1) 100 mM NH_4HCO_3, 2) 0.1% CHAPS, 3) 25 mM $NaHCO_3$, 4) 0.2% SDS.

100 mM NH_4HCO_3, six aliquots were spotted on PSF-b-PEO film and washed with 1.0 μL 50% aqueous ACN solution containing 0.1% trifluoroacetic acid. Then the rinsed solutions were spotted on stainless steel targets and analyzed by MALDI. It can be seen that the number of peaks assigned to MYO digests (Fig. 2b) is almost identical to those directly analyzed after the aliquot samples were spotted onto PSF-b-PEO (Fig. 1b), indicating the salts were removed by being absorbed into the PSF-b-PEO. Fig. 2c shows ATR-FT-IR spectra of pristine PSF film (upper) and PSF-b-PEO film (lower), in which both films were treated with a couple of drops of either pure water (as pristine polymer film), 100 mM

NH_4HCO_3, 0.1% CHAPS, 25 mM $NaHCO_3$, or 0.2% SDS solutions, respectively on the film surface. As shown, contaminants were clearly detected on pristine PSF polymer film with characteristic saline bands (NH_4HCO_3, 1669 cm^{-1}; CHAPS, 2867 cm^{-1} and 2924 cm^{-1}; $NaHCO_3$, 1680 cm^{-1}; SDS, 2855 cm^{-1} and 2923 cm^{-1}). In contrast, no salt peaks were displayed in corresponding ATR-FT-IR spectra on the PFS-b-PEO film, implying that only the PEO domain can absorb and embed contaminants dramatically. Furthermore, the SEM spectra (data not shown) also demonstrated that there was no significant difference between two PFS-b-PEO films which were respectively salt-free and treated with 100 mM NH_4HCO_3. These results suggest that almost all contaminants in the sample can be absorbed into the PSF-b-PEO substrate. Furthermore, the interaction is fairly strong as the contaminants are unable to be washed away from the PSF-b-PEO substrate.

The present WISE method provides both higher intensity and higher reproducibility as shown in Table S2 in ESI.† The S/N was obtained when 20 aliquots of 50 fmol μL^{-1} MYO peptides without salts were parallel spotted onto PSF-b-PEO film, and when the same samples were spotted onto a stainless steel target. The enhanced signal and reproducibility are likely attributable to the more hydrophobic nature of the PSF-b-PEO substrate than the stainless steel target which can concentrate peptides onto a small area. Therefore, the WISE method provides high reproducibility for two reasons: (1) a large portion of the sample can be laser irradiated simultaneously due to analytes being concentrated in a small area;[23,24] (2) well-proportioned, homogeneous crystals can be formed on top of the copolymer, ensuring a uniform analyte-to-matrix ratio in each valid zone.[24] Furthermore, the comparison of the mass spectra (Figure S2 in ESI†) from 10 fmol μL^{-1} MYO digests in the presence of 50 mM NH_4HCO_3 desalted by WISE and normal C18Ziptip methods shows that the S/N ratios using normal Ziptip are only 10–20% of those using the WISE method.

We have successfully utilized the WISE method in an analysis of gel-separated low abundance proteins. The proteins extracted from the liver of house mouse were separated by 2DE, and 10 protein spots were selected and labeled with Arabic numerals from strong to weak optical density of gel spots (Figure S3 and related experiment in ESI†). All the samples were divided into two equal aliquots which were spotted onto a PSF-b-PEO substrate and a stainless steel target, respectively. The results are shown in Table S3 in ESI.† For the strongest spots 1, 2 and 3, the resulting protein scores did not differ significantly between the two methods. However, for the weaker protein spots 4 and 5, the WISE method obtained significantly higher scores compared with those from the conventional method. For the low-concentration spots from 6 to 10, the proteins could barely be identified (based on the threshold of a combined matching score of >62 with 95% confidence level) by the conventional method, especially for spots 8 and 9. In contrast, all five low abundance proteins were identified confidently with high scores by the proposed WISE method. Therefore, the success rate for identification of low-concentration proteins has dramatically improved with the WISE method, indicating its promising potential in the analysis of low abundance proteins.

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