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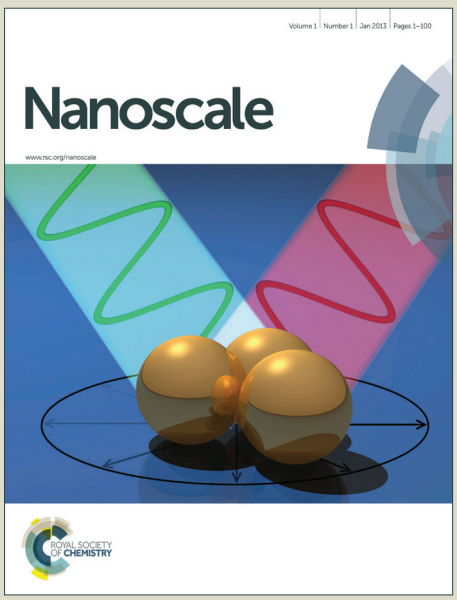

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A Designed Combo-Pore Approach for Programmable Extraction of Peptides/Proteins

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A novel combo-pore approach is designed to achieve programmable purification, minimisation of sample complexity, enrichment and sensitive detection of peptides in bio-samples, showing superior performance compared to conventional protocols and commercial products.

Bioactive peptides can be used as vaccines, hormones, cytokines, toxins, etc., which play essential roles in human health and disease treatment. Nowadays analysis of peptide has received prominence in proteomic[1, 2] and peptidomic study.[3, 4] The comprehensive peptide analysis in biological systems (so called *Peptidomics*) has led to the discovery of novel biomarkers and new diagnostic approaches, since the amount and repertoire of peptides in the circulation change dynamically according to the physiological or pathological state of an individual.[5, 6] However, the detection of peptides is a challenge considering their low abundance and strong disturbance from other bio-molecules (mostly proteins) in complex bio-mixtures. To date, the purification and extraction of peptides has become a key step in the pre-treatment of biological samples before analysis, where the enrichment of peptides is required to increase the concentration of peptides and avoid the influence from other unwanted molecules.[5-7]

Antibody can be used to recognise and pull-out peptides from bio-samples, however it is impractical to make antibody for every peptide in discovery phase experiments.[8, 9] Due to this limitation as well as the relatively high costs, many nanomaterial based protocols have been developed to capture peptides/proteins, e.g., zeolites,[10] poly(methyl methacrylate) (PMMA) type materials[7, 11-13] and ordered mesoporous materials[7, 14-21] followed by mass spectrometry (MS) analysis.[22] Particularly, the "small pore" (pore size < 6 nm)[7, 14-16] mesoporous materials can be used as molecules sieves for peptides due to the size-exclusive effect. Despite the success in the optimisation of surface chemistry,[7, 17, 23] wall composition[14, 19] and pore structure,[24, 25] in all these reports it is still hard to exclude the interference of proteins against the detection of peptides, especially low molecular weight (LMW) proteins. Even using mesoporous materials with small pore sizes of 2.05-4.7 nm, the LMW proteins such as cytochrome (MW 10-20 kDa) cannot be efficiently separated from peptides.[7, 14-16] Therefore, a new approach to replace the conventional size-selective sieving method[7, 14-16] is in demand to reduce the interference of LMW proteins towards sensitive peptide enrichment and detection.

In parallel with the applications of "small pore" materials, the "big pore" mesoporous/macroporous materials (pore size in the range of 10-100 nm)[19, 26-31] have been successfully applied in protein immobilization, enrichment and digestion. Recent studies suggest that the adsorption and digestion of proteins can be greatly enhanced by using macroporous silica foams (MOSF) when the pore size increases to ~ 100 nm.[19, 29-31] In previous studies where "big pore" materials were used as nano-reactors for protein immobilisation and digestion, accumulated evidences have shown that the peptides after digestion are readily released from the nanopores,[26, 28, 29] indicating that in general "big pore" exhibits stronger affinity towards proteins but relatively weaker interaction with peptides. However, such observations have not been systematically studied for advanced applications such as separation and purification. More importantly, the adsorption behaviour of "small pore" (favouring peptides) is inversely complementary to that of "large pore" materials (favouring proteins). Therefore, we hypothesize that a careful combination of "big pore + small pore" approach will make best use of advantages of each material, but overcome the disadvantages of each part.

In this study, a novel "big pore + small pore" combinational approach (hereafter referred to as combo-pore approach) is developed for programmable and selective peptide extraction from biological complex samples, showing superior performance compared to current methods and commercial products. The combo-pore approach is rationally designed through our systematic studies: (1) The "big pore" MOSF have a higher immobilization capacity towards a LMW protein (cytochrome c), but limited adsorption

ability towards a peptide (E7 as the target peptide in this study, see Figure 1a). (2) The "small pore" periodic mesoporous organosilica (PMO) materials possess a higher enrichment capacity to the peptide, but a limited adsorption amount to the protein. (3) To take advantages of both "big pore" and "small pore", in the combo-pore approach the "big pore" is used in the protein extraction step (I) while the "small pore" sequentially in the peptide enrichment step (II). As a result, the interference of LMW proteins can be significantly suppressed and the target peptide can be isolated from complex bio-samples and detected with a high efficiency.

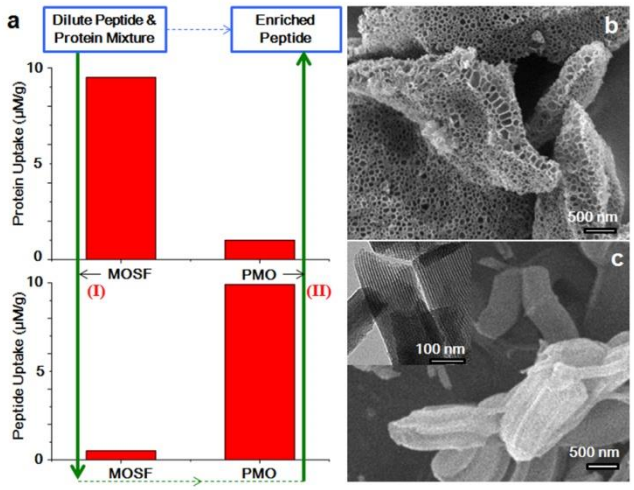

Figure 1. a) Comparison of the adsorption capacity between MOSF and PMO materials towards protein (up) and peptide (down). The green arrows indicate the sequence of combo-pore approach; b) SEM image of MOSF; c) SEM and TEM (inset) images of PMO.

The MOSF and PMO used in this study were synthesized according to the reported methods (see Experimental Section in SI for details). As shown in the scanning electron microscopy (SEM) image in Figure 1b, MOSF exhibited typical foam-like structure with roughly 100 nm macropores.[29-31] Nitrogen adsorption analysis revealed that MOSF displayed a type IV isotherm with an H1 hysteresis loop in high relative pressure (P/P_0) range (Figure S1). The surface area and pore volume were calculated to be 425 m^2/g and 1.7 cc/g, respectively. The PMO chosen in this study had a pore size of 5.8 nm with *p6mm* structural symmetry and rod-like morphology (Figure 1c), specifically designed for the enrichment of E7 peptide.[25] The inset of Figure 1c was a typical TEM image, showing that the ordered stripe-like one-dimensional meso-channels are parallel to the long axis of rods, in accordance with a highly ordered hexagonal mesostructure.

To investigate the immobilization ability of the chosen PMO and MOSF, standard bio-molecules (cytochrome c, MW ~ 12 kDa, denoted Cyt-c, E7, sequence RAHYNI**V**TF, a peptide vaccine to eliminate tumour growth and introduce immune responses) were chosen.[32] Cyt-c type proteins are typical LMW proteins and generally exist in bio-extracts, which severely affect the analysis and suppress the signals of other functional bio-molecules such as peptides due to their high abundance. As displayed in Figure 1a, MOSF had a high immobilization capacity of Cyt-c (>9.5 μM/g) with over 95% proteins extracted (detected by UV-Vis method). For comparison, PMO immobilised only 10% proteins (1 μM/g), much lower compared to MOSF. However, PMO showed advantages over MOSF in the extraction of peptides (Figure 1a) with over 99% of E7 peptides extracted (>9.9 μM/g), whereas MOSF only adsorbed ~ 5 % of peptides (<1 μM/g). In the peptide enrichment tests, a signature peptide was used for label-free MS quantification of E7 using a reported protocol (see Experimental Section and Figure S2 in SI).[24] Our quantitative analysis results have demonstrated the opposite biomolecule immobilization behaviour between MOSF and PMO: MOSF favours a high adsorption capacity towards LMW proteins, but can not effectively trap peptides such as E7 due to the ultra-large pore size; on the other hand, PMO is ideal for peptide capture but has limited protein adsorption due to the small pore size.

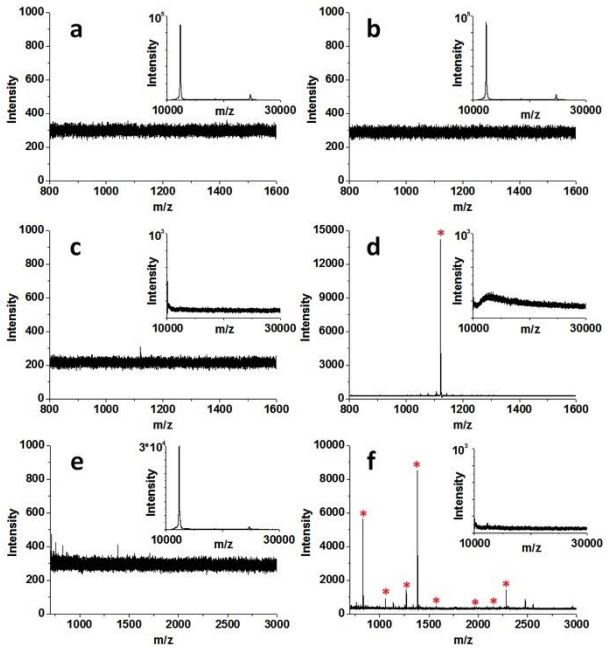

Figure 2. MS spectra from 89 nM E7 peptides in 0.1 mg/mL Cyt-c solution a) directly, b) after enrichment by PMO, c) after extraction by MOSF and d) after MOSF extraction followed by PMO enrichment (combo-pore approach); MS spectra from 20 nM casein digests in 0.1 mg/mL Cyt-c solution e) before treatment and f) after combo-pore treatment. The * stands for the peptides signal and inset shows the high mass range.

The combo-pore approach (Figure 1a) was applied for selective peptide extraction in a mixture of E7 (89 nM) and Cyt-c (peptide/protein, ~1/90, molar ratio). As shown in Figure 2a, no peptide signals can be found in the low mass range (m/z 800-1200), but strong protein peaks from Cyt-c can be viewed (inset of Figure 2a) at the high mass range (m/z 10,000-30,000) without any pre-treatment. When only PMO was employed for enrichment, similar results were obtained (Figure 2b). This is because the size-selective extraction by PMO itself cannot completely eliminate the interference from the LMW proteins (Cyt-c in this case). Consequently, the detection of E7 is failed. When only MOSF was used to extract Cyt-c, the protein peaks were not observed in the high mass range (inset of Figure 2c), indicating that most of the Cyt-c were removed from the mixture. However, the E7 signal cannot be clearly observed (Figure 2c) due to the low abundance of E7 and lack of enrichment procedures. Only in the combo-pore approach after extraction by MOSF (inset of Figure 2d) followed by PMO enrichment (Figure 1d), the E7 peptide signal can be observed (m/z of 1120.6) with strong signal intensity (~ 14,000) and high signal-to-noise (S/N) ratio. With the combo-pore approach, E7 can be detectable at a concentration down to 4.5 nM (Figure S3, peptide/protein, ~1/1800, molar ratio).

The performance of the combo-pore approach was compared to commercial 10 kDa and 3 kDa MW cut-off membranes to test the peptide separation efficiency from the same bio-mixtures studied before. As shown in Figure S4a and inset, no peptide signals were detected and the protein peaks were present, indicating that Cyt-c cannot be separated from peptides after the filtration by the 10 kDa membrane. After filtration by the 3 kDa membrane, although the proteins are blocked, the peptide signal still cannot be observed (Figure S4b and inset), which can be explained by the fact that the membrane filtration itself has no enrichment effects. Our results have shown that the combo-pore approach is advantageous over the conventional methods using one material, or using the commercial MW cut-off products.

The combo-pore approach was further applied to investigate its performance to detect peptides mixtures in the presence of LMW Cyt-c. Figure 2e showed the MS spectrum from casein digests (20 nM) mixed with Cyt-c before extraction at a molar ratio of ~1/400 (peptide/protein). The protein peak with strong intensity can be observed, while only one very weak peptide signal can be found with intensity less than 500. As a result, the identification of casein protein failed. By the combo-pore approach, the proteins were removed (Figure 2f, inset), and a fruitful of peptides can be detected with high S/N ratio (Figure 2f) through which the casein protein can be identified successfully. Similar results can be obtained dealing with other protein digests mixed with LMW proteins (Figure S5). It is concluded that the combo-pore approach can be applied in the extraction of diverse peptides, allowing better application in peptidomics and proteomics.

Complex bio-samples were also examined using a further designed combo-pore approach. Figure 3a showed the MS spectra from the 89 nM E7 peptides mixed with various proteins at a molar ratio of ~1/60 (1/200, peptide/each protein, w/w) including Cyt-c, myoglobin (MW 17 kDa), beta casein (CAS, MW 24 kDa), horseradish peroxidase (HRP, MW 44 kDa), ovalbumin (OVA, MW 45 kDa) and bovine serum albumin (BSA, MW 66 kDa).[29, 30] No peptides were detected and strong protein signals can be viewed before treatment. After the extraction by MOSF (Figure 3b), the acid proteins including CAS (pI~5.3), OVA (pI~4.6) and BSA (pI~4.9) were observed in solution, although the other proteins were removed. On the contrary, after extraction by NH_2-MOSF (a modified MOSF with positively charged surface), the basic proteins like Cyt-c (pI~10.8) and HRP (pI~8.8) retained in the solution, whereas the acid proteins were purified (Figure 3c). The phenomenon was attributed to the electrostatic interaction between the materials and proteins.[29] After an optimised combo-pore extraction using both MOSF and NH_2-MOSF, all the proteins can be well extracted (Figure 3d,) and the E7 peptides can be detected after the PMO enrichment (inset of Figure 3d).

The optimised combo-pore approach was applied in the detection of E7 spilled (4.5 μM) serum sample. Serum tests are fundamentally important in clinical diagnostics but often affected by unwanted hemolysis. As shown in Figure 3e (control sample without any treatment), strong signals of serum proteins (e. g. serum albumin, hemoglobin and its sub-chains) can be observed in the high mass range with absence of peptide signal in the low mass range (inset of Figure 3e). Using PMO enrichment alone provided similar results and failed for detection of E7 as shown in Figure 3f and the inset. By using MOSF and NH_2-MOSF, the proteins can be efficiently purified (Figure 3g, see also the colour change in Figure 3e, g), whereas the E7 signal cannot be detected (inset of Figure 3g). Only after the optimised combo-pore approach (extraction by MOSF and NH_2-MOSF, followed by PMO enrichment), E7 can be successfully detected (Figure 3h). It is noteworthy that using the optimised combo-pore approach, E7 was detected with at a low concentration down to 89 nM in serum (inset of Figure 3h), almost 2 magnitudes lower compared to a previous report (detection-of-limit over 45 μM).[24] Additionally, many signal peptides existed in the serum sample. Analysis of these peptides is one of the most important targets in peptidomics and requires specific sample treatment even before enrichment and detection (e. g. pre-denaturing steps[18] and other methods[33]). Our current extraction/detection is performed in native serum without such treatments thus difficult to detect other peptides simultaneously.

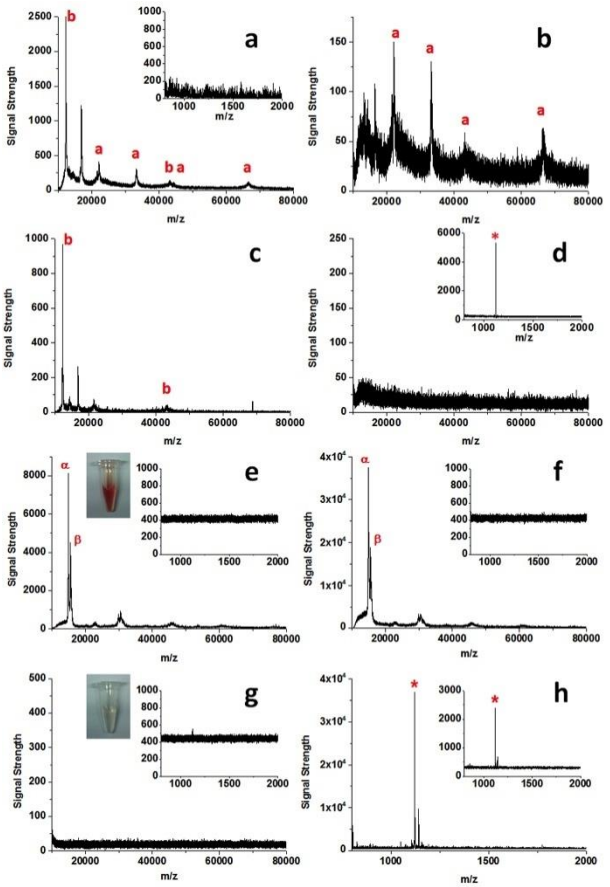

Figure 3. MS spectra from 89 nM E7 peptides in 0.1 mg/mL complex proteins solution a) directly, b) after extraction by MOSF, c) after extraction by NH_2-MOSF and d) after extraction by MOSF and NH_2-MOSF followed by PMO enrichment; MS spectra of E7 (4.5 μM) spilled serum samples e) before treatment, f) after enrichment by PMO, g) after purification by MOSF and NH_2-MOSF, h) after combo-pore treatment. The digital images in e) and g) showed the solution colour change before and after purification, respectively. The inset of h shows the enrichment results with initial E7 concentration of 89 nM after the combo-pore treatment. a and b stand for the acid and basic protein signals, respectively; α and β stand for the sub-chains from the hemoglobin and the * stands for the peptides signal.

Conclusions

In summary, a novel combo-pore platform technology has been developed for programmable separation and detection of peptides with high sensitivity in complex bio-samples, showing better performance compared to conventional protocols and commercial products. This concept of designed combinational approach is not

limited "big pore + small pore" (MOSF+PMO in our study), "negatively and positively charged surface" (MOSF and NH_2-MOSF in our case). It is expected that many other types of functional materials with complementary advantages can be rationally assembled into various combo-pore approaches (e.g., introducing surface modification to extract information on post-translational modifications of peptides/proteins[19, 31]), providing a versatile platform technology for the sensitive detection of biomolecules in biological samples with broad applications.

Notes and references

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